Identification of Asp218 and Asp326 as the Principal Mg^{2+} Binding Ligands of the Homing Endonuclease PI-*Sce*I[†,‡]

Sylvia Schöttler, Wolfgang Wende, Vera Pingoud, and Alfred Pingoud*

Institut für Biochemie, FB 08, Justus-Liebig-Universität, Heinrich-Buff-Ring 58, D-35392 Giessen, Germany

Received July 28, 2000; Revised Manuscript Received October 24, 2000

ABSTRACT: The monomeric homing endonuclease PI-*Sce*I harbors two catalytic centers which cooperate in the cleavage of the two strands of its extended recognition sequence. Structural and biochemical data suggest that catalytic center I contains Asp218, Asp229, and Lys403, while catalytic center II contains Asp326, Thr341, and Lys301. The analogy with I-*Cre*I, for which the cocrystal structure with the DNA substrate has been determined, suggests that Asp218 and Asp229 in catalytic center I and Asp326 and Thr341 in catalytic center II serve as ligands for Mg^{2+}, the essential divalent metal ion cofactor which can be replaced by Mn^{2+} in vitro. We have carried out a mutational analysis of these presumptive Mg^{2+} ligands. The variants carrying an alanine or asparagine substitution bind DNA, but (with the exception of the D229N variant) are inactive in DNA cleavage in the presence of Mg^{2+}, demonstrating that these residues are important for cleavage. Our finding that the PI-*Sce*I variants carrying single cysteine substitutions at these positions are inactive in the presence of the oxophilic Mg^{2+} but active in the presence of the thiophilic Mn^{2+} suggests that the amino acid residues at these positions are involved in cofactor binding. From the fact that in the presence of Mn^{2+} the D218C and D326C variants are even more active than the wild-type enzyme, it is concluded that Asp218 and Asp326 are the principal Mg^{2+} ligands of PI-*Sce*I. On the basis of these findings and the available structural information, a model for the composition of the two Mg^{2+} binding sites of PI-*Sce*I is proposed.

The genes encoding homing endonucleases can be considered to be selfish elements, as the activity of their gene products results in self-propagation of these genes (*1*). Homing endonucleases are usually encoded by introns and inteins and occur in prokaryotes and eukaryotes as well as in archeae. By making a specific double-strand cut in a homologous intron- or inteinless allele, they initiate a recombination event which leads to the "homing" of their coding sequence (reviews in refs *2*−*5*).

Homing endonucleases can be grouped into four families, characterized by the sequence motifs LAGLIDADG, GIY-YIG, H-N-H, and the His-Cys box (reviews in refs *6*−*8*), the latter two belonging to the $\beta\beta\alpha$Me family of endonucleases (*9*). The LAGLIDADG enzymes constitute the largest family of homing endonucleases (*10*). Prominent members are the intein-encoded PI-*Sce*I (from *Saccharomyces cerevisiae*) and PI-*Pfu*I (from *Pyrococcus furiosus*) and the intron-encoded I-*Cre*I (from *Chlamydomonas reinhardtii*) and I-*Dmo*I (from *Desulfurococcus mobilis*), whose crystal structures are known and which have been studied biochemically in considerable detail. I-*Cre*I is a homodimer with one LAGLIDADG motif in each subunit (*11*), while PI-*Sce*I (*12*), I-*Dmo*I (*13*), and PI-*Pfu*I (*14*) are monomers which have a catalytic domain with two subdomains, each containing a LAGLIDADG motif, arranged in a pseudosymmetrical manner. For I-*Cre*I, a cocrystal structure has been determined (*15*) which shows that the two symmetry-related LAGLIDADG motifs are part of two catalytic centers responsible for cleavage of the two strands of the DNA. The penultimate Asp residue of the LAGLIDADG motif in I-*Cre*I is one of the ligands for the divalent metal ion cofactor (Mg^{2+} or Mn^{2+}, substituted by the inactive analogue Ca^{2+} in the cocrystal). This Asp residue is conserved in the monomeric relatives of the homodimeric I-*Cre*I, namely, PI-*Sce*I (Asp218 in one LAGLIDADG motif and Asp326 in the other), I-*Dmo*I (Asp21 and Glu117), and PI-*Pfu*I (Asp149 and Glu250). The catalytic importance of this acidic residue has been demonstrated by a mutational analysis of I-*Cre*I (*16*), PI-*Sce*I (*17*), and PI-*Pfu*I (*18*). The I-*Cre*I cocrystal structure shows that in each subunit Gln47 serves as a second ligand for the divalent metal ion cofactor. In PI-*Sce*I Asp229 and Thr341 are found in a similar position in the two subdomains, in I-*Dmo*I Gln42 and Gln129, and in PI-*Pfu*I Asp173 and Met263. Gln47 in I-*Cre*I (*16*), Asp229 and Thr341 in PI-*Sce*I (*19*, *20*), and Asp173 in PI-*Pfu*I (*18*) have been shown to be catalytically important by mutational analysis.

Although it is clear from the cocrystal structure of I-*Cre*I that Asp20 and Gln47 are Mg^{2+} binding ligands, it is only inferred by analogy to I-*Cre*I that the homologous amino acid residues have the same function for the other members of the LAGLIDADG family of homing endonucleases. We

[†] This work has been supported by grants from the Deutsche Forschungsgemeinschaft (Pi 122/13-2 and Pi 151/2-1). S.S. is a recipient of a scholarship of the Graduiertenkolleg "Biochemie von Nukleoproteinkomplexen".

[‡] This paper is dedicated to Dr. Andrea Parmeggiani on the occasion of his 70th birthday.

* To whom correspondence should be addressed: Institut für Biochemie, FB 08, Justus-Liebig-Universität, Heinrich-Buff-Ring 58, D-35392 Giessen, Germany. Telephone: +49 641 9935400. Fax: +49 641 9935409. E-mail: alfred.m.pingoud@chemie.bio.uni-giessen.de.

10.1021/bi001775n CCC: $19.00
Published on Web 11/29/2000

had shown previously that PI-*Sce*I, which is a monomeric enzyme, harbors two catalytic centers and thus functionally is similar to the homodimeric I-*Cre*I. The results of DNA cleavage and metal ion mapping experiments with PI-*Sce*I variants carrying amino acid substitutions in the presumptive active site allowed us to suggest that catalytic center I directed against the top strand consists of Asp218, Asp229, and Lys403, and that catalytic center II directed against the bottom strand consists of Asp326, Thr341, and Lys301. To demonstrate directly that Asp218, Asp229, Asp326, and Thr341 in the PI-*Sce*I homing endonuclease are the Mg^{2+} binding ligands in the two catalytic centers, as suggested in a previous work (*20*), we have now subjected these residues to a detailed mutational analysis and measured the DNA cleavage activity of the different variants in the presence of Mg^{2+} and Mn^{2+}. Our results support the notion that these residues are involved in Mg^{2+} binding. The finding that the D218C and D326C variants are completely inactive in the presence of the oxophilic Mg^{2+} and even more active than the wild-type enzyme in the presence of the thiophilic Mn^{2+} allows us to conclude that in PI-*Sce*I Asp218 and Asp326 are the principal ligands for the divalent metal ion cofactor, and most likely form inner sphere complexes with the Mg^{2+} ion.

EXPERIMENTAL PROCEDURES

Mutagenesis and Purification of PI-SceI. Site-directed mutagenesis of the PI-*Sce*I gene was performed by a PCR[1]-based technique essentially as described by Kirsch and Joly (*21*). The sequences of the mutated genes were confirmed by sequencing. Wild-type PI-*Sce*I and the PI-*Sce*I variants were purified to >95% homogeneity as described by Wende et al. (*22*).

DNA Cleavage Experiments. DNA cleavage experiments were carried out with 7 nM linearized or supercoiled plasmid DNA pBSVDEX (*22*) as the substrate. The reactions were performed in 10 mM Tris-HCl (pH 8.5) and 100 mM KCl containing 2.5 mM $MgCl_2$ or in 10 mM Tris-HCl (pH 7.5) and 100 mM KCl containing 2.5 mM $MnCl_2$. After defined time intervals, aliquots were withdrawn and the reaction was stopped with $^1/_5$ volume of 250 mM EDTA (pH 8.0), 25% sucrose, 1.2% SDS, 0.1% bromophenol blue, and 0.1% xylene cyanol. The substrate and products were separated by electrophoresis on 1.2% agarose gels in 80 mM Tris-phosphate (pH 8.2) and 2 mM EDTA. After being stained with ethidium bromide, the gels were analyzed using a gel documentation system (Intas) and the cleavage rates were determined using the program TINA (Raytest).

Electrophoretic Mobility Shift Experiments. For electrophoretic mobility shift experiments (*22*), a ^{32}P-labeled 311 bp DNA fragment (7 nM) derived from pBSVDEX and containing a single PI-*Sce*I site was incubated with increasing amounts of enzyme in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM $CaCl_2$, 1 mM DTT, 0.05% nonfat dry milk, 5% glycerol, and 0.1 μg/10 μL poly(dI-dC) (Pharmacia) for 30 min at room temperature. After incubation, 3 μL of 10% Ficoll, 15% glycerol, 0.2% bromphenol blue, and 0.2% xylene cyanol was added to the 15 μL reaction mixture and electrophoresis was carried out on 6% polyacrylamide gels in 50 mM Tris-borate (pH 8.3) and 2 mM Ca^{2+}. The gels were analyzed using an Instant Imager (Canberra Packard).

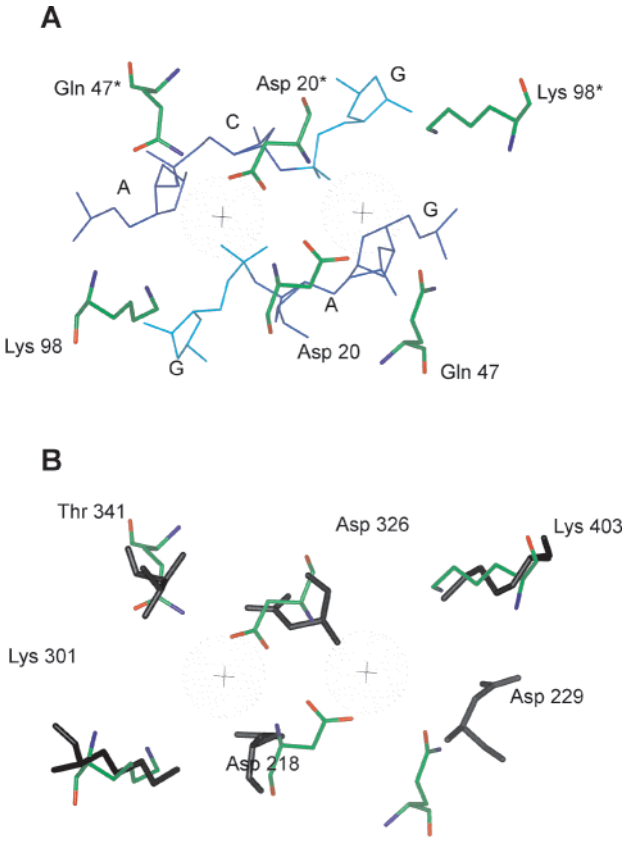

FIGURE 1: Comparison of the catalytic centers of the homing endonucleases PI-*Sce*I (PDB entry 1VDE) and I-*Cre*I (PDB entry 1BP7). Shown in panel A is a detail of the I-*Cre*I−DNA complex structure. In the active site of I-*Cre*I, the two Ca^{2+} ions are complexed by Asp20 (homologous to Asp218 and Asp326 in PI-*Sce*I) and Gln47 (homologous to Asp229 and Thr341 in PI-*Sce*I). Lys98 (homologous to Lys301 and Lys403 in PI-*Sce*I) is interacting with the labile phosphate. Only the sugar−phosphate backbone at the site of cleavage is depicted, the 5′-part darkly colored and the 3′-part lightly colored. Shown in panel B is the superposition of the active site amino acid residues of I-*Cre*I with the corresponding residues of PI-*Sce*I which was carried out with the backbone atoms of the amino acid residues displayed. The amino acid residues of PI-*Sce*I are shown in black, and the corresponding amino acid residues of I-*Cre*I (Asp20, Gln47, and Lys98) are shown colored by atom type.

Photo-Cross-Linking Experiments. Photo-cross-linking experiments were carried out with 20 μM wild-type PI-*Sce*I, 20 μM D218C or D326C variant, and 20 μM double-stranded oligodeoxynucleotide comprising the PI-*Sce*I recognition site and substituted with iododeoxyuridine at position 9 in the bottom strand (*23*). The samples were irradiated for 90 min with a 40 mW HeCd laser (λ = 325 nm). Following irradiation, aliquots of the reaction mixtures were analyzed by SDS−PAGE. The gels were silver stained and subjected to autoradiography.

RESULTS

Identification of Presumptive Mg^{2+} Binding Ligands of PI-SceI by Structure Comparisons. Figure 1 shows a superposi-

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; 5-IdU, 5-iododeoxyuridine; lc, lower complex; lin, linear; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PDB, Protein Data Bank; sc, supercoiled; SDS, sodium dodecyl sulfate; uc, upper complex.

Table 1: Catalytic Centers of LAGLIDADG Homing Endonucleases[a]

I-*Cre*I		PI-*Sce*I		I-*Dmo*I		PI-*Pfu*I	
I	II	I(B)[b]	II(A)[b]	I(A)[b]	II(B)[b]	I	II
Asp20	Asp20	Asp218	Asp326	Asp21	Glu117	Asp149	Glu250
Gln47	Gln47	Asp229	Thr341	Asp42	Gln129	Asp173	?
Lys98	Lys98	Lys403	Lys301	?	Lys120	Lys322	?

[a] Based on the cocrystal and crystal structures of I-*Cre*I (*15*), PI-*Sce*I (*12*), I-*Dmo*I (*13*), and PI-*Pfu*I (*14*). [b] It was pointed out by Silva et al. (*13*) that the catalytic centers I and II of PI-*Sce*I are homologous to catalytic centers II and I, respectively, of I-*Dmo*I.

tion of the active sites of the homodimeric I-*Cre*I [from the structure of the enzyme−DNA complex (*15*) determined in the presence of Ca^{2+}, an inactive analogue of the essential cofactor Mg^{2+}] and the monomeric PI-*Sce*I [from the structure of the free enzyme (*12*)]. The two catalytic centers of I-*Cre*I, one in each subunit, are composed of Asp20 and Gln47, which are ligands for the divalent cation, and Lys98 which could serve to stabilize the extra negative charge at the phosphate attacked in the transition state. In PI-*Sce*I, Asp218, Asp229, and Lys403 in one catalytic center and Asp326, Thr341, and Lys301 in the other are found in an arrangement similar to that of the corresponding amino acid residues in I-*Cre*I. As a matter of fact, if one also considers the structures of the two other monomeric homing endonucleases of the LAGLIDADG family (Table 1), whose structures are known, namely, I-*Dmo*I (*13*) and PI-*Pfu*I (*14*), it becomes clear that in each catalytic center, the presumptive Mg^{2+} binding site is formed by an acidic and a polar amino acid residue, and in addition that a lysine residue is present in most cases. This comparison emphasizes that in PI-*Sce*I Asp218 and Asp229 as well as Asp326 and Thr341 are good candidates to be the ligands for Mg^{2+} on the protein side in the enzyme−substrate complex. To demonstrate this, we have carried out a mutational analysis at these positions with a particular emphasis on a comparison between Mg^{2+}- and Mn^{2+}-supported DNA cleavage by the cysteine variants that were produced. The rationale of this comparison is that the thiophilic Mn^{2+} should be able to "rescue" a cysteine variant which is inactive in the presence of the oxophilic Mg^{2+}, because of the fact that sulfur is a good ligand for Mn^{2+} but not Mg^{2+} (*24*). Indeed, using this comparison, Mg^{2+} ligands were identified in ribozymes (*25*, *26*) and more recently in enzymes with nucleolytic activity, viz., Tn7 transposase (*27*), Tn10 transposase (*28*), and RAG1 and RAG2 (*29*), which are involved in V(D)J recombination.

DNA Binding Experiments with PI-SceI Variants. The PI-*Sce*I variants with alanine, asparagine, and cysteine substitutions at positions 218, 229, 326, and 341 were produced, purified to homogeneity, and characterized with respect to DNA binding and DNA cleavage activity. Gel shift experiments (representative examples are shown in Figure 2) demonstrated that all variants are able to bind a 311 bp PCR fragment with a central PI-*Sce*I recognition site with the same or almost the same affinity as wild-type PI-*Sce*I, with the exception of the T341A, -N, and -C variants which display an approximately 10-fold lower affinity than the other variants. It is noteworthy that all variants such as the wild-type enzyme form the so-called upper and lower complex, in which the DNA bend angles differ (*22*, *30*). It is thought that the upper complex, which has a lower electrophoretic

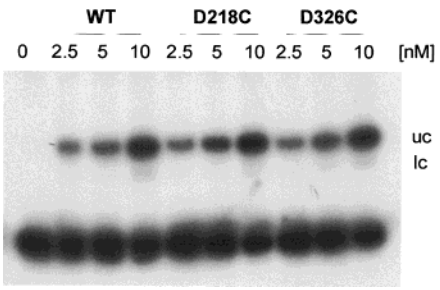

FIGURE 2: DNA binding of wild-type PI-*Sce*I and the PI-*Sce*I variants D218C and D326C. The analysis was carried out with a gel shift assay with a 311 bp DNA fragment containing a single PI-*Sce*I binding site. Aliquots of 7 nM substrate were incubated with increasing amounts of enzyme. Complex formation was analyzed on a 6% polyacrylamide gel. The positions of the upper complex (uc) and lower complex (lc) are indicated on the right.

mobility than the lower complex, is the productive enzyme−substrate complex (*22*, *30*), as variants which only form the lower complex are inactive. The results of the gel shift experiments demonstrate that the catalytic inactivity is not due to a drastic decrease in affinity or to an inability to form the upper complex.

Photo-Cross-Linking Experiments with Selected PI-SceI Variants. To obtain further support for the assumption that the variants investigated here are able to bind their substrate in the same manner as the wild-type enzyme, a prerequisite for our analysis, we have performed photo-cross-linking experiments with the D218C and D326C variants. It had been shown before that wild-type PI-*Sce*I can be cross-linked in good yield with an oligodeoxynucleotide carrying a thymidine to iododeoxyuridine substitution at position 9 in the bottom strand of the PI-*Sce*I recognition sequence (*31*) and that the cross-linked complex is enzymatically active (*20*). Figure 3 shows that D218C and D326C can be cross-linked with the same yield as the wild-type enzyme, making it very likely that the aspartic acid to cysteine exchange has not affected the ability of these two variants to form an enzyme−substrate complex with a similar if not the same conformation as the wild-type enzyme.

DNA Cleavage Activity of the PI-SceI Variants in the Presence of Mg^{2+} and Mn^{2+}. The DNA cleavage activity of the different variants was determined with a plasmid DNA with a single PI-*Sce*I recognition site in the presence of Mg^{2+} and Mn^{2+}. Figure 4 shows the kinetics of cleavage of linearized pBSVDEX by wild-type PI-*Sce*I and the D218C and D326C variants. Table 2 summarizes the results obtained for the cleavage of the linearized as well as the supercoiled plasmid DNA by the different variants. With the exception of the D229N, all variants are inactive in the presence of Mg^{2+}, even when a substrate under torsional stress (supercoiled plasmid DNA) is used (data not shown), which in some cases leads to cleavage of substrates that are not cleaved in a relaxed state (linear plasmid DNA) (*32*). In the presence of Mn^{2+}, differential effects are observed for the different variants: the D218A and -N, D229A, and D326A and -N variants are also inactive in the presence of Mn^{2+}. The D218C, D229C, and D326C as well as the T341A, -N, and -C variants, in contrast, are activated by Mn^{2+}. For two variants, D218C and D326C, the effect of Mn^{2+} is dramatic, as they are activated to levels exceeding wild-type activity levels (Figure 4). For these two variants, we have shown that their Mn^{2+}-dependent cleavage activity can be inhibited

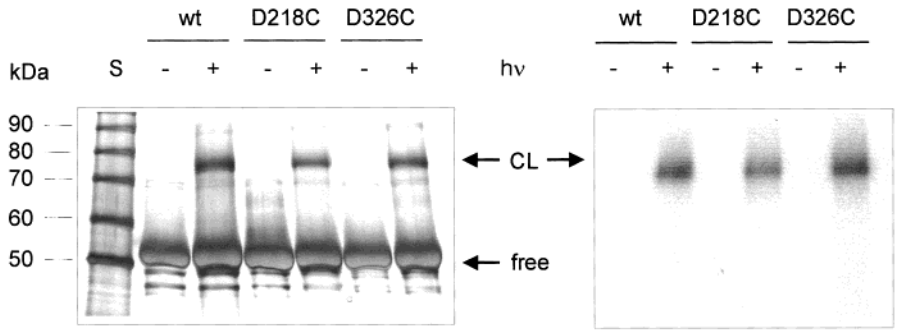

FIGURE 3: SDS−PAGE analysis of the photo-cross-linking reactions of wild-type PI-*Sce*I and the variants D218C and D326C with a double-stranded oligodeoxynucleotide substituted with 5-IdU at position T+9 in the bottom strand. Shown on the left is the silver-stained gel and on the right the autoradiogram. − and + denote the sample before and after irradiation. The positions of cross-linked PI-*Sce*I (CL) and un-cross-linked PI-*Sce*I (free) are indicated. S represents the molecular mass standard. The identity of the upper band as representing the cross-linked complex was established by demonstrating that this band is shifted to a lower position when the reaction mixture is incubated with Mn^{2+} after irradiation as described by Christ et al. (*20*).

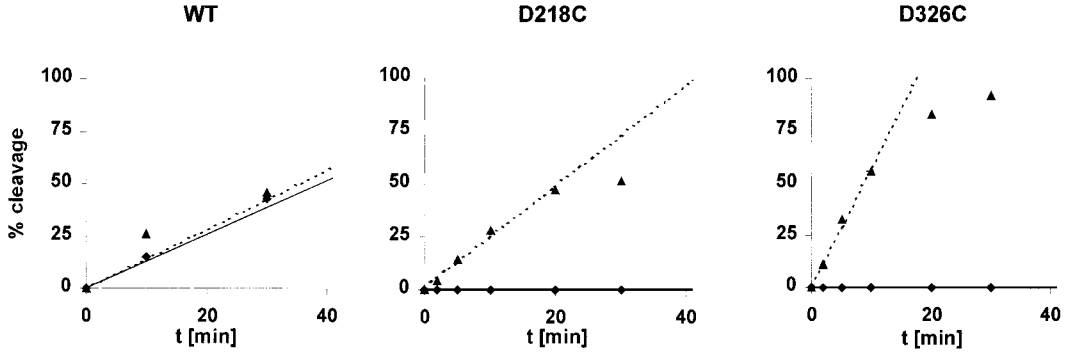

FIGURE 4: Cleavage activity of wild-type PI-*Sce*I and the PI-*Sce*I variants D218C and D326C. Cleavage reactions were performed using 7 nM linearized plasmid DNA and 100 nM enzyme in the presence of $MgCl_2$ (◆) or $MnCl_2$ (▲). The product mixture was analyzed on a 1.2% agarose gel. Curves representing initial rates are given for $MgCl_2$ (—) and $MnCl_2$ (···).

Table 2: DNA Cleavage Activity of PI-*Sce*I Variants with Amino Acid Substitutions in Catalytic Centers I and II

	relative cleavage activity			
	lin substrate		sc substrate	
variant	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
wild type	1[a]	1[b]	1[c]	1[d]
D218A	inactive	inactive	inactive	inactive
D218N	inactive	inactive	inactive	inactive
D218C	inactive	1.6	inactive	1.3
D229A	inactive	inactive	inactive	inactive
D229N	0.4	0.5	0.4	0.5
D229C	inactive	0.2	inactive	0.3
D326A	inactive	inactive	inactive	inactive
D326N	inactive	inactive	inactive	inactive
D326C	inactive	3.7	inactive	5.2
T341A	inactive	0.6	inactive	0.7
T341N	inactive	0.8	inactive	0.9
T341C	inactive	0.6	inactive	0.8

[a] $k = (0.7 \pm 0.08) \times 10^{-3}$ min^{-1}. [b] $k = (0.8 \pm 0.02) \times 10^{-3}$ min^{-1}. [c] $k = (0.7 \pm 0.10) \times 10^{-3}$ min^{-1}. [d] $k = (0.7 \pm 0.07) \times 10^{-3}$ min^{-1}.

by Ca^{2+} and even better by Zn^{2+}, two divalent cations that also inhibit the Mg^{2+}- or Mn^{2+}-dependent cleavage activity of the wild-type enzyme (data not shown). In contrast to that, Mg^{2+} in a 10-fold excess over Mn^{2+} does not inhibit DNA cleavage by D218C and D326C (data not shown), demonstrating that these variants do not bind Mg^{2+} with measurable affinity at the presumptive metal ion cofactor binding sites. It must be emphasized that we have not seen preferential nicking with any variant under any conditions described here, in agreement with results obtained previously by Gimble's group and our group for wild-type PI-*Sce*I (*22*, *33*). This is evidence for a tight coupling of the two catalytic centers: if one is knocked out, the other is also impaired.

DISCUSSION

Most enzymes that catalyze phosphoryl transfer reactions, like nucleases, require divalent metal ion cofactors (*34*). For restriction endonucleases and homing endonucleases, magnesium is the metal ion of choice. Its precise role in catalysis of phosphodiester bond cleavage, however, is not clear. It is not even clear whether related enzymes, for example, the members of the PD...D/EXK family of restriction enzymes, follow the same catalytic mechanism and require one or two Mg^{2+} ions for phosphodiester bond cleavage (*35*, *36*). In principle, the same uncertainty exists among the LAGLIDADG family of homing endonucleases. The crystal structures of four different members of this family are known. They show that the catalytic centers are similar but not identical (cf. Table 1). For only one homing endonuclease, I-*Cre*I, has a cocrystal structure with the DNA substrate in the presence of Ca^{2+} ions (which do not support cleavage) been determined, which allows us to suggest also for related enzymes which amino acid residues might be involved in Mg^{2+} binding. Such a suggestion, however, must be tested experimentally, in particular as I-*Cre*I is a homodimeric enzyme which recognizes and cleaves an almost symmetric sequence, whereas PI-*Sce*I is a monomeric enzyme with two active sites which recognize a largely asymmetric sequence whose top and bottom strands are cleaved by two different catalytic centers. On the basis of the comparison of the

catalytic centers of I-*Cre*I and PI-*Sce*I (Figure 1), we have identified four amino acid residues, two for each catalytic center of PI-*Sce*I, as candidate ligands for Mg^{2+}: Asp218 and Asp229 in catalytic center I and Asp326 and Thr341 in catalytic center II. These residues were exchanged for alanine, asparagine, and cysteine, with no dramatic effect on DNA binding and bending by the resulting variants. The D218A and -N as well as the D326A and -N variants had been produced previously by Gimble and Stephens (*30*) and shown to be inactive in DNA cleavage, in the presence of both Mg^{2+} and Mn^{2+}; they were included in our mutational analysis only for the sake of comparison. Our results show that not only Asp218 and Asp326 but also Asp229 and Thr341 are required for activity, as their substitution with alanine and in the case of Thr341 also with asparagine leads to binding competent but catalytically inactive enzymes with Mg^{2+} as a cofactor. The substitution of Asp229 with asparagine, in contrast, is well tolerated. This result, as well as the finding that the T341A and -N variants are active in the presence of Mn^{2+}, suggests that Asp229 and Thr341 are not as essential as Asp218 and Asp326, which can only be replaced with glutamic acid, however, with a great reduction in catalytic activity (*30*). All these results demonstrate that Asp218, Asp229, Asp326, and Thr341 are more or less essential for the catalytic activity of PI-*Sce*I, but do not allow us yet to conclude that they are involved in Mg^{2+} binding. This conclusion is only justified for Asp218 and Asp326 when considering our finding that Mn^{2+} can rescue the activity of the D218C and D326C variants, not, however, of D218A and -N or D326A and -N. For Asp229 and Thr341, this conclusion cannot be drawn with the same confidence, because at these positions the D229A and -N and T341A and -N variants are also activated by Mn^{2+}, albeit not to such an extent. The difference in behavior of the D218 and D326 variants on one side and the D229 and T341 variants on the other side may be due to the fact that Asp218 and Asp326 are inner sphere ligands for Mg^{2+} (the Cys−Mn^{2+} interaction in the D218C and D326C variants argues for that) whereas Asp229 and Thr341 are outer sphere ligands for Mg^{2+}.

Nevertheless, the identification of Asp218 (catalytic center I) and Asp326 (catalytic center II) as the principal Mg^{2+} binding ligands makes it likely that the structurally adjacent residues, Asp229 (I) and Thr341 (II), also participate in Mg^{2+} binding. The binding sites presumably are completed by a nonbridging oxygen of the phosphate to be attacked in each strand, by the backbone carbonyl of Gly325 (I) and Ser217 (II), and, most importantly, by a water molecule which may be the attacking nucleophile in phosphodiester bond cleavage. The speculative assignment of the main chain carbonyl at positions 325 and 217 as ligands of the Mg^{2+} ion is suggested by the superposition of the active sites of I-*Cre*I and PI-*Sce*I (Figure 1) which puts the main chain carbonyls within approximately 2 Å of the divalent metal ions in catalytic centers I and II, respectively. Main chain carbonyls are not unusual as ligands for divalent metal ions, recent examples being provided by the enzyme−substrate complexes of the restriction endonucleases *Bam*HI (*37*), *Bgl*I (*38*), *Eco*RI (*39*), *Eco*RV (*40*), *Bgl*II (*41*), and *Pvu*II (*42*) and the enzyme−product complex of *Eco*RV (*43*) and *Ngo*MIV (*44*). It is interesting to note that in PI-*Sce*I the strict coupling of the two catalytic centers which is responsible for the concerted cleavage of the two strands of the DNA (*22*) could be mediated by the adjacent amino acid residues serving as ligands for the Mg^{2+} ion in the two catalytic centers: Gly325 for catalytic center I and Asp326 for catalytic center II. A similar arrangement is seen in the I-*Cre*I−DNA structure (*7*), and has also been observed for other endonucleases, e.g., T4 endonuclease VII with a domain-swapped dimer architecture (*45*).

We believe that the proximity of the two catalytic centers in PI-*Sce*I allows for an intricate hydrogen-bonding network between all catalytic residues, which on the one side guarantees that the two phosphodiester bond cleavage events are strictly coupled, but which on the other side is easily disturbed by single amino acid substitutions in one catalytic center or the other. This would explain that all efforts to uncouple the two cleavage events, either by amino acid substitutions (*17*, *46*) or by changing reaction conditions, viz., choice of metal ion, metal ion concentration, ionic strength, pH, or addition of organic solvents (F. Christ, personal communication), were unsuccessful so far.

There is another important conclusion to be drawn from our experiments: although an aspartic acid residue neither is isosteric nor carries the same charge as a cysteine residue, the D218C and D326C variants of PI-*Sce*I in the presence of Mn^{2+} are more efficient catalysts for phosphodiester bond cleavage than the wild-type enzyme. This argues for a great plasticity of the two catalytic centers of PI-*Sce*I. An essentially similar conclusion could be drawn for RNase H, which by substitution of the two acidic amino acid residues in the active site by arginine residues became a Mg^{2+}-independent enzyme with wild-type activity (*47*). RNase H, however, is a nonspecific enzyme, in contrast to PI-*Sce*I which is one of the most specific enzymes known.

ACKNOWLEDGMENT

We thank Dr. Frauke Christ for drawing our attention to the Mn^{2+} rescue experiments with RAG1 and RAG2 and Dr. Xiadong Cheng, Dr. Kosuke Morikawa, Dr. John Rosenberg, Dr. Virginius Siksnys, and Dr. Barry Stoddard for sending us manuscripts prior to publication.

REFERENCES

1. Goddard, M. R., and Burt, A. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 13880−13885.
2. Dujon, B. (1989) *Gene 82*, 91−114.
3. Lambowitz, A. M., and Belfort, M. (1993) *Annu. Rev. Biochem. 62*, 587−622.
4. Belfort, M., and Perlman, P. S. (1995) *J. Biol. Chem. 270*, 30237−30240.
5. Gimble, F. S. (2000) *FEMS Microbiol. Lett. 185*, 99−107.
6. Belfort, M., and Roberts, R. J. (1997) *Nucleic Acids Res. 25*, 3379−3388.
7. Jurica, M. S., and Stoddard, B. L. (1999) *Cell. Mol. Life Sci. 55*, 1304−1326.
8. Aggarwal, A. K., and Wah, D. A. (1998) *Curr. Opin. Struct. Biol. 8*, 19−25.
9. Kuhlmann, U. C., Moore, G. R., James, R., Kleanthous, C., and Hemmings, A. M. (1999) *FEBS Lett. 463*, 1−2.
10. Dalgaard, J. Z., Klar, A. J., Moser, M. J., Holley, W. R., Chatterjee, A., and Mian, I. S. (1997) *Nucleic Acids Res. 25*, 4626−4638.
11. Heath, P. J., Stephens, K. M., Monnat, R. J., and Stoddard, B. L. (1997) *Nat. Struct. Biol. 4*, 468−476.
12. Duan, X., Gimble, F. S., and Quiocho, F. A. (1997) *Cell 89*, 555−564.

13. Silva, G. H., Dalgaard, J. Z., Belfort, M., and Van Roey, P. (1999) *J. Mol. Biol. 286*, 1123−1136.
14. Ichiyanagi, K., Ishino, Y., Ariyoshi, M., Komori, K., and Morikawa, K. (2000) *J. Mol. Biol. 300*, 889−901.
15. Jurica, M. S., Monnat, R. J., Jr., and Stoddard, B. L. (1998) *Mol. Cell 2*, 469−476.
16. Seligman, L. M., Stephens, K. M., Savage, J. H., and Monnat, R. J. (1997) *Genetics 147*, 1653−1664.
17. Gimble, F. S., and Stephens, B. W. (1995) *J. Biol. Chem. 270*, 5849−5856.
18. Komori, K., Ichiyanagi, K., Morikawa, K., and Ishino, Y. (1999) *Nucleic Acids Res. 27*, 4175−4182.
19. He, Z., Crist, M., Yen, H., Duan, X., Quiocho, F. A., and Gimble, F. S. (1998) *J. Biol. Chem. 273*, 4607−4615.
20. Christ, F., Schoettler, S., Wende, W., Steuer, S., Pingoud, A., and Pingoud, V. (1999) *EMBO J. 18*, 6908−6916.
21. Kirsch, R. D., and Joly, E. (1998) *Nucleic Acids Res. 26*, 1848−1850.
22. Wende, W., Grindl, W., Christ, F., Pingoud, A., and Pingoud, V. (1996) *Nucleic Acids Res. 24*, 4123−4132.
23. Christ, F., Steuer, S., Thole, H., Wende, W., Pingoud, A., and Pingoud, V. (2000) *J. Mol. Biol. 300*, 841−849.
24. Pecoraro, V. L., Hermes, J. D., and Cleland, W. W. (1984) *Biochemistry 23*, 5262−5271.
25. Dahm, S. C., and Uhlenbeck, O. C. (1991) *Biochemistry 30*, 9464−9469.
26. Piccirilli, J. A., Vyle, J. S., Caruthers, M. H., and Cech, T. R. (1993) *Nature 361*, 85−88.
27. Sarnovsky, R. J., May, E. W., and Craig, N. L. (1996) *EMBO J. 15*, 6348−6361.
28. Allingham, J. S., Pribil, P. A., and Haniford, D. B. (1999) *J. Mol. Biol. 289*, 1195−1206.
29. Landree, M. A., Wibbenmeyer, J. A., and Roth, D. B. (1999) *Genes Dev. 13*, 3059−3069.
30. Gimble, F. S., and Wang, J. (1996) *J. Mol. Biol. 263*, 163−180.
31. Pingoud, V., Thole, H., Christ, F., Grindl, W., Wende, W., and Pingoud, A. (1999) *J. Biol. Chem. 274*, 10235−10243.
32. Wende, W., Schoettler, S., Grindl, W., Christ, F., Steuer, S., Noel, A. J., Pingoud, V., and Pingoud, A. (2000) *Mol. Biol. (Moscow)* (in press).
33. Gimble, F. S., and Thorner, J. (1993) *J. Biol. Chem. 268*, 21844−21853.
34. Cowan, J. A. (1998) *Chem. Rev. 98*, 1067−1087.
35. Pingoud, A., and Jeltsch, A. (1997) *Eur. J. Biochem. 246*, 1−22.
36. Galburt, E. A., and Stoddard, B. L. (2000) *Nat. Struct. Biol. 7*, 89−91.
37. Newman, M., Strzelecka, T., Dorner, L. F., Schildkraut, I., and Aggarwal, A. K. (1995) *Science 269*, 656−663.
38. Newman, M., Lunnen, K., Wilson, G., Greci, J., Schildkraut, I., and Phillips, S. E. V. (1998) *EMBO J. 17*, 5466−5476.
39. McClarin, J. A., Frederick, C. A., Wang, B. C., Greene, P., Boyer, H. W., Grable, J., and Rosenberg, J. M. (1986) *Science 234*, 1526−1541.
40. Horton, N. C., Newberry, K. J., and Perona, J. J. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 13489−13494.
41. Lukacs, C. M., Kucera, R., Schildkraut, I., and Aggarwal, A. K. (2000) *Nat. Struct. Biol. 7*, 134−140.
42. Horton, J. R., and Cheng, X. (2000) *J. Mol. Biol. 300*, 1051−1058.
43. Kostrewa, D., and Winkler, F. K. (1995) *Biochemistry 34*, 683−696.
44. Deibert, M., Grazulis, S., Sasnauskas, G., Siksnys, V., and Huber, R. (2000) *Nat. Struct. Biol. 7*, 792−799.
45. Raaijmakers, H., Vix, O., Toro, I., Golz, S., Kemper, B., and Suck, D. (1999) *EMBO J. 18*, 1447−1458.
46. Gimble, F. S., Duan, X., Hu, D., and Quiocho, F. A. (1998) *J. Biol. Chem. 273*, 30524−30529.
47. Casareno, R. L. B., Li, D. W., and Cowan, J. A. (1995) *J. Am. Chem. Soc. 117*, 11011−11012.

BI001775N