The monomeric homing endonuclease PI-SceI has two catalytic centres for cleavage of the two strands of its DNA substrate

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The monomeric homing endonuclease PI-SceI cleaves the two strands of its DNA substrate in a concerted manner, which raises the question of whether this enzyme harbours one or two catalytic centres. If PI-SceI has only one catalytic centre, one would expect that cross-linking enzyme and substrate should prevent reorientation of the enzyme required to perform the second cut after having made the first cut: PI-SceI, however, when cross-linked to its substrate, is able to cleave both DNA strands. If PI-SceI has two catalytic centres, one would expect that it should be possible to inactivate one catalytic centre by mutation and obtain a variant with preference for a substrate nicked in one strand; such variants have been found. The structural homology between the catalytic domain of PI-SceI having a pseudo 2-fold symmetry, and I-CreI, a homodimeric homing endonuclease, suggests that in PI-SceI active site I, which attacks the top strand, comprises Asp218, Asp229 and Lys403, while Asp326, Thr341 and Lys301 make up active site II, which cleaves the bottom strand. Cleavage experiments with modified oligodeoxynucleotides and metal ion mapping experiments demonstrate that PI-SceI interacts differently with the two strands at the cleavage position, supporting a model of two catalytic centres.

Keywords: intein/metal ion footprinting/phosphorothioates/photocross-linking

Introduction

Homing endonucleases are enzymes which catalyse a highly specific double strand cleavage within an extended recognition sequence and thereby induce a double strand break repair that leads to insertion of their gene into an allele which lacks it (Lambowitz and Belfort, 1993; Mueller *et al.*, 1993). Homing endonucleases are encoded in introns or as protein introns (inteins) of prokaryotes, eukaryotes and archaea (reviewed in Dujon, 1989; Belfort and Roberts, 1997). The intein-encoded homing endonucleases combine two catalytic functions in one molecule, endonucleolytic activity and protein splicing activity (Cooper and Stevens, 1995; Shao *et al.*, 1995), with which they liberate themselves from a precursor protein, leading to the generation of two functional and independent proteins (Gimble, 1998; Perler, 1998).

The heterogeneous family of homing endonucleases can be divided into four sub-families which are characterized by one of the following sequence motifs: LAGLIDADG, GIY-YIG, H-N-H or the His-Cys box (Belfort and Perlman, 1995; Belfort and Roberts, 1997). The LAGLIDADG sequence is the most common motif and is not only found in homing endonucleases but also in other proteins interacting with nucleic acids (Dalgaard *et al.*, 1997), e.g. HO-endonuclease (Russell *et al.*, 1986). The intein-encoded PI-SceI from *Saccharomyces cerevisiae* (Gimble and Thorner, 1992, 1993) belongs to this subfamily of homing endonucleases and contains two copies of the LAGLIDADG motif. PI-SceI interacts with DNA as a monomer and recognizes an extraordinarily long DNA sequence with no apparent symmetry. Depending on the substrate, the minimal sequence that is recognized exceeds 35 bp. Whereas binding to the recognition site, which is accompanied by a distortion of the DNA, occurs also in the absence of the cofactor Mg²⁺, specific cleavage requires the presence of this bivalent metal ion. PI-SceI cleaves the two DNA strands in a highly concerted reaction, with no detectable accumulation of a nicked intermediate, to produce specific DNA fragments with 3'-OH and 5'-phosphate ends. Replacement of Mg²⁺ by Mn²⁺ leads to a relaxed specificity and increased rate of reaction, which is nevertheless relatively slow (Gimble and Wang, 1996; Wende *et al.*, 1996; Grindl *et al.*, 1998). In contrast to PI-SceI (Duan *et al.*, 1997) and I-DmoI (Silva *et al.*, 1999), some other members of the LAGLIDADG family are homodimers, e.g. I-CreI (Heath *et al.*, 1997), and recognize pseudo-palindromic sequences. These homodimeric homing endonucleases contain one LAGLIDADG motif per subunit, each of which is involved in phosphodiester bond cleavage in one DNA strand. For the monomeric homing endonuclease PI-SceI, it is still a controversial issue as to whether it has one (Gimble and Stephens, 1995; Duan *et al.*, 1997) or two active sites (Wende *et al.*, 1996; Pingoud *et al.*, 1998). Substitution of Asp218 or Asp326 in PI-SceI leads to complete inactivation of the nucleolytic function, a result which has been interpreted to mean that these residues together make up one single catalytic centre responsible for cleaving both strands of the DNA substrate (Gimble and Stephens, 1995), a feature without precedence among site-specific nucleases. On the other hand, the fact that the two strands are cleaved in a concerted manner argues for the existence of two catalytic centres in the monomeric enzyme (Wende *et al.*, 1996). Support for this conjecture comes from structural comparisons. There is a significant structural similarity between I-CreI, which is a homodimer, and PI-SceI as well as I-DmoI, both of which are monomeric enzymes and have a catalytic domain with a pseudo 2-fold symmetry axis (Silva *et al.*, 1999).

Here we present evidence that PI-SceI has two catalytic

Fig. 1. Cleavage of a radioactively labelled oligodeoxynucleotide substrate by PI-SceI tethered to its substrate as indicated at the bottom of the figure. PI-SceI was covalently linked via His333 to thymine +9 in the bottom strand by photocross-linking. The DNA in the PI-SceI-DNA complex was cleaved by incubation in the presence of Mn^{2+} . The products of irradiation and cleavage were analysed by SDS-PAGE. (A) Silver-stained gel: lane S, molecular weight standard; lanes 1 and 2, PI-SceI-DNA complex before and after irradiation; lane 3, cross-linked PI-SceI-DNA complex after incubation with Mn^{2+} . (B) Autoradiogram of lanes 1, 2 and 3.

centres which are responsible for the cleavage of the top and the bottom strand, respectively, of the double-stranded DNA substrate. This evidence rests on three observations, namely: (i) that PI-SceI tethered to one strand of the DNA is able to cleave both strands; (ii) that active site variants of PI-SceI exist that cleave one strand of the DNA substrate, but not, or hardly at all, the other one; and (iii) that the transition states for cleavage of the two strands are very different.

Results

In the following section, we present the results of different types of experiments designed to find out whether PI-SceI has one or two active sites to cleave the two strands of its DNA substrate, and, if two sites are present, to characterize these sites and to assign the cleavage events at the top and bottom strands to one or the other active site.

Cleavage experiments with PI-SceI tethered to one strand of its substrate by a photocross-link

If PI-SceI has only one catalytic centre to cleave the two strands of its double-stranded DNA substrate, this would mean that after cleavage of one strand the catalytic centre must undergo a major conformational transition in order to be able to attack the other strand. For an in-line attack on the phosphodiester bond, as observed for EcoRI, EcoRV, HpaII and SfiI (Connolly *et al.*, 1984; Grasby and Connolly, 1992; Mizuuchi *et al.*, 1999), this would literally mean that the enzyme has to 'turn around' its active site between the two cleavage events. To find out whether such major movements are part of the mechanism of double strand cleavage, we have tethered PI-SceI via His333 to thymine +9 in the bottom strand by photocross-linking (Pingoud *et al.*, 1999) and analysed whether such a tethered complex is enzymatically active. As shown in Figure 1, the cross-linked PI-SceI complex is able to cleave its DNA substrate. That cleavage occurs in both

strands was established by an independent experiment, in which PI-SceI was first incubated with the DNA in the presence of Mn^{2+} overnight in order to obtain cleavage, and then irradiated to cross-link the product to the enzyme. Cleavage followed by irradiation (which cross-links the downstream product to the protein) resulted in the same cross-linked product as irradiation (which cross-links the substrate to the protein) followed by cleavage (which releases the upstream product; data not shown). Although the kinetics of cleavage has not been analysed in detail for the tethered complex, the rate of cleavage must be similar for the free and the tethered enzyme as complete cleavage is observed by incubation overnight at room temperature in the presence of Mn^{2+} . As His333 is located in the catalytic domain of PI-SceI (Duan, *et al.*, 1997), this result demonstrates that the relative positions of the catalytic domain and the substrate remain largely unaltered between the two cleavage events.

Cleavage experiments with PI-SceI and substrates carrying a single strand break in the scissile phosphodiester bond in the top or bottom strand

If PI-SceI has two active sites for cleavage of the two strands of its double-stranded DNA substrate, it should, in principle, be possible to inactivate one catalytic centre and preserve the activity of the other. Such a PI-SceI variant, produced by site-directed mutagenesis of a presumptive catalytic residue, should be able to introduce a specific nick into one strand of a double-stranded DNA substrate and/or to cleave a DNA substrate with a specific nick in the other strand. Ideally, two sets of variants should be produced, with substitutions in one or the other catalytic centre. Obvious candidates for such substitutions are the Asp residues of the two LAGLIDAG motifs, which have been shown to be essential for catalysis in PI-SceI and related enzymes (Gimble and Stephens, 1995; Lykke-Andersen *et al.*, 1996; Seligman *et al.*, 1997). However, it had been shown previously that the replacement of Asp218 and Asp326 by Ala and Asn led to completely inactive enzyme variants concerning double-stranded DNA substrates (Gimble and Stephens, 1995; S.Schoettler, unpublished).

In order to analyse the influence of single strand breaks in the PI-SceI cleavage positions of the top and bottom strand of the DNA substrate, cleavage experiments with fully double-stranded and nicked substrates were carried out. These experiments were performed with PI-SceI and variants carrying amino acid substitutions at catalytically important positions, other than Asp218 and Asp326. We selected Asp229, Thr341 and Lys403 because they are located close to Asp218 and Asp326 but, in contrast to these residues, do not lead to completely inactive PI-SceI variants when substituted by Asn (S.Schoettler, unpublished) or Ala (Gimble *et al.*, 1998).

As shown in Table I, a substrate with a nick at the cleavage position in the top strand is cleaved faster by wild-type PI-SceI than the intact double-stranded oligodeoxynucleotide or an oligodeoxynucleotide with a nick in the bottom strand. These data may indicate that the rate-limiting step for double strand cleavage by wild-type PI-SceI is the cleavage of the top strand, which means that the bottom strand can be cleaved more readily when the top strand is already nicked. A different result is

Table I. Cleavage of nicked substrates by PI-SceI and PI-SceI variants

	k^{app} (per h) ^a			
	Double-stranded ^b	Nicked in bottom strand ^b	Double-stranded ^c	Nicked in top strand ^c
Wild-type	2.6 ± 0.03	2.7 ± 0.45	3.0 ± 0.12	9.9 ± 0.3
D326A	–	–	–	–
K301A	–	–	–	–
T341N	+	++	+	(+)
D218A	–	–	–	–
D229N	+	(+)	+	++
K403A	+	–	+	+

Cleavage experiments with PI-SceI variants were only evaluated in a semi-quantitative manner by measuring product concentration at single time points, taken after 30 min or 2 h, respectively: ++, wild-type activity; +, reduced activity; (+), minimal activity; –, no activity.

^aApparent first order rate constant for cleavage of double-stranded and nicked substrates, obtained by assuming that a 1:1 enzyme–substrate complex is the relevant catalytic species in the single turnover experiments.

^bThe radioactive phosphate label is at the 5' end of the top strand.

^cThe radioactive ddAMP label is at the 3' end of the bottom strand.

obtained with PI-SceI variants with single amino acid substitutions in the presumptive catalytic centres (Table I). While completely inactive variants such as D218A, K301A and D326A (Gimble and Stephens, 1995; He *et al.*, 1998) do not exhibit any cleavage activity with the nicked substrates, D229N, T341N and K403A exhibit residual activity on fully double-stranded substrates and, intriguingly, show a pronounced preference for a substrate with a nick in one strand: D229N and K403A in the top strand, T341N in the bottom strand. This suggests that Asp229 and Lys403 are involved mainly in the cleavage of the scissile phosphodiester bond in the top strand. When this bond is already cleaved, the functions of Asp229 (absent in D229N) and, to a lesser extent, of Lys403 (absent in K403A) are dispensable for the cleavage of the bottom strand. In contrast, Thr341 seems to be responsible mainly for cleavage of the bottom strand, as the T341N variant (which lacks the Thr functionality) prefers to cleave a substrate already nicked in the bottom strand. Our data suggest that the top strand is attacked first and that this is the rate-limiting step for double strand cleavage. If an oligodeoxynucleotide with a nick in the top strand is offered as a substrate, cleavage of the bottom strand is fast, presumably because of relief of conformational stress.

Identification of the two catalytic centres of PI-SceI by homology considerations

PI-SceI has considerable structural homology with two other members of the LAGLIDADG family of homing endonucleases, namely I-CreI (Heath *et al.*, 1997) and I-DmoI (Silva *et al.*, 1999). The catalytic centres of these enzymes can be superimposed and corresponding residues identified in the homodimeric enzyme I-CreI and the two symmetry-related subdomains of the catalytic domain of the monomeric enzymes PI-SceI and I-DmoI (cf. Silva *et al.*, 1999). The very good fit of these superpositions is a strong argument that these enzymes have a similar mechanism of action, which implies that the monomeric homing endonucleases PI-SceI and I-DmoI have two catalytic centres like their homodimeric relative I-CreI.

The superposition of the catalytic domain of PI-SceI on the I-CreI–DNA complex allows a suggestion to be made about which presumptive catalytic amino acid residues of PI-SceI belong to which catalytic centre and are responsible for cleavage of the top and bottom strand. Figure 2 shows the superposition of the catalytic centres of I-CreI and PI-SceI together with the DNA substrate of I-CreI. In Figure 2 are also shown two Ca²⁺ ions from the I-CreI–DNA structure. In the hydration sphere of one Ca²⁺ ion, there is a water molecule that is in an ideal position for an in-line attack on the scissile phosphodiester bond of the top strand. The superposition suggests that in PI-SceI this Ca²⁺ ion would be bound by Asp218 and would be involved in cleavage of the top strand.

Cleavage experiments with PI-SceI and phosphorothioate-substituted oligodeoxynucleotides

PI-SceI recognizes an asymmetric sequence of >35 bp, with only little symmetry at the site of cleavage, i.e.

Thus, one would not expect that an enzyme with only one catalytic centre makes similar base contacts with both strands at the sites of cleavage. However, for the phosphate contacts at the site of cleavage, it can be expected that the structural requirements must be similar for the two cleavage events, as the transition state involves not only the functional groups of the enzyme but also the phosphates being attacked. It is very likely, therefore, that phosphorothioate for phosphate substitutions at the site of cleavage should have a similar effect in both strands when one catalytic centre is responsible for cleavage of both strands; in contrast, when two catalytic centres are involved, one would expect differences.

In order to analyse the substrate requirements of the homing endonuclease PI-SceI at and next to the site of cleavage, we have produced 12 different 47 bp oligodeoxynucleotides comprising the PI-SceI recognition sequence and containing single stereochemically pure phosphorothioate substitutions at positions located either at the cleavage position or 5' and 3' to this position in the top and the bottom strand, respectively. These oligodeoxynucleotides are bound as well as unmodified oligodeoxynucleotide substrates by PI-SceI (data not shown).

Figure 3 shows the kinetics of cleavage of oligonucleotides modified in the top (oligodeoxynucleotides UcpR and UcpS) and the bottom strand (LcpR and LcpS), in comparison with non-modified substrates. Substitution of a non-bridging oxygen by sulfur at the site of cleavage in the top strand decreases the rate of cleavage by more than a factor of 25 in comparison with the non-modified substrate and with no stereoselectivity for the R_p and S_p diastereomer. In contrast, the oligodeoxynucleotide carrying the S_p phosphorothioate substitution in the cleavage position in the bottom strand (LcpS) is cleaved much better than that carrying the corresponding substitution in the top strand (UcpS). The R_p diastereomer (LcpR) is cleaved less efficiently (by a factor of 6) than the S_p diastereomer (LcpS) (Figure 4).

Sulfur substitution of a non-bridging oxygen atom, in

Fig. 2. Stereo view of the presumptive catalytic residues of PI-SceI (Brookhaven Protein Data Bank entry: 1VDE) superimposed on parts of the I-CreI-DNA co-crystal structure (Brookhaven Protein Data Bank entry: 1BP7) including the top and bottom strands of the recognition sequence (in dark and light blue, respectively), with the scissile phosphates (in yellow), the catalytic residues of I-CreI (in green) homologous to the corresponding residues in PI-SceI, two Ca^{2+} ions and one H_2O molecule (blue cross) which is in a position to attack in-line the phosphodiester bond of the top strand. Indicated in red are Asp218, Asp229 and Lys403, and Asp326, Lys301 and Thr341 of PI-SceI, which define the two catalytic centres.

Fig. 3. Kinetics of cleavage of non-modified (▲), R_P -substituted (◆) and S_P -substituted (■) oligodeoxynucleotides, with the phosphorothioate at the cleavage position, by PI-SceI. The top strand is radioactively labelled and substituted in the panel on the left, while in the panel on the right it is the bottom strand which is radioactively labelled and substituted.

Fig. 4. Relative cleavage efficiencies of PI-SceI for R_P and S_P phosphorothioate-substituted oligodeoxynucleotide substrates. The substrates were incubated overnight with PI-SceI under standard conditions in the presence of Mn^{2+} . U and L denote substitutions in the top and bottom strand, respectively; cp refers to the cleavage position, and 3' and 5' to the respective flanking positions.

both the R_P and S_P position, 3' adjacent to the scissile phosphodiester bond in the top strand (U3'R and U3'S) does not significantly affect the extent of cleavage, demon-

strating that PI-SceI does not exhibit stereoselectivity regarding the sulfur substitution at this position within the recognition sequence (Figure 4). As similar extents of cleavage were observed for the oligodeoxynucleotides U5'R and U5'S, PI-SceI also does not discriminate between the diastereomers in this position of the phosphodiester group, although the yield of product formation is 25% lower with U5'R and U5'S than with the unmodified oligodeoxynucleotide. Oligodeoxynucleotides substituted by phosphorothioate in the bottom strand at the position 5' and 3' to the scissile phosphodiester bond are accepted as substrates by PI-SceI. There is a 2-fold preference for L3'S over L3'R and a 3-fold preference for L5'R over L5'S (Figure 4).

Thus, the most pronounced differences between the two strands regarding the effects of phosphorothioate for phosphate substitutions are observed at the position of cleavage where a precise geometry and polarity are critical for the formation of the transition state. This finding is much more compatible with a mechanism of cleavage involving two catalytic centres, which are likely to have different structural requirements regarding their substrates, than with a mechanism involving only one catalytic centre,

Fig. 5. Metal ion mapping at the PI-SceI cleavage site. (A) Fe^{2+} -induced hydroxyl radical cleavage of the DNA in the wt PI-SceI-oligodeoxynucleotide complex analysed by denaturing PAGE. Lanes a and b show a standard ranging from 8 to 32 nucleotides, radioactively labelled with either $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (a) or $[\alpha\text{-}^{32}\text{P}]\text{ddATP}$ (b). In lanes 1–6, the results of cleavage experiments with a PI-SceI substrate labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the top strand and in lanes 7–12 labelled with $[\alpha\text{-}^{32}\text{P}]\text{ddATP}$ in the bottom strand are shown. Lanes 1 and 7, the oligodeoxynucleotide without further incubation; lanes 2 and 8, cleavage by *AclI*; lanes 3 and 9, cleavage by PI-SceI; lanes 4 and 10, metal ion mapping with PI-SceI; lanes 5 and 11, metal ion mapping without addition of PI-SceI; lanes 6 and 12, metal ion mapping with PI-SceI in the absence of H_2O_2 . (B) Metal ion mapping with Ca^{2+} competing for Fe^{2+} . Lanes 10 and 12 correspond to lanes 10 and 12 in (A): lane 10, metal ion mapping with PI-SceI and Mg^{2+} ; lane 12, metal ion mapping with PI-SceI in the absence of H_2O_2 ; lane 13, metal ion mapping with PI-SceI and an equimolar mixture of Ca^{2+} and Fe^{2+} . (C) Sequence of the oligodeoxynucleotide used in this study. The PI-SceI cleavage positions are indicated with solid lines, the *AclI* cleavage positions with dotted lines. The metal ion-induced cleavages are indicated by arrows. (D) Comparison of the results of the metal ion footprinting for the wt-PI-SceI, the D326A and the D218A variant. + denotes strong hydroxyl radical cleavage and – no cleavage.

for which one would assume that it must adopt a similar transition state for the two cleavage events.

Metal ion mapping experiments with PI-SceI and an oligodeoxynucleotide substrate

Divalent metal ions, such as Mg^{2+} or Mn^{2+} , are obligatory cofactors for PI-SceI. They are likely to be involved directly in catalysis. In order to determine to which phosphate residues the essential divalent metal ions are bound in the PI-SceI–DNA complex, metal ion mapping experiments were performed similarly to previous descriptions (Farber and Levine, 1986; Ettner *et al.*, 1995; Zaychikov *et al.*, 1996). As shown for the homing endonucleases I-PorI and I-DmoI (Lykke-Andersen *et al.*, 1997), PI-SceI accepts Fe^{2+} as a cofactor and displays with Fe^{2+} a similar activity to that seen with Mg^{2+} (data not shown). For the metal ion mapping experiments, the 47mer oligodeoxynucleotide was incubated in the presence or absence of PI-SceI and $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ as well as in the presence of PI-SceI and Fe^{2+} , under conditions (low temperature, short incubation time) where hardly any cleavage occurs due to the endonuclease activity of PI-SceI. In order to determine the size of the fragments generated by hydroxyl radical attack in the metal ion mapping experiments, the 47mer was also cleaved with PI-SceI and with the restriction endonuclease *AclI*. In the presence of Fe^{2+} and H_2O_2 , specific hydroxyl radical-induced cleavages could be detected in the PI-SceI–DNA

complex but not in the free DNA (which is degraded non-specifically) (Figure 5). They occur at two major sites in the bottom strand and at three major sites in the top strand. Taking into account that hydroxyl radical-induced cleavage generates phosphate ends differing from the 5'-phosphate ends generated by PI-SceI and *AclI* cleavage (Hertzberg and Dervan, 1984; Stubbe and Kozarich, 1987), the sites of hydroxyl radical attack can be identified. Both strands were cleaved by hydroxyl radicals at the scissile phosphates. In the bottom strand, an additional cleavage is observed 3' to the cleavage position, whereas for the top strand one additional cleavage occurs 5' and one 3' to the scissile phosphodiester bond (Figure 5C). These hydroxyl radical-induced cleavages can also be observed, albeit only as faint bands, in the absence of H_2O_2 , as some Fe^{2+} -induced hydroxyl radical formation takes place in H_2O . The observed hydroxyl radical-induced cleavages are due to catalytically relevant metal ions as they can be suppressed by the addition of Ca^{2+} , a specific inhibitor of the enzymatic cleavage reaction (Figure 5B). In hydroxyl radical footprinting experiments, Gimble and Stephens (1995) had observed a hypersensitive site in the top strand (presumably corresponding to site +3 in our Figure 5) and two hypersensitive sites in the bottom strand (presumably corresponding to sites –2 and –3 in our Figure 5), but did not demonstrate that these hypersensitive sites are due to Fe^{2+} ions specifically bound to PI-SceI.

In order to identify amino acid residues responsible for

metal ion co-ordination, PI-SceI variants with single amino acid substitutions were used for the metal ion mapping (Figure 5D). An Asp to Ala substitution at position 326 leads to the disappearance of the hydroxyl radical-induced cleavage at position +1 in the top strand and -3 in the bottom strand, whereas the cleavage at position -2 in the bottom strand is reduced. In contrast, with the D218A variant, the hydroxyl radical cleavages at position +3 of the top strand and -2 of the bottom strand are not observed. These data indicate that hydroxyl radical formation is caused by at least two metal ions, one bound to Asp326 leading to cleavage at positions +1 in the top strand and -2 as well as -3 of the bottom strand, and the other bound to Asp218 leading to cleavage at position +3 of the top strand and -2 of the bottom strand. Gimble and Stephens (1995) had also observed that the hydroxyl radical cleavage pattern shown for the PI-SceI-DNA complex changed when substituting Asp218 and Asp326 by Ala. The hypersensitivity in the top strand of one site (presumably our site +3) disappeared in the presence of the D218A variant, and that of one site in the bottom strand (presumably our site -3) disappeared in the presence of the D326A variant. Qualitatively, their observations agree with ours, in particular that the D218A and D326A variants protect different sites in the top and bottom strand, respectively. The results can be explained by binding of two Fe^{2+} ions, one to Asp218 and the other to Asp326, with slight differences regarding the orientation of the two metal ions towards the two strands of the DNA substrate. This explains the different sensitivities of the two strands towards hydroxyl radical attack.

Discussion

The monomeric homing endonuclease PI-SceI recognizes an extended and asymmetric double-stranded DNA sequence with extreme specificity and cleaves the two strands of the DNA in a highly concerted reaction. This raises the question of how this enzyme can cleave both strands of the DNA, which requires two catalytic events. In the case of the homodimeric homing endonuclease I-CreI, which recognizes a pseudo-palindromic sequence, this is accomplished by a pair of catalytic centres, formed by the two LAGLIDADG motifs, one in each subunit. Double strand cleavage of an asymmetric site by a monomeric enzyme can, in principle, be achieved by two different mechanisms: (i) involving a single catalytic site which acts at both cleavage sites or (ii) making use of two sites which deal with the separate strand cleavage events. The type II restriction enzyme FokI seemed to be the paradigm for a monomeric enzyme which has only one catalytic centre, but nevertheless makes a double strand cut. However, it was shown recently that FokI must dimerize for DNA cleavage (Bitinaite *et al.*, 1998). Indeed, based on the structure of the FokI dimer, a model for the cleavage domain dimer bound to the FokI cleavage site was proposed, which shows the two catalytic centres in close proximity to the scissile bonds (Wah *et al.*, 1998). In contrast to FokI, there is no evidence that PI-SceI dimerizes in the presence of DNA (Wende *et al.*, 1996), which makes the use of FokI as a model for PI-SceI questionable. In addition, FokI has only one catalytic motif (PD...D/EXK) typical of many restriction enzymes

(Thielking *et al.*, 1991; Anderson, 1993), while PI-SceI has two catalytic motifs (LAGLIDADG) characteristic for this class of homing endonucleases. From the co-crystal structure of the I-CreI-DNA complex (Jurica *et al.*, 1998), it is quite clear that each LAGLIDADG sequence is involved in one cleavage event. The structural homology between I-CreI and the catalytic domain of PI-SceI, which is characterized by a pseudo 2-fold symmetry of two subdomains, each with one LAGLIDADG sequence, strongly suggests that PI-SceI has two catalytic centres. However, of course, it cannot be excluded that similar structures are used for different purposes, as pointed out by Jurica *et al.* (1998).

In the present study, we have presented experimental evidence that supports a model of two catalytic centres in PI-SceI. The strongest evidence is that the catalytic domain of PI-SceI can be cross-linked to one strand of the DNA without affecting double strand cleavage. This excludes the possibility that PI-SceI undergoes major conformational changes between the two cleavage events, as expected for one catalytic centre cleaving the two strands of the antiparallel DNA duplex. Further evidence is provided by the finding that two PI-SceI variants with substitutions of residues located at or close to the presumptive catalytic centres, i.e. D229N and K403A, and T341N, respectively, show a strong preference for a substrate nicked in the top and bottom strand, respectively. This indicates that Asp229 and Lys403 are involved mainly in cleavage of the top strand, while Thr341 participates in cleavage of the bottom strand. It is not unexpected that these variants preserve some activity towards the other strand, as we had selected variants for this experiment that have a residual activity, due to the fact that Asp229, Thr341 and Lys403, unlike Asp218 and Asp326, do not have an absolutely essential function. It had been shown by Gimble and Stephens (1995) that the D218A or N and the D326A or N variants of PI-SceI are inactive in double strand cleavage as well as in nicking. This has been taken as evidence that Asp218 and Asp326 are part of one active centre. We prefer to argue that removal of one negative charge in the centre of the active site of PI-SceI has a deleterious effect on both catalytic centres, presumably by preventing the exact positioning of the essential divalent metal ion cofactor. Removal of a more peripheral negative or positive charge, as in D229N, T341N and K403A, will be more critical for one catalytic centre than for the other.

Each catalytic event is characterized by a defined mechanism and involves a particular transition state. The mechanism of cleavage of a phosphodiester bond is characterized by a general base that has to activate the attacking nucleophile, a Lewis acid, for stabilization of the transition state (for an in-line mechanism, the transition state is characterized by a pentaco-ordinated phosphorus) and a general acid to protonate the leaving group. If only one catalytic centre is involved, the mechanism must be essentially the same and, more importantly here, the transition state will be similar for the two cleavage events. Similar transition states mean that substitution of individual non-bridging atoms by sulfur at the scissile phosphates, the most conservative phosphate modification one can make, should have similar effects. This is not observed for PI-SceI: both the R_p and S_p diastereomers are hardly accepted as substrates when the modification

Fig. 6. Schematic drawing of the catalytic centres of PI-SceI. The two catalytic centres of PI-SceI are depicted by a 'jing-jang' motif, each responsible for the cleavage of one strand of the double-stranded recognition sequence. Asp218, Lys403 and Asp229 presumably are part of the catalytic centre I responsible for the cleavage of the top strand, whereas Asp326, Lys301 and Thr341 as part of catalytic centre II are likely to co-operate in cleaving the bottom strand. The arrows indicate the attack on the scissile phosphate.

is in the cleavage position in the top strand, while the S_P diastereomer, but not, however, the R_P diastereomer, is a good substrate when the bottom strand is modified in the cleavage position. Thus, the different sensitivities of the scissile phosphate in the two strands towards subtle chemical modifications also suggest that PI-SceI has two catalytic centres.

Metal ion experiments were carried out to obtain an estimate of how many and where metal ions are bound at the active site of PI-SceI. We employed a hydroxyl radical mapping technique, using Fe^{2+} specifically bound at the catalytic centres of PI-SceI, as recently described by Lykke-Andersen *et al.* (1997) for I-PorI and I-DmoI. Specific binding was demonstrated by showing that Fe^{2+} could replace Mg^{2+} as the essential divalent metal ion cofactor and that Ca^{2+} , a specific inhibitor of PI-SceI-catalysed DNA cleavage, could suppress the specific hydroxyl radical cleavage pattern. Our results suggest that at least two Fe^{2+} are present in the PI-SceI-DNA complex and are located close to the sites cleaved by PI-SceI. The hydroxyl radical-induced cleavage patterns produced by Fe^{2+} ions bound to wild-type PI-SceI are not identical in the top and bottom strands. This suggests that the binding modes of the divalent metal ions are slightly different for the two binding sites, which we interpret as the two catalytic centres. As the DNA is partially protected against hydroxyl radical attack in the presence of the D218A and D326A variants, we conclude that Asp218 and Asp326 participate in Mg^{2+} binding at the two catalytic centres.

Our results, taken together, allow us to conclude that PI-SceI has two distinct catalytic centres for cleavage of the two strands of its double-stranded DNA substrate and, based on the homology with I-CreI, that Asp218, Asp229 and Lys403 are responsible for cleavage of the top strand, while Asp326, Thr341 and Lys301 are involved in cleavage

of the bottom strand (Figure 6). While the role of these individual residues is not yet clear, it is likely that both Asp218 and Asp326 co-ordinate the essential divalent metal ion cofactors, presumably one per catalytic centre. The results of cleavage experiments with nicked substrates suggest that the top strand is attacked first, but slowly, and that the bottom strand is cleaved quickly, but only after cleavage of the top strand, leading to a concerted double strand cleavage. We finally conclude that probably all members of the LAGLIDADG family of homing endonucleases follow basically the same mechanism, regardless of whether they are monomeric, like PI-SceI and I-DmoI, or homodimeric, like I-CreI.

Materials and methods

Mutagenesis and purification of PI-SceI

Site-directed mutagenesis of the PI-SceI gene was performed by a PCR-based technique according to Ito *et al.* (1991). The sequences of the mutated genes were confirmed by sequencing. Wild-type PI-SceI and PI-SceI variants were purified to >95% homogeneity as described previously (Wende *et al.*, 1996) using Ni-NTA affinity and phosphocellulose chromatography.

Synthesis of phosphorothioate-substituted oligodeoxynucleotides

Oligodeoxynucleotides were synthesized on a solid support with a Milligen cyclone plus DNA synthesizer using β -cyanoethylphosphoramidites obtained from PerSeptive Biosystems. Phosphorothioate substitutions were introduced by interrupting the automated synthesis immediately after the coupling step, removing the column and manually treating the phosphite triester with 0.5 M tetraethylthiuram disulfide in acetonitrile for 15 min at room temperature as described previously (Vu and Hirschbein, 1991). After rinsing the column with 3×5 ml of acetonitrile, it was flushed with argon and the synthesis was resumed. At the end of the synthesis, the dimethoxytrityl (DMTr) group was left on the oligodeoxynucleotide. Oligodeoxynucleotides were cleaved off the column and deprotected in 30% ammonium hydroxide. After lyophilization, the oligodeoxynucleotides were dissolved in water. Non-modified oligodeoxynucleotides (s1, s2, FCU and FCL oligodeoxynucleotides, see below) were obtained from MWG Biotech. The sequences of the oligodeoxynucleotides synthesized or purchased to produce double-stranded oligodeoxynucleotide substrates for PI-SceI (S denotes the position of the phosphorothioate substitution) were, for the top strand: s1, 5'-CATTATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGGCAGAAG-3'; FCU5, 5'-CATTATCTATGTCGGGTGC-3'; FCU, 5'-CATTATCTATGTCGGGTG-3'; FCU3, 5'-CATTATCTATGTCGGGT-3'; u5', 5'-G S GAGAAAGAGGTAATGAAATGGCAGAAG-3'; ucp, 5'-C S GGAGAAAGAGGTAATGAAATGGCAGAAG-3'; and u3', 5'-G S C-GGAGAAAGAGGTAATGAAATGGCAGAAG-3'. The sequences of the oligodeoxynucleotides used for the bottom strand were: s2, 3'-GTAATAGATACAGCCACGCCTCTTTCTCCATTACTTTACCGTCTTC-5'; FCL5, 3'-CGCCTCTTTCTCCATTACTTTACCGTCTTC-5'; FCL3, 3'-CACGCCTCTTTCTCCATTACTTTACCGTCTTC-5'; FCL, 3'-ACGCCTCTTTCTCCATTACTTTACCGTCTTC-5'; 15', 3'-GTAATAGATACAGCC S A-5'; lcp, 3'-GTAATAGATACAGCC S C-5'; and 13', 3'-GTAATAGATACAGC S C-5'.

HPLC purification and deprotection of the DMTr-containing phosphorothioate-substituted oligodeoxynucleotides

The separation of the respective R_P and S_P diastereomers of the phosphorothioate-substituted oligodeoxynucleotides (1 μ mol) was performed on a reversed phase HPLC (RP-HPLC) column (Apex ODS, 5 μ m, Jones Chromatography) using a two-component buffer system with buffer A [0.1 M triethyl ammonium acetate (TEAA) pH 6.5, 0.5 mM L-cysteine, 1% acetonitrile] and buffer B (0.1 M TEAA pH 6.5, 0.5 mM L-cysteine, 50% acetonitrile). For separation, a linear gradient was applied (0–40% buffer B in 5 min, 40–75% buffer B in 35 min, 75–100% buffer B in 5 min). As the oligodeoxynucleotides still carried the DMTr group, they could be well separated from shorter synthesis products lacking this group. Depending on the length of the oligodeoxynucleotide, the isomers were eluted in a concentration range of 50–70% buffer B. Fractions containing the two isomers were collected separately

and lyophilized overnight. During lyophilization, 50% ethanol (v/v) was added several times in order to accelerate the process. After lyophilization, the oligodeoxynucleotides were dissolved in 100 µl of distilled water. To this solution were added 400 µl of acetic acid (87%), the DMTr group was cleaved off by incubation for 40 min at room temperature and the oligodeoxynucleotide was purified using an NAP5™ column (Pharmacia). The eluates were dried in a Speedvac and the precipitates were dissolved in 50 µl of distilled water.

Stereochemical characterization of the phosphorothioate-substituted oligodeoxynucleotides by nuclease P1 digestion and RP-HPLC analysis of the digestion products

A 3 µl aliquot of the respective oligodeoxynucleotide solution (~1 mM) was incubated with 1 µl of nuclease P1 (1 mg/ml) in a total volume of 10 µl at 37°C. After 3 h, P1 was inactivated by heating the reaction mixture for 10 min at 65°C. After cooling on ice, 1 U of alkaline phosphatase (USB) and the buffer were added as recommended by the supplier. The dephosphorylation was performed for 15 min at 37°C. The reaction was stopped by heating for 5 min at 95°C. The standard mixture of 1 mM each of 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine and thymidine (Sigma Aldrich) was treated in the same manner. The products of the P1 cleavage reaction after phosphatase treatment, i.e. mononucleosides and dinucleotides, were analysed on an RP-HPLC column (Apex ODS, 5 µm, Jones Chromatography) using a two-component gradient system (buffer A: 0.1 M TEAA pH 6.5; buffer B: 0.1 M TEAA pH 6.5, 30% acetonitrile; gradient: 0–15% B in 30 min; 15–25% B in 10 min; 25–100% B in 5 min). As nuclease P1 cannot cleave phosphodiester bonds with an R_p phosphorothioate substitution (Potter *et al.*, 1983), the nuclease P1 reaction yields a dinucleotide in this case. While mononucleosides under these conditions elute between 10 and 30 min, dinucleotides elute after 40 min. The identity of the mononucleoside peaks was confirmed by comparison with the nucleoside standard.

Generation and purification of double-stranded full-length phosphorothioate-substituted oligodeoxynucleotide substrates

In order to generate a PI-SceI oligodeoxynucleotide substrate covering the complete recognition sequence and containing a single phosphorothioate substitution, the R_p and S_p diastereomeric oligodeoxynucleotides were ligated with non-modified oligodeoxynucleotides consisting of the missing part of the recognition sequence. Before ligation, the phosphorothioates were first phosphorylated at the 5' end with 5 U of T4 polynucleotide kinase (MBI Fermentas) [1 mM dithiothreitol (DTT), 1 mM ATP, 2 h at 37°C] and then precipitated by adding 1/10 vol. of ammonium acetate (10 M) and 3 vols of ethanol. The phosphorylated oligodeoxynucleotides subsequently were radioactively labelled at the 3' end using 5 U of terminal transferase and 10 µCi of [α -³²P]ddATP (1 h at 37°C). The oligodeoxynucleotides were separated from non-incorporated [α -³²P]ddATP by ammonium acetate/ethanol precipitation. Then 625 pmol of the labelled oligodeoxynucleotides were incubated at 95°C with an excess of the respective partner oligodeoxynucleotide and the complementary oligodeoxynucleotide. After cooling to room temperature, the annealed oligodeoxynucleotides were ligated overnight with T4 DNA ligase (5 U, 16°C): FCU3 + u3' + s2 → U3'; FCU + ucp + s2 → Ucp; FCU5 + u5' + s2 → U5'; FCL3 + l3' + s1 → L3'; FCL + lcp + s1 → Lcp; FCL5 + l5' + s1 → L5'.

The products of the ligation reactions were analysed by denaturing gel electrophoresis (7 M urea, 20% acrylamide, 0.5× TBE). The top and the bottom strand of the PI-SceI recognition sequence migrate at the same position in the gel. The bands consisting of the ligated oligodeoxynucleotides with the phosphorothioate substitution at the defined position and the non-modified oligonucleotides were cut out of the gel and eluted into 500 µl of distilled water (12 h at room temperature). After elution, the oligodeoxynucleotides were precipitated twice with ammonium acetate/ethanol in order to remove the urea completely. The strands were dissolved in water and annealed by heating to 95°C and slowly cooling to room temperature, giving a 1 µM oligodeoxynucleotide solution with a specific radioactivity of 2000 c.p.m./µl.

Non-modified oligodeoxynucleotides were used as standards for the cleavage experiments. For this purpose, double-stranded substrates were generated with a radioactive label at the 3' end either in the top or the bottom strand: radioactively labelled s1 + s2 → S1; s1 + radioactively labelled s2 → S2.

Cleavage experiments with oligodeoxynucleotide substrates

Cleavage reactions with unmodified and modified oligodeoxynucleotides (100 nM) and PI-SceI (1 µM) were performed in cleavage buffer (10 mM Tris-HCl pH 8.5, 100 mM KCl) containing 2.5 mM MnCl₂ at 37°C. The cleavage reaction was stopped by adding 1 vol. of formamide/500 mM EDTA (80%:20%) and heating the mixture for 5 min at 95°C prior to cooling on ice. The progress of the cleavage reaction was analysed by gel electrophoresis (20% acrylamide, 0.5× TBE, 7 M urea). The radioactivity was quantified using an Instant Imager™ (Canberra Packard) and the progress of the cleavage reaction determined by calculating the percentage of radioactivity within the band of the cleavage product in comparison with the substrate band.

Cleavage of the oligodeoxynucleotide cross-linked via thymine +9 in the bottom strand to His333 in domain II of PI-SceI

A 36 bp double-stranded oligodeoxynucleotide covering the PI-SceI recognition site from position -12 to +24 and carrying a single 5-iododeoxyuridine substitution at position +9 in the bottom strand (Pingoud *et al.*, 1999) was radioactively labelled at its 5' ends using T4 polynucleotide kinase and [γ -³²P]ATP. Equimolar amounts of PI-SceI (20 µM) and the double-stranded oligodeoxynucleotide T+9 (20 µM) were incubated in 10 mM Tris-HCl pH 7.5 and 100 mM KCl in a volume of 25 µl for 5 min at ambient temperature. Photocross-linking was carried out as described previously (Pingoud *et al.*, 1999). Samples of 5 µl were withdrawn before and after cross-linking. Cleavage of the covalently linked oligodeoxynucleotide was performed in a buffer containing 20 mM Tris-HCl pH 8.5, 100 mM KCl, 1 mM DTT and 2.5 mM MnCl₂ at 37°C overnight. Samples were analysed on a 15% SDS-polyacrylamide gel. The gel was silver stained and dried; radioactive bands were visualized by autoradiography with intensifying screens.

Metal ion mapping experiments

In order to determine the metal ion co-ordination sites on the DNA substrate, the individual strands, s1 or s2 (see above), of the 47mer substrate containing the cleavage site for PI-SceI were radioactively labelled as described above. Approximately 10 000 c.p.m. were incubated with PI-SceI (7.5 µM), 10 mM Tris-HCl pH 7.0, 50 mM KCl and 2 mM Fe(NH₄)₂(SO₄)₂ for 10 min at room temperature in order to form the protein-DNA complex. Subsequently, 1% H₂O₂ and 100 mM sodium ascorbate were added and the mixture was incubated for 4 min at 4°C. The reaction was stopped by adding 1 mM DTT, 25 mM EDTA, 10% (v/v) ammonium acetate (10 M) and 3 vols of ethanol. The precipitate was washed with 80% ethanol and then dissolved in 10 µl of formamide/500 mM EDTA (80%/20%). After denaturation by incubation for 2 min at 95°C, the cleavage products were analysed by electrophoresis on a 20% acrylamide gel containing 0.5× TBE and 7 M urea together with a standard obtained by incubation of 10 000 c.p.m. of the oligodeoxynucleotide substrates for 1 h with 2 U of *AclI* under the conditions recommended by the supplier. The yield of cleavage products was quantified using an Instant Imager™ (Canberra Packard).

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