Structural and Functional Analysis of the Homing Endonuclease PI-*Sce*I by Limited Proteolytic Cleavage and Molecular Cloning of Partial Digestion Products†

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ABSTRACT: PI-*Sce*I is a member of an unusual class of rare cutting homing endonucleases produced by an autocatalytic protein splicing from a precursor. To analyze the structural and functional domain organization of the endonuclease PI-*Sce*I and to examine whether the DNA binding activity can be structurally separated from the catalytic activity, we performed limited proteolytic digestion experiments with various proteases. Two protease-resistant fragments spanning the N- and C-terminal halves of the nuclease were identified using different proteases which cleave the protein in the same region. Each fragment contains one of the two conserved LAGLIDADG motifs. The products of the limited proteolytic digests were shown to remain associated and to exhibit specific DNA binding but to be inactive in DNA cleavage. Different from what is observed with native PI-*Sce*I, only one complex is formed as shown in an electrophoretic mobility shift assay. Expression clones for the N- and C-terminal protein fragments obtained by tryptic digestion were constructed, and the proteins PI-*Sce*I-N and PI-*Sce*I-C were purified. Only PI-*Sce*I-N exhibits DNA binding activity. Bending experiments with PI-*Sce*I-N, a mixture of PI-*Sce*I-N and PI-*Sce*I-C, as well as the products of the limited tryptic digest show that a DNA substrate with the full length recognition sequence is bent by 45°. This degree of bending is also observed with a DNA containing only the right side of the recognition sequence, corresponding to one of the DNA cleavage products of PI-*Sce*I. Our results demonstrate that the N-terminal half of PI-*Sce*I which lacks one of the two LAGLDADG motifs is able to bind to DNA specifically and to induce one of the distortions observed to occur in the process of DNA binding by PI-*Sce*I. These results are discussed in light of the recently solved crystal structure of PI-*Sce*I and used to refine a model for the mechanism of DNA binding and cleavage by PI-*Sce*I.

Homing endonucleases catalyze a highly specific DNA double-strand cleavage and thereby initiate a double strand break repair which leads to the insertion by recombination of the coding sequence for these enzymes into alleles that lack them (*1*−*3*). Homing endonucleases are frequently encoded by introns or are derived from protein introns (inteins) within precursor proteins (*4*−*6*). The majority of homing endonucleases can be divided into four families which are characterized by one of the following sequence motifs: LAGLIDADG, GIY-YIG, H-N-H, and the His-Cys box (*1, 5, 7*).

PI-*Sce*I[1] from *Saccharomyces cerevisiae* (*8*−*10*) is a member of the LAGLIDADG family of homing endonucleases. It is produced as a precursor protein from which it is autocatalytically spliced out to give the catalytic subunit of the vacuolar membrane H^+-ATPase and the mature endonuclease which was originally called the vacuolar membrane H^+-ATPase derived endonuclease (VDE, *10*). Like other homing endonucleases, it recognizes an extraordinarily long DNA sequence with no apparent symmetry (*10*−*12*). Depending on the substrate, the recognition sequence of PI-*Sce*I may exceed 40 base pairs (*13*). Upon specific DNA binding, PI-*Sce*I distorts the DNA. In gel shift experiments, two kinds of complexes are observed, one with a 45° and the other with a 75° bend of the DNA (*13, 14*). It has been suggested that initial binding occurs at the right side of the recognition sequence and leads to a small distortion of the DNA (*13*). Subsequently, contacts also to the left side of the recognition sequence are formed which induce a larger distortion of the DNA and position the scissile phosphodiester bonds into the active site(s). In the presence of Mg^{2+}, PI-*Sce*I cleaves the two strands of the DNA in a concerted reaction to produce two DNA fragments with 3′-OH and 5′-phosphate ends with a 3′ overhang of four nucleotides (*10, 11*). The replacement of Mg^{2+} by Mn^{2+} leads to a relaxation of specificity and an increase in activity which nevertheless is very slow, in part because the enzyme has a strong affinity to one of its products (*13, 14*).

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[1] We will throughout the text refer to PI-*Sce*I, PI-*Sce*I-N, and PI-*Sce*I-C, when experiments with the His_6-tagged variants are described or discussed.

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The nucleolytic activity of PI-*Sce*I is associated with the two LAGLIDADG motifs, as shown in a mutational analysis (*12*): substitution by alanine of Asp218 in the first LAGLIDADG motif, or of Asp326 in the second LAGLIDADG motif, leads to complete inactivity of the enzyme but does not affect specific DNA binding. PI-*Sce*I is not only a nucleolytic enzyme, but in the context of additional N- and C-terminal extensions, as in the precursor protein, it is also a protein splicing enzyme. The amino acid residues involved in this activity have been mapped mainly to N- and C-terminally located regions of PI-*Sce*I (*16, 17*). Deletion mutants coding for PI-*Sce*I variants without the regions comprising the two LAGLIDADG motifs are fully active in their protein splicing activity (*18*). Similar results were obtained with the *Mtu* recA intein which was reduced to a functional mini-intein (*19*). These findings taken together suggest that PI-*Sce*I, a protein of 454 amino acid residues, is composed of two functional parts.

To find out to what extent the nucleolytic activity depends on regions of the protein involved in protein splicing, we analyzed the structural and functional organization of the nucleolytic activity of PI-*Sce*I. For this purpose, we have subjected PI-*Sce*I to a limited digest with trypsin and other proteases and analyzed the DNA binding and cleavage activity of the products of the protease digests. Our results show that PI-*Sce*I is highly susceptible to cleavage by trypsin at Arg277 and by other proteases at residues nearby. The resulting N- and C-terminal fragments, which we have cloned, expressed, and purified, have no nucleolytic activity; the N-terminal fragment, however, is active in specific DNA binding. It induces a distortion in the DNA similar to the one observed with the native PI-*Sce*I upon initial complex formation. Like the full-length protein, it also binds and bends one of the products of the DNA cleavage reaction. These results are discussed in light of the recently solved crystal structure of PI-*Sce*I (*20*).

EXPERIMENTAL PROCEDURES

Cloning of PI-SceI and PI-SceI Fragments. Cloning of His_6-tagged PI-*Sce*I[1] and the His_6-tagged N-terminal fragment PI-*Sce*I-N[1] (i.e., a PI-*Sce*I variant which comprises amino acids 1−277) was performed as described by Wende et al. (*13*). The His_6-tagged C-terminal fragment PI-*Sce*I-C[1] (i.e., a PI-*Sce*I variant which comprises amino acids 278−454 and starts with an additional methionine residue) was prepared according to the following procedure: The plasmid pHisPI-*Sce*I-C coding for the C-terminal fragment of PI-*Sce*I was prepared using the plasmid pHisPI-*Sce*I as a template in a PCR[2] reaction with *Pfu*-DNA-polymerase (Stratagene) and the primers 5′-GCGCGGATCCAATAATCTTAATACTGAGAA-3′ and 5′-CCGGCGTCGACGTCAGCAATTATGGACGACAACCTGG-3′. The subcloning of the purified PCR product as well as the expression and purification of the encoded C-terminal fragment was performed as described for PI-*Sce*I-N (*13*).

[2] Abbreviations: bp, base pair(s); BSA, bovine serum albumin; ds, double-stranded; CD, circular dichroism; DTE, dithioerythritol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; KPi, potassium phosphate; Ni^{2+}-NTA, Ni-nitrilotriacetic acid; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

Fermentation of Recombinant E. coli Strains and Purification of PI-SceI, PI-SceI-N, and PI-SceI-C. The fermentation of recombinant *E. coli* strains expressing PI-*Sce*I and its variants, as well as the purification of PI-*Sce*I, PI-*Sce*I-N, and PI-*Sce*I-C, was carried out as described before (*13*). The protein concentration was determined from the absorbance at 280 nm. The extinction coefficients of the PI-*Sce*I fragments were calculated according to Pace et al. (*21*).

Tryptic Digestion in the Absence and Presence of DNA. Purified PI-*Sce*I (20 μM) was treated in the absence and presence of a synthetic 66 bp oligodeoxynucleotide (20 μM) containing the PI-*Sce*I recognition site (5′-GATCTGACGCCATTATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGGCAGAAGTCTTGATGT-3′) with trypsin at a substrate:protease ratio of 200:1 (w/w) at ambient temperature. The buffer used for digestion was 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM DTE, and 0.01% (w/v) Lubrol. The digestion time was up to 46 h. Aliquots of the trypsinization reaction were withdrawn after the time intervals indicated, the reaction was terminated with 50 μg/mL aprotinin, and aliquots were analyzed on 15% (w/v) SDS−polyacrylamide gels (*22*). The silver staining was performed as described in ref *23*. The tryptic digestion of PI-*Sce*I in the absence of DNA with parallel measurement of circular dichroism was performed in 50 mM Tris/HCl, pH 8.0, at a substrate:protease ratio of 500:1 (w/w) at ambient temperature. At the time points indicated, aliquots of the reaction mixture were withdrawn, the reaction was terminated by the addition of 5 mM PMSF, and the mixture was diluted with dialysis buffer [10 mM KPi, pH 7.4, 200 mM KCl, 0.1 mM EDTA, 1 mM DTE, and 50% (v/v) glycerol] to a final concentration of 5 μM or 1 μM.

Cleavage of DNA by PI-SceI and PI-SceI Fragments. Cleavage reactions were performed with a ^{32}P-labeled 311 bp PCR-generated DNA fragment derived from pBSVDEX containing a central PI-*Sce*I cleavage site (*13*) as substrate (5 nM) in 10 μL of cleavage buffer (10 mM Tris/HCl, pH 8.5, 100 mM KCl, 1 mM DTT, 100 μg/mL BSA, 2.5 mM $MgCl_2$) for 1.5 h at 37 °C. The reactions were terminated by the addition of 3 μL of stop buffer [100 mM EDTA, pH 8.0, 25% Ficoll (w/v), 0.1% (w/v) bromophenolblue, 0.1% (w/v) xylene cyanol]. The cleavage reactions were analyzed on 10% (w/v) polyacrylamide gels in 1 × TPE buffer (80 mM Tris−phosphate, pH 8.2, 2 mM EDTA). Following electrophoresis, the gels were dried, and radioactive bands were visualized by autoradiography with intensifying screens or by using an imager.

Electrophoretic Mobility Shift Assay. For electrophoretic mobility shift assays, PI-*Sce*I, proteolytically partially digested PI-*Sce*I, PI-*Sce*I-N, and PI-*Sce*I-C, respectively, were mixed with binding buffer [10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 0.05% (w/v) nonfat dry milk, 5% (v/v) glycerol, 10 mM DTT] in a total volume of 10 μL containing in addition 0.1 μg of the nonspecific DNA carrier poly(dI-dC) (Pharmacia), and 10 000 cpm of the ^{32}P-labeled 311 bp DNA substrate (3−7 nM) or ^{32}P-labeled ds oligodeoxynucleotides, 29-mer and 39-mer (2.5 nM) corresponding to the cleavage products F_I and F_{II} of the ds oligodesoxynucleotide substrate F (*13*). The mixtures were incubated for 30 min at ambient temperature; subsequently, 3 μL of loading buffer [10% (w/v) Ficoll, 15% (v/v) glycerol, 40 mM EDTA, 0.2% (w/v) bromophenolblue, 0.2% (w/v) xylene

cyanol] was added. Samples were loaded onto a native 6% or 12% (w/v) polyacrylamide (acrylamide:bisacrylamide ratio 19:1) minigel containing TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA). Gels were prerun at 80 V for 30 min and, after loading, run for additional 4 or 2 h at ambient temperature. Following electrophoresis, gels were dried and radioactive bands visualized by autoradiography. For preparative isolation of tryptic fragments from a shift gel, essentially the same procedure was applied at a 50-fold larger scale. After electrophoresis, the shifted band was cut out of the gel and eluted with 10 mM Tris/HCl, pH 8.0, 1 mM EDTA. The proteins eluted were precipitated with a 4-fold volume of cold acetone, solubilized in Laemmli sample buffer (*22*), and analyzed on a 15% (w/v) SDS−polyacrylamide gel.

Sequencing of the 20 kDa and the 30−32 kDa Tryptic Fragments of PI-SceI. The reaction products obtained by a limited tryptic digestion were separated by SDS−PAGE on a 15% (w/v) polyacrylamide gel. Following electrophoresis, proteins were transferred onto an Immobilon P (Millipore) membrane by the semi-dry procedure using a discontinuous buffer system (anode solution I, 0.3 M Tris, pH 10.4; anode solution II, 25 mM Tris, pH 10.4; cathode solution, 4 mM 6-amino-*n*-hexanoic acid, pH 7.6). The blotting procedure was performed in the Nova Blot electrophoretic transfer unit (Pharmacia) at 0.8 mA/cm^2 for 1 h. The blot was stained with 0.1% (w/v) Coomassie Brilliant Blue R 250 in 40% (v/v) methanol, and 1% (v/v) acetic acid, destained with 50% (v/v) methanol and washed with distilled water at 4 °C overnight. The 20 kDa and the 30−32 kDa peptides (about 30 pmol) were cut out and sequenced on a pulsed liquid-phase sequenator Model 477A (Applied Biosystems) with a 120A on-line HPLC system.

Gel Filtration of PI-SceI and Its Tryptic Digest. Size-exclusion HPLC was performed on a 30 cm × 1.0 cm Superdex HR 75 10/30 column. In a control run, about 15 μg intact PI-*Sce*I was passed over the column which had been equilibrated with 30 mM KPi, pH 7.0, and 150 mM NaCl. The flow rate was 0.5 mL/min, and the absorbance was measured at 280 nm. PI-*Sce*I was digested with trypsin for 30 min under the same conditions as described for the digestion in the absence of DNA. An equivalent amount of protein as in the control run was applied to the column. Fractions of 0.5 mL were collected. Aliquots of individual fractions were precipitated with 4 volumes of acetone and analyzed on a 15% (w/v) SDS−polyacrylamide gel.

Other Protease Digestions. PI-*Sce*I was digested with chymotrypsin, subtilisin, or proteinase K under identical conditions as with trypsin. The proteases were used at different substrate:protease ratios of 600:1 (w/w) (subtilisin), 200:1 (w/w) (proteinase K), and 60:1 (w/w) (chymotrypsin). Sixty microliter samples containing 1 mg/mL PI-*Sce*I in 50 mM Tris/HCl (pH 8.0) and the protease were incubated for 2.5−120 min at room temperature. Six microliter aliquots were quenched by the addition of 2 mM PMSF and 5 mM EDTA and analyzed by separation on 15% (w/v) SDS−polyacrylamide gels. Aliquots for binding and cleavage assays were diluted with dialysis buffer [10 mM KPi, pH 7.4, 200 mM KCl, 0.1 mM EDTA, 1 mM DTE, 50% (v/v) glycerol, 100 μg/mL BSA] 1:3 and stored frozen.

Circular Dichroism Spectroscopy. CD spectra of 20 μM PI-*Sce*I and PI-*Sce*I/protease incubation mixtures, as well as 7 μM PI-*Sce*I-N and PI-*Sce*I-C, were measured in DNA cleavage buffer on a JASCO J-710 spectrophotometer at ambient temperature. The melting curves of PI-*Sce*I and nicked PI-*Sce*I were recorded with a temperature rise of 20 °C/h at a fixed wavelength of 210 nm.

Circular Permutation Assay. Substrates for the circular permutation assay were obtained from the plasmids pBend2-F, which contains a 76 bp fragment of the PI-*Sce*I recognition sequence (pBend2VMA1Δvde in ref *13*), and pBend2-FII, which contains the right half of the recognition sequence corresponding to one of the cleavage products. This plasmid was prepared by insertion of the fragment FII (Figure 3, ref. *13*) into the *Sal*I site of plasmid pBend2 (*24*) after conversion of overhanging termini of plasmid and insert to blunt ends by Klenow polymerase. Each plasmid was used as a template in a PCR which was performed with the primers 5′-GAGGCCCTTTCGTCTTCAAGAATTC-3′ and 5′-GTGATAAACTACCGCATTAAAGCTT-3′. PCR was carried out in the presence of [α-^{32}P]dATP to produce radioactively labeled substrates. The actual probes used in bending experiments were generated by digestion of the labeled PCR products with the restriction enzymes (*Mlu*I, *Spe*I, *Pvu*I, *Sma*I, and *Bam*HI), resulting in 201 bp fragments from pBend2-F and in 160 bp fragments from pBend2-FII.

RESULTS

Limited Trypsinization of PI-SceI in the Absence and Presence of DNA. Protein domains involved in DNA binding are likely to become less susceptible to protease digestion upon DNA binding. To identify regions involved in DNA binding, PI-*Sce*I was subjected to tryptic digestion in the absence and presence of a 66 bp DNA substrate containing the recognition site of PI-*Sce*I. These experiments were carried out in the absence of Mg^{2+} which is not required for specific DNA binding (*12*). Protein fragments were separated on SDS−polyacrylamide gels which were subjected to silver staining. Figure 1 shows the characteristic pattern of fragments obtained by limited proteolysis of PI-*Sce*I (Figure 1A) and the PI-*Sce*I−DNA complex (Figure 1B). A striking feature of this pattern is the relatively small number of tryptic fragments, indicating that many Lys and Arg residues of PI-*Sce*I are not readily accessible. Similar results were obtained using other proteases (data not shown). During the first hour of tryptic digestion of PI-*Sce*I, a double band in the molecular mass range of 30−32 kDa and a single band in the molecular mass region of 20 kDa dominate the digestion pattern (Figure 1A). Upon longer trypsin treatment (up to 46 h), PI-*Sce*I was split into small fragments which migrate near the front of the gel. The addition of the 66 bp DNA substrate containing the PI-*Sce*I recognition sequence in equimolar amounts prior to proteolysis stabilizes the 30−32 and 20 kDa fragments (Figure 1B). These fragments are present even after 46 h of trypsinization, when in the absence of DNA mainly small peptides are left over. Edman degradation of the 20 kDa fragment reveals that this fragment represents the C-terminal fragment of PI-*Sce*I, extending from Asn278 to Cys454 (analyzed sequence: NNLNTENPLWDAIVGLGFLK). The N-terminal fragments (30−32 kDa) were also sequenced. The upper fragment (32 kDa) contains the complete N-terminus including the His_6-tag, whereas the lower fragment (30 kDa) lacks a decapeptide at its amino

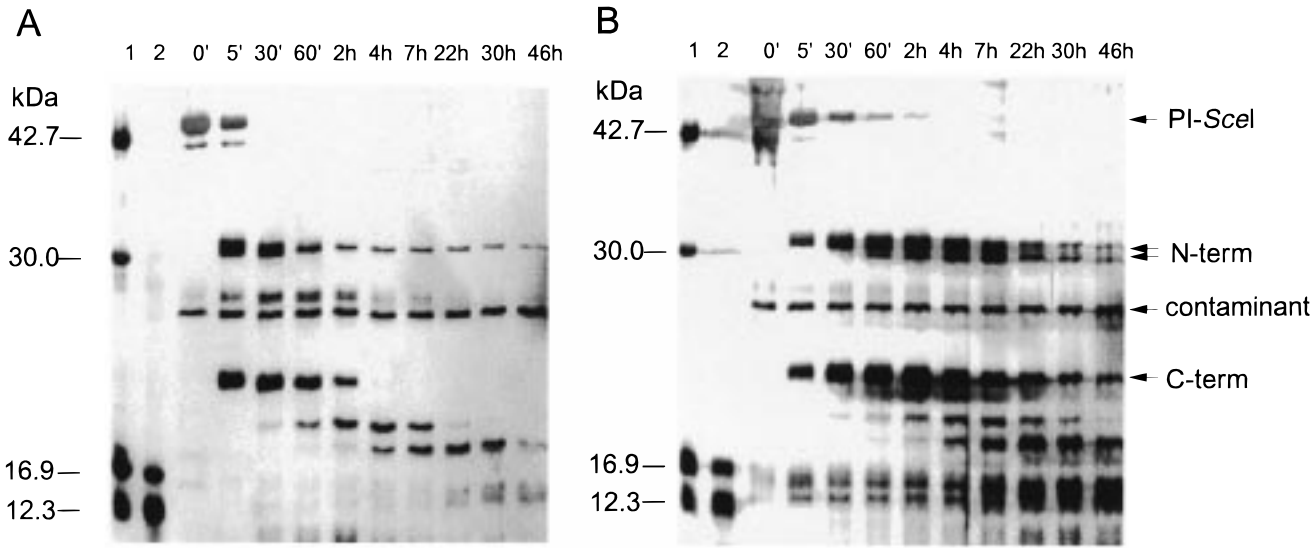


FIGURE 1: Tryptic digestion of PI-*Sce*I in the absence and presence of specific DNA. An SDS−PAGE analysis is shown of the time course of the digestion of PI-*Sce*I (20 μM) with trypsin at a substrate:protease ratio of 200:1 (w/w) without (A) or with (B) a synthetic 66 bp oligodeoxynucleotide (20 μM) containing the PI-*Sce*I recognition site. Lanes 1 and 2, molecular mass standards; arrows indicate intact PI-*Sce*I and initial cleavage products, as well as a 26 kDa contaminant present in the PI-*Sce*I preparation.

terminus and starts with Cys. In highly resolving SDS−PAGE, we observe a double band for PI-*Sce*I after Ni^{2+}-NTA purification. This means that the PI-*Sce*I preparation itself contains a degradation product of slightly lower molecular mass, the self-splicing product of the His_6-tagged PI-*Sce*I. The 10 amino acid shorter version of PI-*Sce*I is generated during purification on a Ni^{2+}-NTA column by elution with a buffer containing imidazole and DTE. Trypsin, therefore, cleaves the PI-*Sce*I molecule (and its shorter version) at Arg277 initially into two halves, the 30−32 kDa N-terminal fragments comprising residues 1−277 (with and without the N-terminal decapeptide) and the 20 kDa C-terminal fragment comprising residues 278−454. Both fragments are effectively protected from further trypsinization by complex formation with specific DNA. Comparison of the digestion patterns of free PI-*Sce*I (Figure 1A) and PI-*Sce*I bound to its recognition sequence (Figure 1B) shows that both patterns are very similar, with the difference that the digestion in the presence of DNA is drastically slowed.

CD Spectroscopy of the Tryptic Digestion of PI-SceI. To find out to what extent the products of a limited proteolysis of PI-*Sce*I preserve the native conformation, we studied the change in secondary structure of PI-*Sce*I during proteolysis. For this purpose, the circular dichroism of the tryptic cleavage reaction mixture was measured after defined time intervals (Figure 2D). The CD spectrum of intact PI-*Sce*I (t = 0 min) is characteristic for an $\alpha\beta$ protein. With increasing time of trypsinization (up to 6 h), the amplitude of the CD signal decreases which might be an indication for digestion to unstructured small peptides. The CD spectrum obtained after 6 h indicates that the remaining large protein fragments have an overall secondary structure composition resembling that of PI-*Sce*I. Inspection of the SDS gel reveals that the enzyme is completely cleaved into the N- and C-terminal fragments after 2−3 h. No nuclease activity can be observed from this time on, whereas binding activity is detectable until 6 h of tryptic digestion. This result implies that the individual secondary structural elements remain largely intact, despite the single cut in the PI-*Sce*I molecule that destroys the covalent connection between the N- and C-terminal halves and leads to catalytic inactivity.

The thermal stabilities of native PI-*Sce*I, and PI-*Sce*I obtained after 2 h of trypsinization (nicked PI-*Sce*I) under limiting conditions, were measured in a melting experiment. The transition temperature determined for PI-*Sce*I and nicked PI-*Sce*I is identical within the limits of experimental error and is about 45 °C. Unfolding of both PI-*Sce*I and nicked PI-*Sce*I is accompanied by aggregation and is irreversible.

Binding and Cleavage Activity of Tryptic Fragments. To measure the DNA binding and cleavage activity of intermediates of the proteolytic digestion, the rate of digestion of PI-*Sce*I by trypsin was reduced by decreasing the trypsin to PI-*Sce*I ratio to 1:500. Figure 2A shows the characteristic pattern of fragments obtained during the 20 h time course of trypsinization. After defined time intervals, the proteolytic digestion products were analyzed for DNA binding and cleavage activity. Binding activity was analyzed in an electrophoretic mobility shift experiment with a radioactively labeled 311 bp substrate with the recognition sequence in the center. After 5 min of digestion, two new bandshifts, B_{II}, with increased mobility appear in addition to bandshift B_I, typical of the native PI-*Sce*I−DNA complex (Figure 2B). The occurrence of the two new bandshifts coincides with the generation of the two N-terminal fragments. This result suggests that the N-terminal fragments, possibly in conjunction with the C-terminal fragment (see below) of PI-*Sce*I, are involved in specific DNA binding. DNA cleavage experiments with the products of the limited tryptic digest clearly showed that specific catalytic activity is only detectable as long as the intact endonuclease is present (Figure 2C). The same result was obtained with Mn^{2+} ions which induce relaxed specificity and increase the activity (data not shown). The primary tryptic cleavage occurs after Arg277 which is between the two LAGLIDADG motifs and separates the two elements which are essential for catalytic activity. It thus appears that catalytic activity is dependent on the covalent connection between these catalytic motifs and that

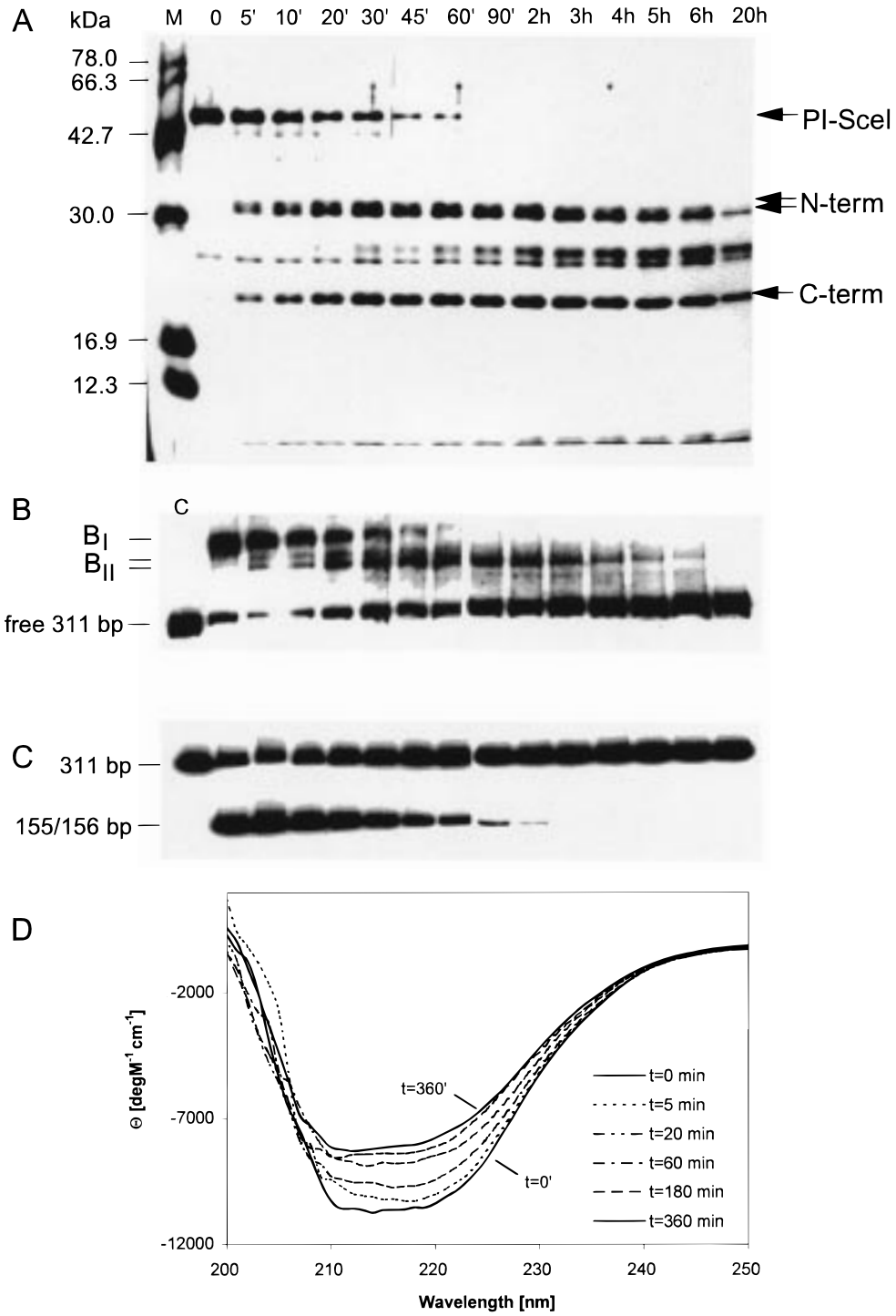
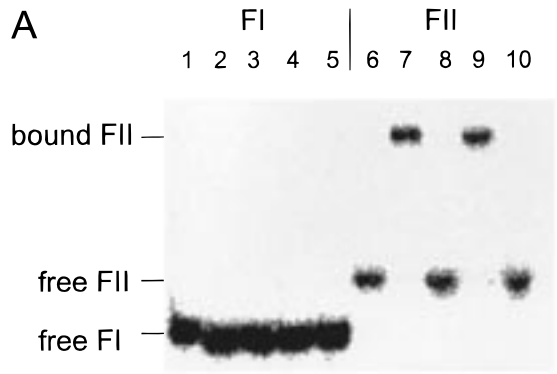


FIGURE 2: Analysis of binding and cleavage activity during tryptic digestion of PI-*Sce*I; correlation with secondary structure. (A) shows the SDS−PAGE analysis of the time course of the digestion of PI-*Sce*I (20 μM) with trypsin at a substrate:protease ratio of 500:1 (w/w). Oligopeptide C-term corresponds to the C-terminal half comprising amino acid residues 278−454, as determined by direct sequencing. Lane M shows the size markers. (B) shows the gel shift analysis of the binding of the products of the partial tryptic digests to a 311 bp DNA substrate. The digestion mixture (50 nM) was incubated with 5 nM ^{32}P-labeled 311 bp substrate in the presence of 10 μg/mL poly-(dI-dC). Bound and unbound species were separated by electrophoresis on a native 6% (w/v) polyacrylamide gel. B_I represents the upper complex and B_{II} the lower complex which is present in two bands. Lane C shows the DNA substrate without protein. In (C), the analysis of the cleavage of the ^{32}P-labeled 311mer DNA substrate (5 nM) with the products of the partial tryptic digest (250 nM) is given. Product analysis was carried out by electrophoresis on a 12% (w/v) polyacrylamide gel. (D) shows the circular dichroism spectra of the products of the tryptic digestion at selected time intervals.

a single cut completely abolishes catalytic activity, but affects specific DNA binding only slightly.

The product mixture obtained after 2 h of tryptic digestion (Figure 2B) is essentially free of intact PI-*Sce*I and contains the N- and C-terminal fragments. This mixture of tryptic fragments was also tested for binding to the cleavage products of the recognition sequence. Specific binding occurs only to one of the products, namely, the one that contains the right half of the PI-*Sce*I recognition sequence (Figure 3A, lane 9). The apparent binding constant is of



B

FI GATCTGACGCCATTATCTATGTCGGGTGC
ACTGCGGTAATAGATACAGCC

FII GGAGAAAGAGGTAATGAAATGGCAGAAGTCT
CACGCCTCTTTCTCCATTACTTTACCGTCTTCAGACTAG

FIGURE 3: Binding of PI-*Sce*I and the products of a partial tryptic digest of PI-*Sce*I to the cleavage products of the recognition site (FI, left half-site; FII, right half-site). In (A), a gel electrophoretic mobility shift assay is shown for PI-*Sce*I and the products of a partial tryptic digest of PI-*Sce*I. 2.5 nM ^{32}P-labeled ds oligodeoxynucleotide FI (lanes 1−5) or FII (lanes 6−10) was incubated with 10 nM PI-*Sce*I without (lanes 2 and 7) or with a 1000-fold molar excess of unlabeled FI (lane 3) or FII (lane 8). Lanes 4 and 9 show the results obtained with trypsinized PI-*Sce*I without and with (lanes 5 and 10) unlabeled FI or FII. In (B), the sequences of the oligodeoxynucleotides used are shown, which represent the cleavage products of the full-length recognition sequence.

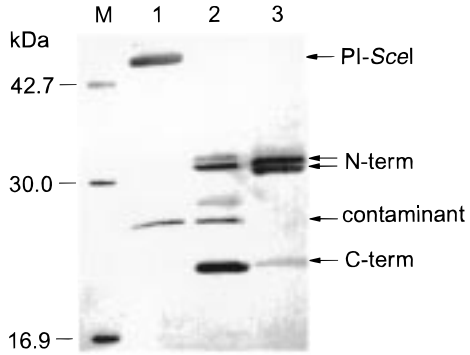


FIGURE 4: SDS−PAGE analysis of the tryptic peptides obtained from a preparative gel shift of a DNA substrate and the products of a partial tryptic digest of PI-*Sce*I. PI-*Sce*I was digested with trypsin for 2 h (see Figure 2) and then mixed with a ^{32}P-labeled 311 bp PCR product. The reaction mixture was loaded onto a preparative shift gel, and the band corresponding to the complex was cut out. After elution, peptides were precipitated and analyzed on a 15% (w/v) SDS−polyacrylamide gel. Lane M shows the size markers; lane 1, PI-*Sce*I (containing a contaminant of 26 kDa); lane 2, trypsinized PI-*Sce*I before and lane 3 after isolation from an electrophoretic mobility shift gel. The gel was silver-stained.

similar magnitude as the one determined for intact PI-*Sce*I (Figure 3A, lane 7; ref *13*), as determined in a titration experiment (data not shown). No interaction between intact PI-*Sce*I (Figure 3A, lane 2) or trypsinized PI-*Sce*I (Figure 3A, lane 4) and the DNA cleavage product corresponding to the left side of the PI-*Sce*I recognition sequence can be detected. From the result of these experiments, we can only conclude that the N-terminal fragment of PI-*Sce*I, possibly in conjunction with the C-terminal fragment (see below) of PI-*Sce*I, is capable of interacting specifically with the right part of the recognition site.

Analysis of the Physical Interaction between the N- and C-Terminal Fragments of PI-SceI . It might well be that after trypsin cleavage the N- and C-terminal fragments interact with each other. To analyze to what extent the N- and C-terminal fragments of PI-*Sce*I cooperate in DNA binding, the protein content of the protein−DNA complexes formed after a 2 h tryptic digestion and purified by an electrophoretic mobility shift experiment was analyzed. The respective bands were cut out from a preparative shift gel and analyzed by SDS−PAGE (Figure 4). Lane 1 represents intact PI-*Sce*I, lane 2 PI-*Sce*I after a 2 h tryptic digestion, and lane 3 the protein composition of the digest after elution from a shifted band. In addition to the N-terminal fragments (30−32 kDa), the C-terminal fragment (20 kDa) can be detected, sometimes in lower amounts than the N-terminal fragment (cf. Figure 4, lane 3) which may indicate that the N-terminal fragment is bound more firmly to the DNA than the C-terminal fragment. These fragments are present in a shifted protein−DNA complex, no matter which DNA substrate is used, the complete recognition sequence or the cleavage product with the right half site (data not shown). Binding of the N- together with the C-terminal fragment to the DNA substrate or the DNA cleavage product may be explained by a strong protein−protein interaction between the two parts of the PI-*Sce*I molecule and/or by a strong binding of both parts to the DNA. Gel filtration experiments with the products of the limited tryptic digestion on a Superdex HR 75 FPLC gel-filtration column under nondenaturing conditions revealed that the 30−32 kDa tryptic fragments eluted in the same fractions as the 20 kDa fragment (Figure 5C) with an elution volume corresponding to that of intact PI-*Sce*I (Figure 5A). The formation of a complex between the N- and C-terminal fragments demonstrates that the two fragments are held together also in the absence of DNA. We conclude from these experiments that the N- and C-terminal fragments remain tightly associated after limited proteolysis and may be both involved in DNA binding.

Proteolytic Fragments of PI-SceI Generated by Different Proteases. We subjected PI-*Sce*I to limited proteolysis also by other proteases than trypsin in order to further specify regions with activity. Reaction conditions for proteolysis by chymotrypsin, proteinase K, and subtilisin that gave stable intermediate products were established. With all proteases, we found a doublet of fragments around 30−32 kDa and a fragment of 20 kDa despite the different specificities of the enzymes which lead to differences in the overall fragment patterns (data not shown). All proteases tested, therefore, seem to attack PI-*Sce*I in the same region as trypsin. The product mixtures obtained with these proteases in partial digests resemble in their DNA binding activity and the absence of DNA cleavage activity the product mixture of trypsinolysis (data not shown). It thus appears that PI-*Sce*I has a hypersensitive region for proteolysis. Cleavage within this region abolishes DNA cleavage activity, but does not dramatically affect the affinity for DNA.

Analysis of DNA Binding by Genetically Engineered PI-SceI-N and PI-SceI-C. The partial amino acid sequence of the C-terminal fragment obtained by limited proteolysis of PI-*Sce*I allowed identification of the position where trypsin proteolytically attacks PI-*Sce*I and the PI-*Sce*I−DNA com-

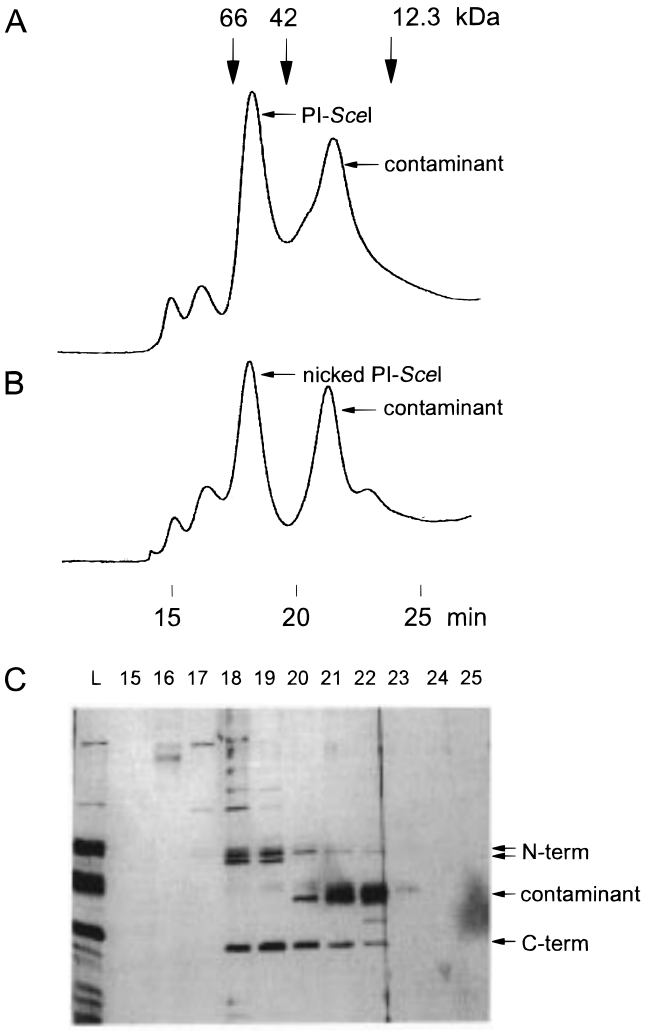


FIGURE 5: Gel filtration of PI-*Sce*I and a tryptic digest of PI-*Sce*I. In (A), the elution profile of native PI-*Sce*I (the preparation contains a contaminant of 26 kDa) on a Superdex HR 75 10/30 gel filtration column is shown. The elution positions of marker proteins are indicated (66 kDa, BSA; 42 kDa, ovalbumin; 12.3 kDa; cytochrome *c*). In (B), the elution profile of PI-*Sce*I after trypsinization for 30 min performed as described in the legend to Figure 1 is shown. (C) SDS−PAGE analysis of the eluted fractions of (B). L represents an aliquot of the tryptic digest before gel filtration.

plex. Proteins corresponding to the N-terminal fragment (PI-*Sce*I-N: amino acids 1−277) and the C-terminal fragment (PI-*Sce*I-C: amino acids 278−454) were molecularly cloned and expressed as His_6-tagged proteins (for details, cf. ref *13*). After affinity chromatography on Ni^{2+}-NTA−agarose, the proteins were over 95% pure and showed an apparent molecular mass of 32.8 kDa (PI-*Sce*I-N) and 20.5 kDa (PI-*Sce*I-C), respectively.

Binding experiments revealed that PI-*Sce*I-N specifically interacts with the full-length recognition sequence (Figure 6A, lanes 10, 11, and 12) as well as with the DNA cleavage product comprising the right part of the recognition site (Figure 6B, lanes 13−17). PI-*Sce*I-C, in contrast, does not bind to the recognition sequence (Figure 6A, lanes 14, 15, and 16) or to parts of it (data not shown). A comparison of the intensity of the bandshifts obtained with PI-*Sce*I and PI-*Sce*I-N shows that PI-*Sce*I binds about 1−2 orders of magnitude more strongly to the PI-*Sce*I recognition site than the recombinant PI-*Sce*I-N. This could reflect that a substantial part of the PI-*Sce*I-N preparation obtained after preparation under denaturing conditions with subsequent renaturing contains uncorrectly folded PI-*Sce*I-N molecules but could also mean that PI-*Sce*I-N needs PI-*SceI*-C to bind DNA with full affinity. The combination of PI-*Sce*I-N and PI-*Sce*I-C (Figure 6A, lanes 18, 19, and 20), which for this experiment were renatured together, indeed demonstrates that PI-*Sce*I-C stimulates DNA binding by PI-*Sce*I-N, but not to the same level as observed with PI-*Sce*I or nicked PI-*Sce*I. This could be due to the fact that recombinant PI-*Sce*I-N and/or PI-*Sce*I-C are not properly folded. The CD measurements with the recombinant PI-*Sce*I-N and PI-*Sce*I-C indicate that PI-*Sce*I-N is well structured, while PI-*Sce*I-C shows only a very small negative CD effect between 200 and 250 nm (data not shown), which demonstrates that it is not a random coil, but also that it does not adopt the same conformation as present in the intact structure of PI-*Sce*I. Neither PI-*Sce*I-N alone nor in combination with PI-*Sce*I-C is catalytically active, not even in the presence of Mn^{2+} ions (data not shown).

Bending of DNA by the Products of a Partial Tryptic Digest and PI-SceI-N. PI-*Sce*I forms two complexes with substrate DNA which differ in the degree of DNA distortion (*13, 14*). In the slowly migrating complex, the bending angle is 75° (±15°), in the quickly migrating complex 45° (±15°). As the products of a partial tryptic digest of PI-*Sce*I as well as the genetically engineered PI-*Sce*I-N bind firmly and specifically to DNA, but only form one complex with DNA, it was of obvious interest to find out whether they distort the DNA and whether both bend the DNA to a similar extent. To analyze the DNA distortion produced by the partial tryptic digest of PI-*Sce*I, PI-*Sce*I-N, and PI-*Sce*I-N in combination with PI-*Sce*I-C, we performed electrophoretic mobility shift assays using DNA substrates of identical size but with the recognition site in different positions (*25*). The electrophoretic mobility shifts obtained with DNA containing the PI-*Sce*I recognition site and native PI-*Sce*I, the products of the partial tryptic digest of PI-*Sce*I, PI-*Sce*I-N, as well as PI-*Sce*I-N in combination with PI-*Sce*I-C, respectively, are shown in Figure 6A. The free 201 bp DNA fragments display the same mobility regardless of the position of the binding site within the probe. Among the bending probes which were incubated with PI-*Sce*I (lanes 2, 3, and 4), the probe with the binding site positioned in the center (lane 3) shows two shifted bands representing the upper protein−DNA complex with the strongly distorted DNA and the lower complex with the weakly distorted DNA (*13, 14*). When PI-*Sce*I after partial digestion with trypsin for 2 h (Figure 2A) is incubated with the same DNA probes, only a quickly migrating complex appears (lanes 6, 7, and 8) whose electrophoretic mobility is comparable to that of the lower complexes produced by intact PI-*Sce*I. As we found that both the N- and C-terminal fragments of PI-*Sce*I are bound to the full-length recognition site, this result suggests that a specific cut after amino acid 277 destroys the ability for the large DNA distortion induced by native PI-SceI. When PI-*Sce*I-N is incubated with the probes containing the full-length recognition site, again only one protein−DNA complex is observed (lanes 10, 11, and 12) with the same mobility as that of the lower PI-*Sce*I−DNA complex or of the complex obtained with the products of the partial tryptic digest of PI-*Sce*I though the molecular mass is much smaller. PI-

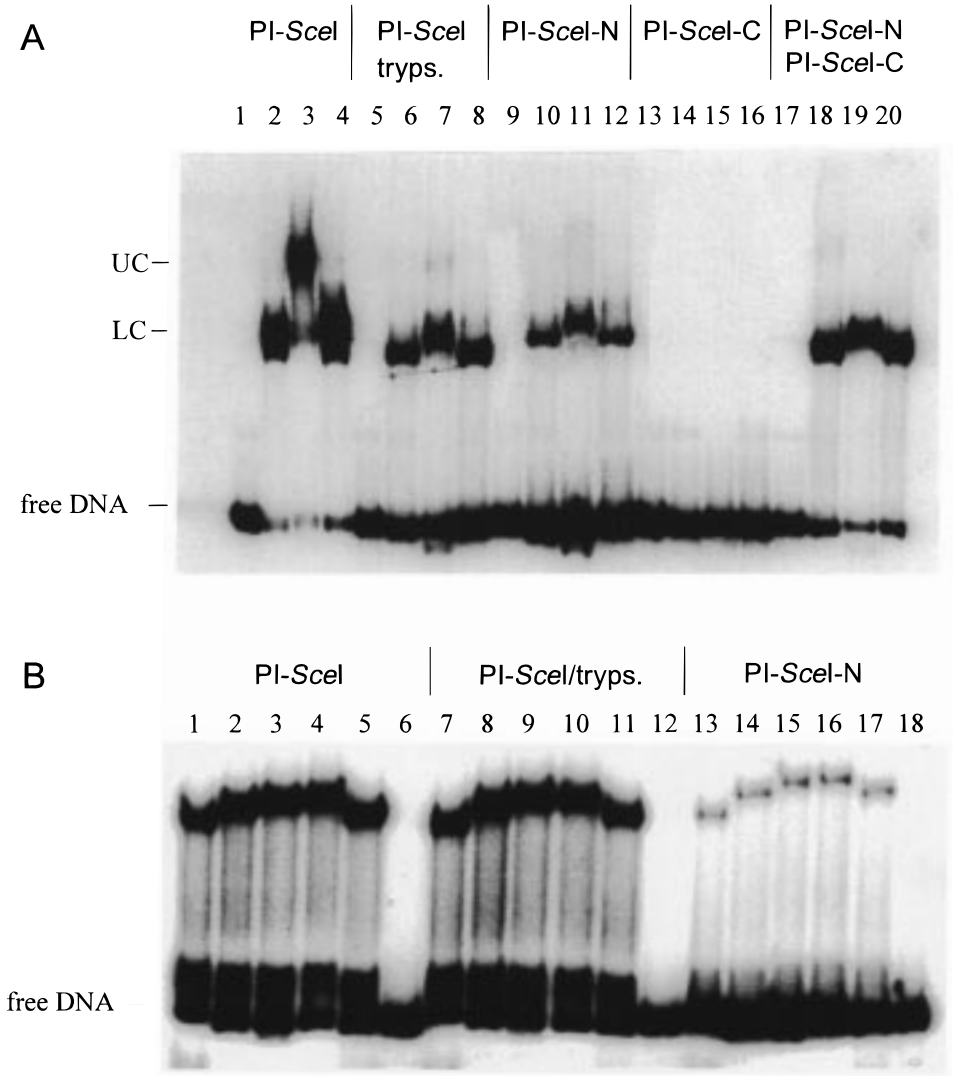


FIGURE 6: Circular permutation analysis of DNA binding by PI-*Sce*I, the products of a partial tryptic digest, as well as the recombinant N- and C-terminal fragments, PI-*Sce*I-N and PI-*Sce*I-C. (A) Circular permutation probes were derived from pBend2-F by cleavage with the restriction enzymes *Mlu*I, *Sma*I, and *Bam*HI containing the full-length PI-*Sce*I recognition sequence in different positions. The electrophoretic mobility shift assay with PI-*Sce*I, 10 nM (lanes 2−4), trypsinized PI-*Sce*I, 25 nM (lanes 6−8), PI-*Sce*I-N, 400 nM (lanes 10−12), PI-*Sce*I-C, 400 nM (lanes 14−16), and a combination of these two, 200 nM each (lanes 18−20), was performed with three different bending probes of 201 bp length. The positions of the upper (UC) and the lower complex (LC) of PI-*Sce*I with DNA are indicated. Lanes 1, 5, 9, 13, and 17 show the respective bending probes without added protein. (B) Shift fragments were derived from pBend2-FII, containing the right half of the recognition site, by cleavage with the restriction enzymes *Mlu*I, *Spe*I, *Pvu*I, *Sma*I, and *Bam*HI. The electrophoretic mobility shift assay was performed with five different bending probes of 160 bp length with PI-*Sce*I (lanes 1−5), trypsinized PI-*Sce*I (lanes 7−11), and PI-*Sce*I-N (lanes 13−18). Lanes 6, 12, and 18 show the free probe without protein.

*Sce*I-C alone does not exhibit any DNA binding activity (lanes 14, 15, and 16), whereas the combination of PI-*Sce*I-N and PI-*Sce*I-C shows the same result as obtained with PI-*Sce*I-N alone (lanes 18, 19, and 20).

The respective shifts with probes containing the right half of the recognition sequence are presented in Figure 6B. Incubation with PI-*Sce*I generates only a single protein−DNA complex (lanes 1−5), in agreement with published results (*14*). The DNA bending was calculated to be approximately 45°, which is comparable to the bending observed in the lower complex produced by PI-*Sce*I and the full-length probes. This result suggests that binding of PI-*Sce*I to the right half of the recognition site DNA produces a minor distortion of 45° (±15°). When we performed these analyses with the products of a partial tryptic digest of PI-*Sce*I, we observed again only one protein−DNA complex (lanes 7−11). The same result was obtained with PI-*Sce*I-N (lanes 13−17). The N-terminal fragment in combination with the C-terminal fragment either generated proteolytically or expressed as recombinant proteins shows identical results: binding to substrate DNA or product DNA leads to very similar protein−DNA complexes with the same slightly distorted DNA. This similarity is suggested by the same electrophoretic mobility of the tryptic digest/full-length DNA complexes and the lower complexes generated with PI-*Sce*I and the full-length substrate, respectively, as well as by the same electrophoretic mobility of the tryptic digest/pBend2-FII complexes and the respective PI-*Sce*I/pBend2-FII complexes, respectively.

DISCUSSION

PI-*Sce*I is a bifunctional protein which harbors catalytic centers for protein splicing and phosphodiester bond cleavage. This dual function suggested that PI-*Sce*I might have

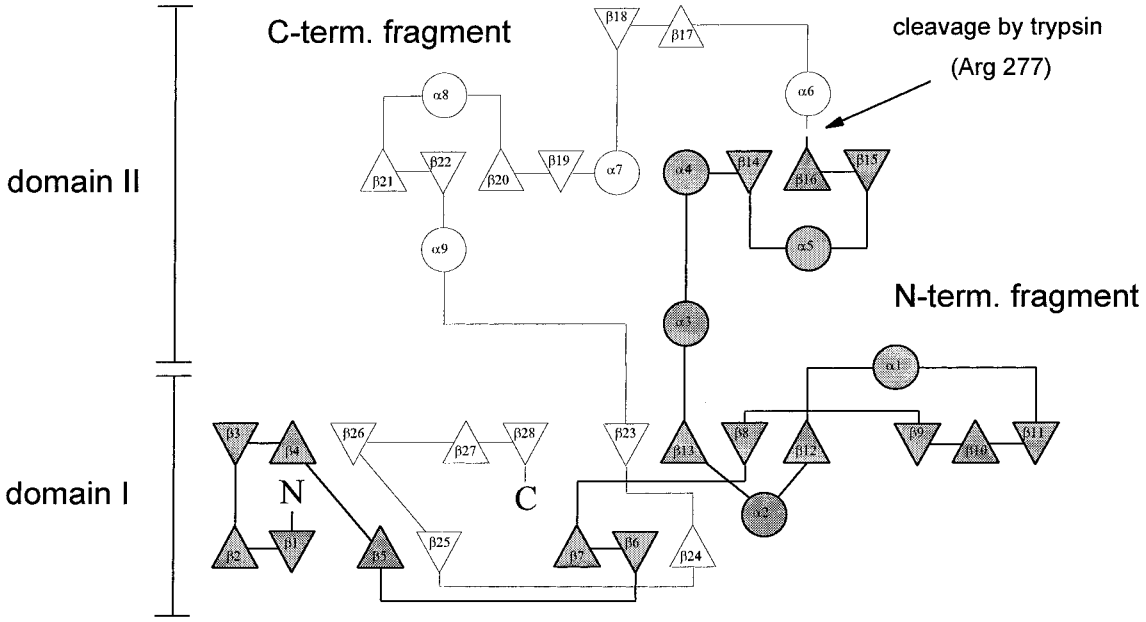


FIGURE 7: Schematic representation of the topological organization of the secondary structural elements of PI-*Sce*I [adapted from Duan et al. (20)]. PI-*Sce*I comprises two domains: domain I containing the N- and the C-terminus in whose proximity the catalytic activity for protein splicing is located; and domain II with the two LAGLIDADG motifs (in the symmetry-related α-helices 4 and 7) involved in the nucleolytic activity. Between *β*-strand 16 and α-helix 6, a protease-sensitive loop region is located where cleavage defines the N- and C-terminal fragments. Note that *β*-strands 24, 25, and 26 of the C-terminal fragment are parts of mixed *β*-sheets which may stabilize the association of the N- and C-terminal fragments after trypsin-catalyzed cleavage at Arg277.

a two-domain structure, with one domain being responsible for the autocatalytic removal of PI-*Sce*I from the precursor protein, and the other domain for the catalysis of DNA cleavage. This suggestion was supported by the finding that deletions of the LAGLIDADG motifs, which are considered to be part of the nucleolytic catalytic center(s), do not affect the protein splicing activity (*18*). Vice versa, amino acid substitutions in the N- and C-terminal regions of PI-*Sce*I, where the catalytic center for protein splicing is located, do not have a major effect on the nucleolytic activity (W. Grindl and W. Wende, unpublished). The recently solved crystal structure (*20*) confirmed the suggestion that PI-*Sce*I is a two-domain protein with clearly separated regions responsible for protein splicing (domain I) and nucleolytic activity (domain II) (Figure 7). It is conceivable, therefore, that the two catalytic activities can be physically separated. On the other hand, PI-*Sce*I, a 454 amino acid protein, recognizes a very large DNA sequence, extending over 40 bp with an oligodeoxynucleotide substrate (*12, 13*). This means that PI-*Sce*I must interact with DNA over a substantial part of its surface. The docking model put forward by the authors of the crystal structure of PI-*Sce*I (*20*) illustrates this suggestion by assuming that the protein splicing domain I and the nucleolytic domain II each interact with almost two turns of the DNA double helix. This situation is reminiscent of type IIs restriction enzymes, like *Fok*I, which bind firmly to their recognition site and cleave the DNA at a distance, thereby covering an extended piece of DNA (2*6, 27*). The results presented here demonstrate that PI-*Sce*I when subjected to limited proteolysis is cleaved at Arg277 into two fragments of not too dissimilar size—with trypsin, fragments of 277 and 177 amino acid residues are produced, which are catalytically inactive but together bind specifically to their substrate and, as native PI-*Sce*I, also to one of the products. The two tryptic fragments of PI-*Sce*I remain associated, which is not surprising considering the fact that the N- and the C-terminal fragments of PI-*Sce*I, according to the crystal structure (*20*), are intimately connected with each other via four *β*-sheets (Figure 7). The two tryptic fragments not only remain associated but also preserve essential features of the structure of native PI-*Sce*I, as demonstrated by the analysis of the circular dichroism. The experimental finding that PI-*Sce*I cleaved at Arg277 ('nicked PI-*Sce*I') is active in DNA binding could mean that both the N- and C-terminal fragments are involved in specific DNA binding. Indeed, in a preparative gel shift experiment, a complex was isolated which contained DNA, the N-terminal fragment, and, in variable amounts, also the C-terminal fragment. To resolve the questions, whether the combination of the fragments or which of the two fragments is responsible for DNA binding, the N- and C-terminal fragments were produced as recombinant proteins, PI-*Sce*I-N and PI-*Sce*I-C, and analyzed with respect to their DNA binding and cleavage activity. The results show that PI-*Sce*I-N, which contains a substantial part of domain I and a small part of domain II, is capable of specific DNA binding, and that both fragments, alone or in combination, are catalytically inactive. A comparison with native and nicked PI-*Sce*I on one side and PI-*Sce*I-N on the other side shows that the recombinant N-terminal fragment binds DNA not as firmly as the intact or the proteolytically cleaved enzyme, which could indicate that in PI-*Sce*I-N the DNA binding site is not quite complete.

Not only the full-length recognition site is bound by PI-*Sce*I, but also the right half-site (*13*) which corresponds to one of the cleavage products. This has also been observed with other LAGLIDADG enzymes, namely, I-*Sce*I (*28*), I-*Dmo*I (*29*), and I-*Cre*I (*30*), but also with the GIY-YIG enzymes I-*Tev*I and I-*Tev*II (*31*), whereas the LAGLIDADG enzyme I-*Por*I (*32*) and I-*Ppo*I (*33*), which is not a member of the LAGLIDADG family, are released after catalysis.

Binding of the endonucleases to one of their cleavage products may have consequences in recombination events (*34*). We have shown here that also nicked PI-*Sce*I and PI-*Sce*I-N, like native PI-*Sce*I, bind firmly to one of the products of the DNA cleavage reaction, corresponding to the right half-site.

DNA binding by proteins often is accompanied by DNA bending. For I-*Tev*I and I-*Tev*II as well as for PI-*Sce*I, two distortions have been observed (*13, 14, 31, 35*), and it was shown that bending is directed into the major groove. While DNA is structurally intact in the bent PI-*Sce*I complexes, one of the distortions induced by I-*Tev*I and I-*Tev*II is accompanied by a nick, which implies a sequential cleavage mechanism. Cleavage in one strand was also detected with other LAGLIDADG homing endonucleases as, for example, I-*Sce*I (*28*), I-*Chu*I, I-*Ceu*I, and I-*Cpa*II (*36*), depending on the reaction conditions, in particular on the Mg^{2+} concentration. Different from native PI-*Sce*I which forms two complexes of different electrophoretic mobility with the DNA substrate [lower and upper complex (*13, 14*)], nicked PI-*Sce*I or PI-*Sce*I-N only form one complex with the DNA substrate. In this complex, the DNA is bent by 45°, similarly as observed for the lower complex. We argue that the N-terminal fragment binds and bends the DNA as does native PI-*Sce*I upon initial complex formation with DNA. PI-*Sce*I then distorts the DNA further to give a bend of 75° which positions the scissile phosphodiester bonds into the active site(s) by making additional contacts which are essential for catalysis (*13, 14*).

Nicked PI-*Sce*I and PI-*Sce*I-N, which like native PI-*Sce*I bind to the right half-site, also bend DNA containing a right half-site, again like PI-*Sce*I by about 45°. Thus, while PI-*Sce*I (two complexes are formed) binds its substrate in a qualitatively different manner from nicked PI-*Sce*I and PI-*Sce*I-N (one complex is formed), native PI-*Sce*I, nicked PI-*Sce*I, and PI-*Sce*I-N seem to interact with their products in the same manner.

Comparison of the results of DNA binding and cleavage experiments obtained with native PI-*Sce*I, nicked PI-*Sce*I, PI-*Sce*I-N, and PI-*Sce*I-C allows the following conclusions to be drawn:

(I) Specific binding tolerates a discontinuity of the peptide backbone in the proteolytically hypersensitive loop between β-strand 16 and α-helix 6 as observed with nicked PI-*Sce*I (Figure 7).

(II) Specific binding is dependent on the presence of a large part of domain I and possibly also a small part of domain II, as observed with PI-*Sce*I-N. Conversely, if large parts of domain II are removed, as in PI-*Sce*I-C, no DNA binding can be detected. In agreement with this conclusion is the finding that the genetically engineered domain I binds DNA specifically and with the same affinity as native PI-*Sce*I, while the isolated domain II is inactive in DNA binding and catalysis (*37*).

(III) A major DNA distortion, bending by 75°, is a prerequisite for DNA cleavage. This distortion is only observed with native PI-*Sce*I (*13, 14*). In nicked PI-*Sce*I, a peptide bond in a loop is cleaved which connects a subdomain comprising β-sheet 7 and α-helix 6 with the catalytic core. This cleavage prevents the conformational transition to a catalytically competent complex. In line with this conclusion is the finding that neither PI-*Sce*I-N (this paper) nor the isolated domain I (*37*), both of which lack this subdomain, is able to bend the DNA by more than 45°, which is the bend angle observed with PI-*Sce*I in the initial enzyme−substrate and the enzyme−product complex (*13, 14*). Intriguingly, the catalytically inactive PI-*Sce*I variant K301A (20) is not able to induce the 75° bend observed with PI-*Sce*I, whereas the D218A and D326A variants in which the catalytically essential aspartic acids at positions 218 and 326 in the two LAGLIDADG motifs (*12*) are substituted by alanine, behave as wild-type PI-*Sce*I in bending experiments (W. Grindl and W. Wende, unpublished). Possibly, Lys301 is part of the molecular lever responsible for this large bending of the substrate molecule, rather than being directly involved in catalysis (*20*). A similar finding was made with I-*Tev*I: when in I-*Tev*I a conserved Arg residue downstream of the GIY-YIG sequence was mutated to Ala (R27A), it was shown that this mutant does not bend the homing site and has no detectable DNA cleavage activity (*38*).

(IV) Nicked PI-*Sce*I and PI-*Sce*I-N, like native PI-*Sce*I (*13*) and the isolated domain I (*37*), also interact specifically with DNA comprising the right side of the recognition site, which corresponds to one of the cleavage products. This binding is accompanied by a bending of the DNA by about 45°. It seems as if bending of the DNA by 45° characterizes not only the process of substrate association but also that of product dissociation. Comparison of PI-*Sce*I-N and domain I (Figure 7 and Figure 2 in ref. *20*), both of which bind DNA specifically, indicates that the right part of domain I is mainly responsible for DNA binding, in agreement with the docking model (*20*).

(V) The fact that nicked PI-*Sce*I and PI-*Sce*I-N are inactive clearly demonstrates that subtle structural perturbations, such as a single proteolytic nick, can abolish both strong DNA bending and catalytic activity. It seems that strong DNA bending is a prerequisite for cleavage. The question, of course, is, why this distortion is required at all. Before a cocrystal structure is available, this is a matter of speculation. However, if one accepts the premise that the two homing endonucleases PI-*Sce*I and I-*Cre*I, both of which belong to the LAGLIDADG family, follow a similar mechanism of action, the requirement for a major distortion of DNA and presumably also of a conformational transition of the protein in the PI-*Sce*I−DNA recognition complex is obvious. Only with a bending of the DNA and presumably a conformational change of the protein can the DNA make extensive contacts to domain II and fit into the saddle groove formed by β-sheets 7 and 9, similarly as suggested for DNA binding to I-*Cre*I (*39*). As I-*Cre*I does not have the equivalent of the PI-*Sce*I domain I, it can be expected that I-*Cre*I does not bend DNA as much as PI-*Sce*I (*39*). That PI-*Sce*I and I-*Cre*I bind DNA in a similar manner is not unlikely, as comparison of the crystal structures of these enzymes (*20, 39*) reveals a striking similarity between domain II of PI-*Sce*I with its local 2-fold symmetry axis and the homodimer of I-*Cre*I. Nevertheless, Gimble, Quiocho, and their colleagues (*20*) and Stoddard and colleagues (*39*) favor different mechanisms of DNA cleavage for PI-*Sce*I and I-*Cre*I, respectively. Gimble and colleagues have suggested that PI-*Sce*I has only one catalytic center, comprising Asp216 and Asp326, that cleaves the two strands of the DNA duplex sequentially (*11, 12, 20*). We proposed that the concerted

cleavage, observed with PI-*Sce*I, argues for two catalytic centers (*13*), one with Asp218, the other with Asp326 as catalytically essential residues. The proposed I-*Cre*I mechanism involves two active sites, one per subunit, with Asp20 at the end of the LAGLIDADG motif (*39*), corresponding to Asp218 and Asp326 of PI-*Sce*I, respectively. With the premise, as mentioned above, that LAGLIDADG homing endonucleases are likely to follow a basically similar mechanism of action, we again suggest that PI-*Sce*I, like the homodimeric I-*Cre*I, has two active sites in which the C-terminal Asp serves to ligand the essential Mg^{2+} ion (see also Dalgaard et al., ref *40*).

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