Sequence Preferences in Cleavage of dsDNA and ssDNA by the Extracellular *Serratia marcescens* Endonuclease†

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ABSTRACT: The preferred cleavage sites in dsDNA and ssDNA for the extracellular *Serratia marcescens* endonuclease (commercially available as BENZONASE) were identified by limited digestion of PCR-generated substrates. Two different dsDNA substrates were synthesized by using either radioactively or fluorescent dye labeled primers. ssDNA of identical sequence to one of the fluorescent dye labeled duplex strands was prepared by affinity chromatography. Cleavage experiments carried out under single hit conditions demonstrate that the enzyme shows preferences for GC-rich regions in dsDNA, in particular d(G)•d(C)-tracts, and avoids cleavage of d(A)•d(T)-tracts. There is a correlation between cleavage at a given position in one strand with cleavage at the same position in the other strand of the duplex. ssDNA cleavage occurs at somewhat different preferred sites than observed in dsDNA. On dsDNA, the *Serratia* nuclease produces a very different cleavage pattern compared to bovine pancreatic DNase I, with the notable exception that both enzymes avoid d(A)•d(T)-tracts. In general, the *Serratia* nuclease compared to DNase I is a slightly more nonspecific endonuclease that attacks a particular substrate more evenly under standard reaction conditions. At high ionic strength or in the presence of DMSO, it becomes more nonspecific. Addition of urea, however, makes the enzyme more selective than observed under standard conditions. From these results which were confirmed by the results of cleavage experiments with synthetic oligodeoxynucleotides, we conclude that the *Serratia* nuclease like DNase I is sensitive to global features of the DNA, for example, the width of the minor groove. In addition, localized sequence-dependent interactions between substrate and nuclease determine whether a site is cleaved preferentially. Some of these interactions seem to be the same for ds- and ssDNA.

The extracellular endonuclease of the pathogenic Gram-negative bacterium *Serratia marcescens* (commercially available as BENZONASE) is produced as a precursor of 266 amino acids that is proteolytically processed upon secretion to give a protein of 245 amino acids (Ball *et al.*, 1987) with 2 disulfide bonds that are required for its activity (Ball *et al.*, 1992). Recently, the structure of the nuclease has been solved by a crystallographic analysis (Miller *et al.*, 1994). The enzyme is a dimer of identical subunits as could be shown by ultracentrifugation analysis (Friedhoff *et al.*, 1994a) and by crystallographic studies (Krause, University of Houston). At higher concentration, the enzyme forms helical fibers (Johannssen, E. Merck Co., Darmstadt, Germany). It is very stable toward chemical denaturation (Yonemura *et al.*, 1983; Biedermann *et al.*, 1989). Its temperature optimum is between 37 and 44 °C. Like many nucleases [for a review, cf. Gerlt (1993) and Fraser & Low (1993)], it requires Mg^{2+} as a cofactor, with an optimum concentration between 5 and 10 mM. Some other divalent cations such as Mn^{2+}, or Ni^{2+} and Co^{2+}, not, however, Ca^{2+}, can substitute for Mg^{2+} (Nestle & Roberts, 1969a; Friedhoff, unpublished results). Little is known so far about its mechanism of action, but several amino acid residues that are involved in catalysis have been identified by a mutational analysis which was based on an alignment of several nucleases of similar function (Friedhoff *et al.*, 1994b). A most striking characteristic of the extracellular *Serratia* nuclease is its broad substrate specificity. The nuclease is able to cleave both single- and double-stranded DNA and RNA, producing 5′-phosphorylated mono-, di-, tri-, and tetranucleotides (Eaves & Jeffin, 1963; Nestle & Roberts, 1969b). The synthetic homopolymers poly(U), poly(G), poly(C), poly[d(A)], poly[d(T)], and poly-[d(A)]•poly[d(T)], however, are resistant to cleavage. Poly-(A)•poly(U) and poly(A) are cleaved only at a very low rate. Poly(I)•poly(C) is cleaved as readily as natural DNA and RNA (Yonemura *et al.*, 1983; Filimonova, 1994). Interestingly, the enzyme cleaves apyrimidinic DNA far better than apurinic DNA (Balaban *et al.*, 1971a,b). So far, no pronounced sequence preferences in cleaving DNA and RNA of natural origin have been reported for the *Serratia* nuclease.

Therefore, we began to analyze in detail the cleavage of polymerase chain reaction (PCR)[1]-generated dsDNA substrates by the *Serratia* nuclease and for comparison by DNase I. These substrates were labeled either radioactively or with

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[1] Abbreviations: bio-, biotin label; bp, base pair(s); DMSO, dimethyl sulfoxide; dsDNA, double-stranded DNA; fluo-, fluorescent dye label; FITC, fluorescein isothiocyanate; li, linear; NHS, *N*-hydroxysuccinimide ester; oc, open circular; PCR, polymerase chain reaction; sc, supercoiled; ssDNA, single-stranded DNA.

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a fluorescent dye. Additionally, a fluorescent dye labeled ssDNA substrate with identical sequence to one of the dye-labeled duplex strands was prepared by affinity chromatography and also analyzed for preferred cleavage sites. The principal aim of this investigation was to find out if the extracellular *Serratia* nuclease exhibits sequence selectivities similarly as reported for bovine pancreatic DNase I (Drew & Travers, 1984; Herrera & Chaires, 1994; Doherty *et al.*, 1995) and whether this selectivity is the same for double- and single-stranded DNA, as well as to try to understand the molecular basis of this selectivity.

Here, we demonstrate that the *Serratia* nuclease exhibits different preferences in cleaving double-stranded DNA when compared to DNase I, indicating that localized interactions which are characteristic for each enzyme/substrate pair have a strong influence on the rate of cleavage. Furthermore, since for the *Serratia* nuclease there exist similar, albeit not identical, preferred cleavage sites on double- and single-stranded DNA, it is likely that some of the sequence-dependent interactions between enzyme and DNA are the same in the double- and single-stranded substrate. On the other hand, for both the *Serratia* nuclease and the DNase I, d(A)•d(T)-tracts are refractory to cleavage, suggesting that global helix parameters have a similar effect on both enzymes.

It must be emphasized that the *Serratia* nuclease is a member of an expanding family of homologous nucleases identified in different species, *viz.*, *Anabaena* (Muror-Pastor *et al.*, 1992), *Syncephalastrum* (Chen *et al.*, 1993), *Saccharomyces* (Zassenhaus & Denniger, 1994), and *Bos* (Cote & Ruiz-Carrillo, 1993). It will be of interest to find out whether these nucleases which share pronounced sequence similarity with the *Serratia* nuclease, but at least in part seem to serve different cellular functions, have similar sequence preferences as the *Serratia* nuclease.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes. Chemicals for electrophoresis were purchased from Gibco-BRL (Gaithersburg, MD) or Baker (Deventer, Holland); chemicals for oligonucleotide synthesis were from Millipore (Eschborn, Germany). Aminolink II and fluorescent dyes were from ABI (Weiterstadt, Germany). FITC, avidin−agarose beads, and biotinyl-ϵ-aminocaproic acid *N*-hydroxysuccinimide ester were obtained from Sigma (Deisenhofen, Germany). All other chemicals were supplied by Merck (Darmstadt, Germany). Restriction enzymes were purchased from Amersham-Buchler (Braunschweig, Germany) or Boehringer-Mannheim (Germany). T4 polynucleotide kinase, *Taq* DNA-polymerase, and Δ*Taq* DNA-polymerase were obtained from Amersham-Buchler. DNase I, T7 DNA-polymerase, and NAP10 columns were purchased from Pharmacia Biotech Europe (Freiburg, Germany). QIAquickspin PCR purification kits for PCR product purification and a Qiagen plasmid kit for plasmid midipreparation were obtained from Qiagen (Hilden, Germany). Centricon 30 microconcentrators were purchased from Amicon (Witten, Germany). Columns for affinity chromatography were obtained from Mobitec (Göttingen, Germany). DEAE-cellulose thin-layer plates were from Macherey-Nagel (Düren, Germany). All enzymes and materials were used according to the manufacturer's protocols. *Serratia* nuclease was purified as described recently (Friedhoff *et al.*, 1994a); the preparation had a specific activity of 122 Kunitz-U/pmol (989 Kunitz-U/μL).

Strains and Plasmids. The *E. coli* strain LK 111(λ) was used to produce plasmids as templates for substrate generating PCR. Plasmid pAfoot (Landgraf, 1987), a derivative of pAT153, was the template used to generate a radioactively labeled 217 bp dsDNA substrate, and pRIF309+ (Wolfes *et al.*, 1986) was used to produce a fluorescent dye labeled 209 bp dsDNA substrate. Plasmid pUC8 was used as scDNA substrate. Bacteria transformed with plasmids were grown at 37 °C in LB-broth containing 100 μg/mL ampicillin. DNA isolation with the Qiagen plasmid kit for midipreparation was carried out as described in the manufacturer's protocol.

Primers. PCR and sequencing primers were synthesized on a Millipore Cyclone Plus DNA synthesizer using the standard β-(cyanoethyl)phosphoramidite chemistry on 0.2 μmol scale. Oligonucleotides for producing radioactive PCR products were synthesized without removing the dimethoxytrityl group after the final coupling step, purified using OligoPak purification cartridges (Millipore) according to the manufacturer's protocol, and detritylated. Oligonucleotides for producing fluorescent dye labeled PCR products were coupled with Aminolink II (ABI, Weiterstadt, Germany) in the final coupling step, and after deprotection and concentration in a Speedvac, they were labeled with four different fluorescent dye *N*-hydroxysuccinimide esters (NHS) according to the manufacturer's protocol. Dyes used were FAM-NHS (5-carboxyfluorescein), JOE-NHS (2′,7′-dimethoxy-4′,5′-dichloro-6-carboxyfluorescein), ROX-NHS (6-carboxyrhodamine X), and TAMRA-NHS (*N,N,N′,N′*-tetramethyl-6-carboxyrhodamine). After the coupling reaction, the fluorescent dye labeled oligonucleotides were purified by reverse-phase HPLC (Landgraf *et al.*, 1991). One oligonucleotide was coupled to the biotin moiety and also purified by reverse-phase HPLC. Extinction coefficients, melting temperatures, and optimal annealing temperatures for PCR were estimated with Oligo-4.0-s (National Bioscience Inc., Plymouth, MA).

Determination of Sequence Preferences. (A) Radioactive dsDNA Substrate. A 217 bp PCR product was synthesized with plasmid pAfoot DNA as template using a Landgraf thermocycler TCV 5*9 according to standard procedures. Only one strand of the 217 bp DNA was radioactively labeled using one primer which had been labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, purified by gel filtration using NAP-10 columns, and concentrated in a SpeedVac. The sequences of the primers were d(pCCCCGAAAAGTGCCACCTGACGTCT) and d(pTCCCCATCGGTGATGTCGGCGATAT), respectively. The PCR product was purified by polyacrylamide gel electrophoresis and eluted from the gel by overnight incubation in a buffer containing 10 mM Tris-HCl, pH 8.0. The eluate was concentrated with Centricon 30 microconcentrators and additionally purified using QIAquickspin PCR product purification columns. Concentrations of purified PCR products were determined by UV spectroscopy. The DNA was then subjected to limited digestion with the *Serratia* nuclease or DNase I, respectively. The cleavage reactions were performed with 20−150 nM DNA and 0.02 Kunitz-U/mL nuclease per 1 nM DNA in 30−80 μL total volume. This provides a more than 1000-fold molar excess of DNA over enzyme. The cleavage reaction was carried out in buffers normally used for the two nucleases. The *Serratia* nuclease buffer was 50 mM

Tris-HCl, pH 8.0, 5 mM $MgCl_2$. The DNase I buffer was 100 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$. Aliquots of the reaction mixture were withdrawn between 15 s and 60 min. The reaction was stopped with 1 volume of SAP [95% formamide, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylenecyanole FF, and 20 mM EDTA, pH 7.5] and 0.5 volume of 100 mM EDTA, pH 7.5. After storage at 0 °C, the samples were heated to 95 °C for 2 min and then subjected to electrophoresis on a denaturing 6% polyacrylamide gel together with the products from a sequencing reaction of the same DNA fragment or its corresponding part on the plasmid. Sequencing was carried out using the dideoxy method with Δ*Taq* DNA-polymerase in a PCR or with T7 DNA-polymerase, respectively.

(B) Fluorescent Dye Labeled dsDNA Substrates. A 209 bp PCR product was synthesized with plasmid pRIF309+ DNA as the template in the same manner as described above. Using one or two primers labeled with different fluorescent dyes, a specific 5′-fluorescent dye labeled substrate with one or two different fluorescent dyes was generated. The oligonucleotides labeled had the sequences d(CGGGAAGCTAGAGTAAGTAG) and d(GCCAACTTACTTCTGACAAC), respectively. The 209 bp PCR product was purified with QIAquickspin PCR product purification columns and preparative polyacrylamide gel electrophoresis, as described above. This DNA was then subjected to a limited digestion by the nucleases as described above; 0.04−0.2 Kunitz-U/mL for 1 nM DNA was used, and the reaction was stopped with 1.5 volumes of formamide/50 mM EDTA (5:1). The DNA was precipitated with ethanol and resuspended in 9 μL of 50% (v/v) formamide. After addition of 1 μL of Genescan ROX 2500 standard, the mixture was boiled, then chilled, and immediately loaded onto a denaturing 6% polyacrylamide gel. Electrophoresis was carried out within the Genescan mode of the ABI 373A-Sequencer. The same PCR product generated with unlabeled primers was used as a template for a Δ*Taq* cycle-sequencing reaction employing dye primers. To identify all fragments of the digest of a specifically labeled substrate, the products of four separate base-specific sequencing reactions with the corresponding dye-labeled primer were loaded onto and separated on the same gel.

(C) Fluorescent Dye Labeled ssDNA. A ssDNA substrate with identical sequence to one of the fluorescent dye labeled duplex strands was prepared by affinity chromatography using an avidin−agarose resin. A duplex DNA was generated in a PCR using one biotinylated d(bio-CGGGAAGCTAGAGTAAGTAG) and one fluorescent dye labeled primer d(fluo-GCCAACTTACTTCTGACAAC). Up to 10 PCR product mixtures obtained in 100 μL reaction volume were pooled and precipitated with ethanol, and the pellet was redissolved in 50 μL of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 2 M NaCl. This solution was repeatedly applied to an avidin−agarose column. After the column was washed twice with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 2 M NaCl, the fluorescent dye labeled strand was eluted from the column with 50 μL of 0.1 N NaOH. The eluted sample was neutralized with 0.1 N HCl and immediately buffered with Tris-HCl, pH 8.0. This solution was then applied to a QIAquickspin PCR product purification column as described in the manufacturer's protocol. Homogeneity was verified by polyacrylamide gel electrophoresis. The typical yield was 50 μL of a 1 μM ssDNA solution. The ssDNA was then subjected to a limited digestion with nuclease as described above.

(D) Supercoiled pUC8 DNA Substrate. Supercoiled pUC8 DNA was prepared as reported earlier (Langowski *et al.*, 1981). 40 nM pUC8 DNA was incubated with 5 pM *Serratia* nuclease in 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, at 37 °C. After defined time intervals, aliquots were withdrawn. The reaction was stopped by addition of 15 mM EDTA, and the reaction products were analyzed by electrophoresis in 0.8% agarose gels. After being stained with ethidium bromide, the gels were photographed and the negatives analyzed by densitometry.

(E) Oligodeoxynucleotide Substrates. Oligodeoxynucleotides were synthesized as described above for the PCR primers. After synthesis, the oligodeoxynucleotides were purified by reverse-phase HPLC and radioactively end labeled in a T4 polynucleotide kinase reaction. To analyze the cleavage of d(AAAAAAAGCTTTTTTT), 1.0 μM of the ^{32}P-labeled oligonucleotide was digested with 0.5 nM (610 Kunitz-U/mL) *Serratia* nuclease or 610 Kunitz-U/mL DNase I in the *Serratia* nuclease buffer and DNase I buffer, respectively. The reaction was performed at 22 °C and stopped by pipetting aliquots of 1 μL onto a DEAE-cellulose thin-layer plate which was then subjected to homochromatography (Jay *et al.*, 1974) and analyzed using an instant Imager (Canberra-Packard). The cleavage experiments with the oligodeoxynucleotides d(GCAAAAAAGCG) and d(CGCTTTTTTGC) were performed at 0 °C in *Serratia* nuclease standard buffer additionally containing 150 mM NaCl in order to stabilize the duplex structure; 1.0 μM of the ^{32}P-labeled oligodeoxynucleotide was digested with 150 nM *Serratia* nuclease. Digestion experiments were performed with the individual single-stranded oligodeoxynucleotides as well as with the duplex DNA in which either the d(A)-stretch-containing strand or the d(T)-stretch-containing strand was labeled.

RESULTS

Qualitative Comparison of dsDNA Cleavage by the Serratia Nuclease and DNase I. Sequence preferences of the *Serratia* nuclease and DNase I in cleaving dsDNA were analyzed with a radioactively end-labeled dsDNA substrate 217 bp in length, obtained by PCR. Both strands of the PCR product were analyzed using either a ^{32}P-labeled upper or a ^{32}P-labeled lower primer, respectively. They were subjected to a partial digestion with the *Serratia* nuclease and DNase I. The cleavage patterns obtained for both strands after incubation with the *Serratia* nuclease and DNase I, respectively, are shown in Figure 1.

On both strands of the duplex DNA, the cleavage pattern obtained with the *Serratia* nuclease and DNase I is very inhomogeneous. It appears that certain regions of the DNA are largely resistant to cleavage, as distinct gaps are seen in the pattern. For the *Serratia* nuclease, the gaps are located in AT-rich regions. It is noteworthy that sites of poor cleavage in one strand often coincide with sites of poor cleavage in the complementary strand of the dsDNA substrate (Figure 1). Furthermore, there are strongly preferred sites. For the *Serratia* nuclease, these sites are located in GC-rich or mixed sequences. It is apparent from Figure 1 that DNase I produces quite a different cleavage pattern than the *Serratia* nuclease, indicating that the *Serratia* nuclease and DNase I

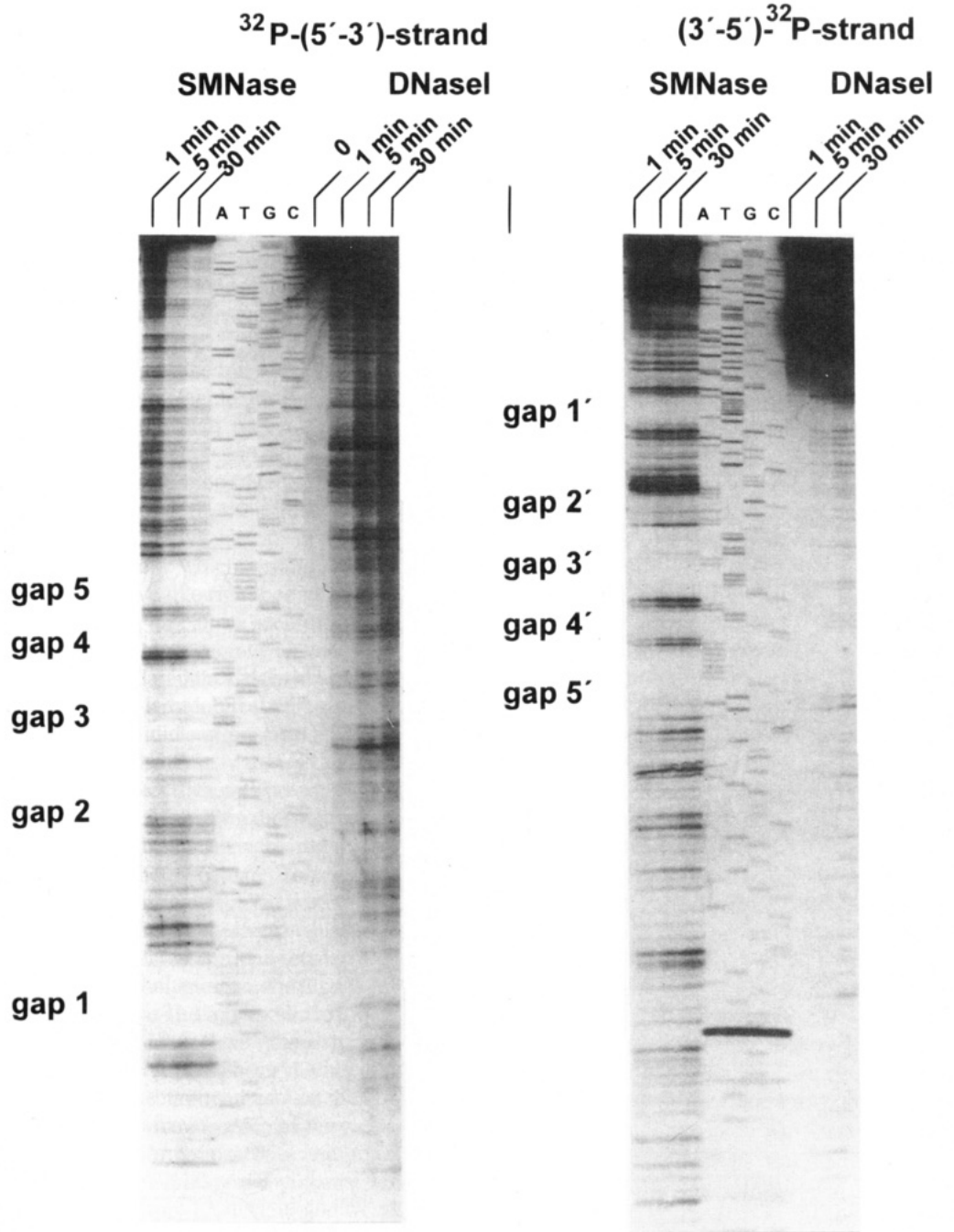

FIGURE 1: Qualitative analysis of preferences for cleavage of a radioactively labeled dsDNA substrate by the *Serratia* nuclease and DNase I, respectively. "^{32}P-(5′-3′)" and "(3′-5′)-^{32}P" indicate which of the two strands of the 217 bp DNA carries the radioactive label. "SMNase" and "DNaseI" identify samples that were subjected to limited digestion with the respective nucleases. The reaction progress after 1, 5, and 30 min is shown. A, T, G, and C identify base-specific sequencing reactions. The sequence of the "^{32}P-(5′-3′)"-strand of this substrate is: 5′-CCCCGAAAAG TGCCACCTGA CGTCTAAGAA ACCATTATTA TCATGACATT AACCTATAAA AATAGGCGTA TCACGAGGCC CTTTCGTCTT CAAGAATTCA AACCGTTAAC GGTTTTTTTC GATATCGGAT CCTCTACGCC GGACGCATCG TGGCCGGCAT CACCGGCGCC ACAGGTGCGG TTGCTGGCGC CTATATCGCC GACATCACCG ATGGGGA-3′. Underlined are gaps 1−5, prominent sites of poor cleavage by the *Serratia* nuclease. Gaps 1′−5′ are corresponding sites of poor cleavage that occur in the other strand of the duplex at complementary positions to gaps 1−5.

interact with their double-stranded substrate in a manner unique for each enzyme.

Quantitative Analysis of dsDNA Cleavage by the Serratia Nuclease under Various Reaction Conditions. For a quantitative comparison of the dsDNA cleavage by the *Serratia* nuclease under a variety of different reaction conditions, a 209 bp fluorescent dye labeled DNA substrate was prepared by PCR using a primer with a fluorescent dye covalently attached to its 5′-end. The substrate had the following sequence: GCCAACTTAC TTCTGACAAC GATCGGAGGA CCGAAGGAGC TAAC***CGCTTT TTTGC***ACAAC ATGGGGGATC ATGTAACTCG CCTTGATCGT TGGGAACCGG AGCTGAATGA AGCCATACCA AACGACGAGC GTGACACCAC GATGCCTGCA GCAATGGCAA CAACGTTGCG CAAACTATTA ACTGGCGAAC TACTTACTCT AGCTTCCCG.

Only the sequence of the upper strand of the fluorescent dye labeled dsDNA substrate is shown here; it also represents the sequence of the 209 bp fluorescent dye labeled ssDNA substrate. The part of the sequence selected in Figures 2

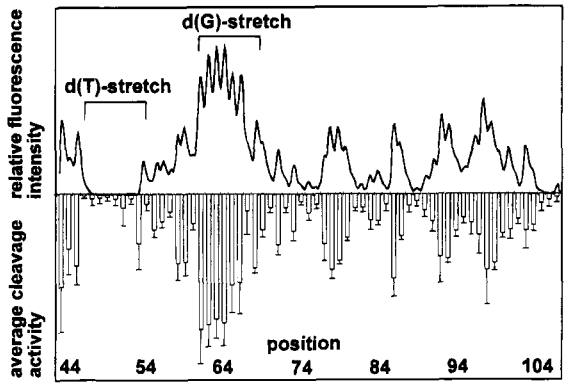

FIGURE 2: Quantitative analysis of preferences for cleavage of a fluorescently labeled dsDNA substrate by the *Serratia* nuclease under standard conditions. One individual experiment is shown in the format obtained as an electrophoretogram from the ABI 373A-sequencer, indicating the relative fluorescence intensities of the cleavage products, juxtaposed with the peak area format of five individual experiments evaluated together to give an average and its standard deviation for cleavage at a particular position. For the sake of clarity, only positions 44−108 of the 209 bp fluorescent dye labeled DNA substrate are shown.

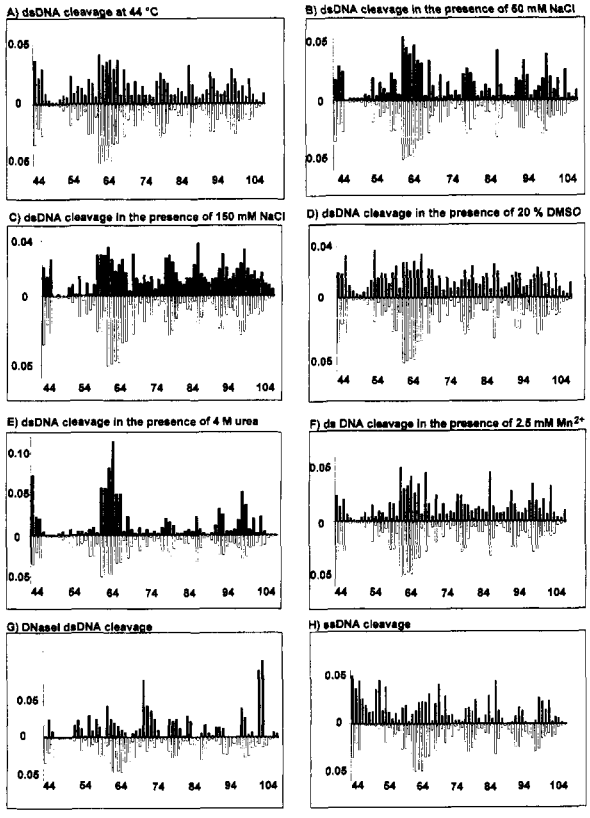

FIGURE 3: Sequence preferences of the *Serratia* nuclease under a variety of conditions. Shown here are the relative cleavage activities at the positions indicated under a variety of different conditions (black bars) in comparison with cleavage under standard conditions (white bars) as introduced in Figure 2. The cleavage of dsDNA by the *Serratia* nuclease is shown in panels A−F under conditions that differ from standard conditions in the parameter indicated (A, 44 °C; B, 50 mM NaCl; C, 150 mM NaCl; D, 20% DMSO; E, 4 M urea; F, 2.5 mM Mn^{2+}). A comparison of dsDNA cleavage by DNase I and the *Serratia* nuclease is presented in panel G. ssDNA cleavage *versus* dsDNA cleavage by the *Serratia* nuclease is compared in panel H.

and 3 to illustrate the results of the quantitative analysis of cleavage preferences (positions 44−108) is underlined. Positions 45−55 (boldface letters) correspond to the oligodeoxynucleotide that was synthesized and subjected to digestion with *Serratia* nuclease to demonstrate poor cleavage in d(A)•d(T)-tracts (see Figure 6).

Table 1: Sequence Selectivities[a] of *Serratia* Nuclease and for Comparison DNase I

condition	selectivity
Serratia Nuclease	
dsDNA cleavage, standard conditions	1.0
dsDNA, 44 °C	0.8
dsDNA, 50 mM NaCl	1.0
dsDNA, 150 mM NaCl	0.7
dsDNA, 20% DMSO	0.6
dsDNA, 4 M urea	1.7
dsDNA, 2.5 mM Mn^{2+}	0.9
ssDNA cleavage, standard conditions	1.0
DNase I	
dsDNA cleavage	1.6

[a] The standard deviation of the fluorescence intensities of all cleavage products in each individual experiment shown in Figure 3 is a measure of the variation of phosphodiester bond cleavage efficiency at any given position and may be used to define the relative sequence selectivity under the particular conditions. The selectivity of the *Serratia* nuclease in dsDNA cleavage under standard conditions is set to 1. By this definition, a totally nonspecific nuclease would have a selectivity of zero.

This fluorescent dye labeled substrate was subjected to limited digestion by the *Serratia* nuclease, and the cleavage products were separated on an ABI 373A-sequencer and analyzed by the Genescan software. The analysis using an automatic sequencer has the advantage of providing resolution down to the nucleotide level and of producing quantitative information directly. Figure 2 shows the digestion pattern obtained with the 209 bp fluorescent dye labeled substrate and the *Serratia* nuclease under the same reaction conditions as used for the qualitative comparison of the dsDNA cleavage by the *Serratia* nuclease and DNase I described above. These conditions are referred to as standard conditions. For the sake of clarity, only a part of the digestion pattern covering positions 40−108 in the sequence is depicted in Figure 2. In this figure, the result of one individual experiment is shown in the conventional format of a densitogram together with the peak area format of five individual experiments evaluated together to give an average and its standard deviation. Figure 2 demonstrates that the cleavage of the fluorescently labeled dsDNA by the *Serratia* nuclease, as the cleavage of the radioactively labeled dsDNA (Figure 1), produces a very inhomogeneous pattern. A distinct gap appears in the peak pattern around positions 48−53 which corresponds to a d(T)-run, while preferred cleavage sites are located in a d(G)-run (positions 63−67). Raising the temperature from 37 to 44 °C only slightly changes the cleavage pattern; the normalized comparison of the 37 and 44 °C data indicates that at higher temperature the step to step variations in cleavage rates are slightly less pronounced (Table 1, Figure 3A). Increasing the ionic strength by addition of 50 mM NaCl does not change the cleavage pattern (Table 1, Figure 3B), while addition of 150 mM NaCl makes the cleavage pattern significantly more even (Table 1, Figure 3C). A similar effect is observed by addition of 20% DMSO, which decreases its selectivity (Table 1, Figure 3D). In contrast, in the presence of 4 M urea the enzyme becomes more selective, as several sites that are well accessible under standard conditions become rather inaccessible (Table 1, Figure 3E). Interestingly, Mn^{2+}, which

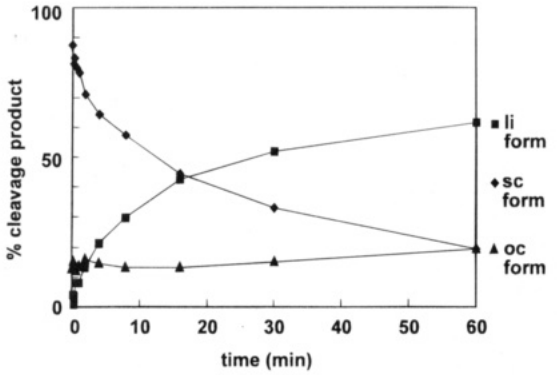

FIGURE 4: Cleavage of supercoiled plasmid DNA by the *Serratia* nuclease. The diagram illustrates that the disappearance of scDNA coincides with the appearance of liDNA. The amount of ocDNA in the preparation of the plasmid does not change significantly during the time course of digestion.

relaxes the specificity of many restriction endonucleases, when substituted for Mg^{2+}, has only a slight effect on the specificity of the *Serratia* nuclease (Table 1, Figure 3F). It should be noted that on the basis of the cleavage results obtained with the fluorescent dye labeled dsDNA substrate the *Serratia* nuclease seems to be slightly more nonspecific (i.e., less selective) than DNase I (Table 1, Figure 3G).

Correlation Analysis of Cleavage in the Two Individual Strands of the dsDNA Substrate. As both strands of the dsDNA substrates could be labeled independently, it was possible to analyze whether on average cleavage in one strand correlates with cleavage in the other. As shown before in Figure 1 for the radioactively labeled 217 bp dsDNA substrate and as was confirmed with the fluorescently labeled 209 bp dsDNA substrate (data not shown), a correlation exists between two cleavage events at a given position in the complementary strands of the dsDNA. This correlation could indicate that cleavages at individual phosphodiester bonds are not independent events, but rather that cleavage in one strand facilitates cleavage in the other strand. This has been confirmed by experiments in which supercoiled (sc) pUC8 DNA was incubated with the *Serratia* nuclease. Figure 4 shows that the initial rates of supercoiled plasmid DNA cleavage and appearance of linearized DNA are identical. Furthermore, there is no detectable lag phase in the production of linearized DNA and no pronounced accumulation of the open circular intermediate, as would be expected when the enzyme would randomly attack phosphodiester bonds, i.e., randomly nick the dsDNA. This finding suggests that at least on a supercoiled substrate the *Serratia* nuclease can produce double-strand breaks. It might be argued that the lack of accumulation of the open circular intermediate could be the result of two independent single-strand cleavage events at two highly preferred sites that are located next to each other on the two strands of the duplex; we regard this alternative, however, as unlikely, as it implies that linearized DNA is produced by nicks in the two strands at only one defined highly preferred site, for which we have no evidence.

Comparison of dsDNA and ssDNA Cleavage by the Serratia Nuclease. Using PCR products with a fluorescently labeled upper primer and a biotin-modified lower primer, it was possible to isolate the fluorescently labeled single strand by affinity chromatography on avidin−agarose columns

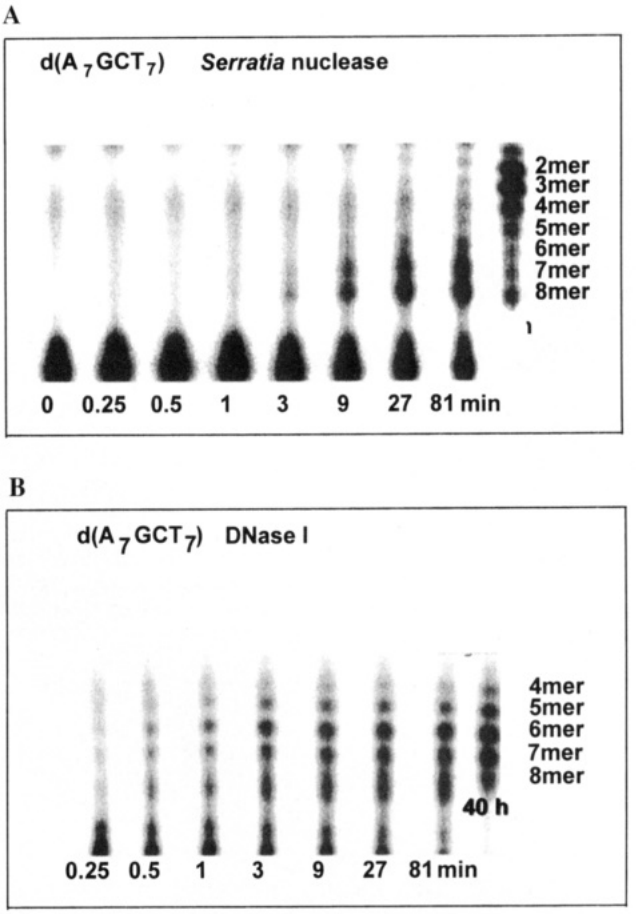

FIGURE 5: Cleavage of the self-complementary oligodeoxynucleotide d(AAAAAAAGCTTTTTTT) by the *Serratia* nuclease. On the basis of the results of the analysis of preferences for cleavage in GC-rich regions on the PCR-generated substrates, the oligodeoxynucleotide $d(A_7GCT_7)$ was synthesized, and its cleavage by the *Serratia* nuclease (A) and, in comparison, by DNase I (B) was analyzed. Cleavage products were identified by *Alu*I cleavage of the substrate (data not shown).

under denaturing conditions. This substrate was subjected to a limited digestion by the *Serratia* nuclease under standard conditions. Figure 3H shows the single-stranded DNA cleavage pattern obtained in comparison with the double-stranded DNA cleavage pattern. With the exception of the d(T)-run (positions 48−53), which is accessible in the single strand but hardly at all in the double strand, there is an overall similarity between the two cleavage patterns, which may indicate that the secondary structure (double strand *vs* single strand) is not the only factor determining the sequence preferences of the *Serratia* nuclease.

Cleavage of the Self-Complementary Oligodeoxynucleotide d(AAAAAAAGCTTTTTTT) by the Serratia Nuclease. The analysis of preferred cleavage sites in double-stranded DNA has demonstrated that cleavage within GC-rich sequences is favored over cleavage in d(A)- or d(T)-runs. With this information, the self-complementary hexadecamer $d(A_7GCT_7)$ was synthesized and ^{32}P-labeled at its 5′-end, and its cleavage by the *Serratia* nuclease was analyzed. Figure 5A shows that this oligonucleotide is cleaved very rapidly at the GC step and less rapidly at the AG step but only very slowly at the other positions. After longer incubation time, these primary products are cleaved by the *Serratia* nuclease to give $d(pA_3)$ and at very much reduced rate the other oligodeoxyadenylates. DNase I catalyzed cleavage of this

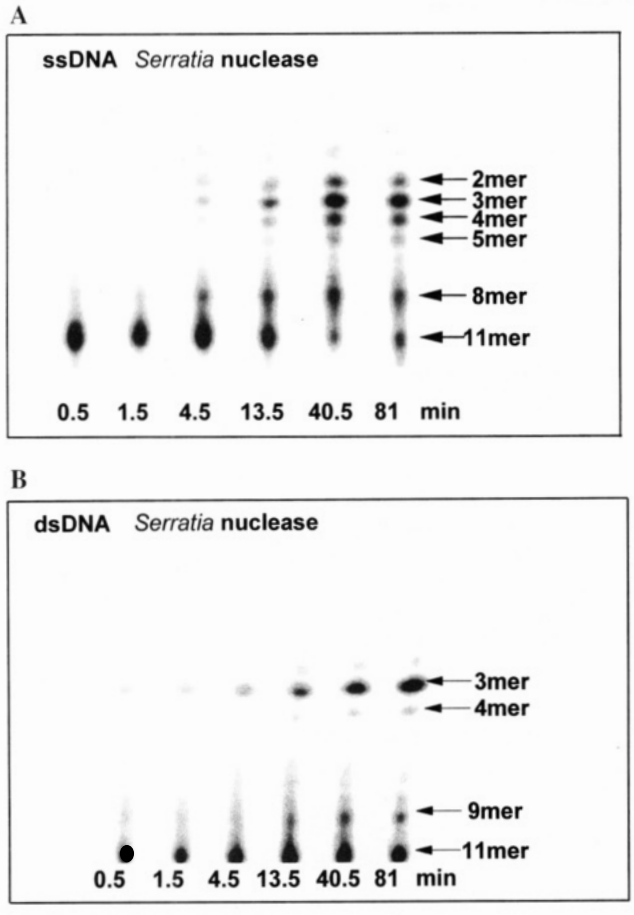

FIGURE 6: Cleavage of the oligodeoxynucleotide d(GCAAA-AAAGCG) in the absence and presence of its complementary strand by the *Serratia* nuclease. The analysis of sequence-dependent preferences of cleavage of the PCR-generated substrates (Figures 1 and 3) revealed poor cleavage in d(A)•d(T)-tracts of dsDNA for both the *Serratia* nuclease and DNase I. To investigate whether this is due to the particular and rigid structure of d(A)•d(T)-tracts, cleavage of d(GCAAAAAAGCG) by the *Serratia* nuclease was analyzed. Experiments were carried out with the individual strand as a ssDNA substrate and in the presence of the complementary strand as a dsDNA substrate. In panel A, it is demonstrated that in the ssDNA substrate cleavage occurs at several sites, including sites within the d(A) stretch. On the dsDNA substrate, however, as shown in panel B, hardly any cleavage occurs within the d(A)-stretch. Cleavage products were identified using a partial snake venom phosphodiesterase I digest of the same oligodeoxynucleotide (data not shown).

oligodeoxynucleotide, in contrast, does not lead to a preferential cleavage at the GC step (Figure 5B).

Cleavage of Double- versus Single-Stranded Oligodeoxynucleotides by the Serratia Nuclease. The comparison of double- *versus* single-stranded DNA cleavage by the *Serratia* nuclease had shown that in dsDNA d(A)- and d(T)-runs are largely resistant to cleavage, but that this resistance is partly relieved in ssDNA. To find out whether this effect is not the consequence of long-range interactions, including fold-back structures of the ssDNA, cleavage experiments were also carried out with synthetic oligodeoxynucleotides. The double-stranded oligodeoxynucleotide d(CGCTTTTTTGC)•d(GCAAAAAAGCG), corresponding in sequence to positions 45−55 in the 209 bp substrate used in the experiment described above, is cleaved almost exclusively at the third phosphodiester bond in both strands, i.e., outside of the d(A)•d(T)-tract (Figure 6A). In contrast, the individual oligodeoxynucleotides are also cleaved within the d(A)-run (Figure 6B) and, although not quite as pronounced, within the d(T)-run (data not shown).

DISCUSSION

The extracellular *Serratia marcescens* endonuclease is presumably one of the most promiscuous endonucleases known, as it cleaves not only single- and double-stranded DNA but also RNA of natural origin, producing 5′-phosphorylated mono-, di-, tri-, and tetranucleotides (Eaves & Jeffin, 1963; Nestle & Roberts, 1969a). This promiscuity is not observed, however, with synthetic homopolynucleotides which are with few exceptions largely resistant to cleavage (Yonemura *et al*., 1983; Filimonova, Balaban, and Leshchinskaya, University of Kazan, personal communication). This finding may at least in part be explained by the particular structures adopted by homopolynucleotides (Saenger, 1984). It appears, therefore, that this enzyme, while being a nonspecific nuclease, nevertheless, does have sequence-dependent preferred cleavage sites. Indeed, as shown in the present paper, cleavage at nearly all positions along a nucleic acid chain is possible, although the rates of cleavage at these positions vary enormously. Similar observations had been made with other nonspecific nucleases, for example, with bovine pancreatic DNase I, whose sequence-dependent variation in its rate of DNA cleavage has been explained to arise from both local and global variations in DNA geometry (Drew & Travers, 1984; Fox, 1992; Herrera & Chaires, 1994), as well as to be modulated by DNA flexibility (Hogan *et al*., 1989) and DNA bending (Brukner *et al*., 1990). For DNase I, structural information is available for enzyme−oligodeoxynucleotide complexes (Lahm & Suck, 1991; Weston *et al*., 1992). It has been possible, therefore, to correlate structural and functional data and, thereby, rationalize at least in part the observed sequence preferences (Weston *et al*., 1992). As for the *Serratia* nuclease, only the structure of the enzyme has been solved (Miller *et al*., 1994); it is not yet possible to explain the observed sequence preferences in structural terms.

The results presented here demonstrate that the *Serratia* nuclease displays different sequence preferences compared to DNase I. Table 2 shows the preferred cleavage sites on one strand of the dsDNA substrate for the *Serratia* nuclease and DNase I, as well as the preferred cleavage sites on ssDNA for the *Serratia* nuclease. There are only a few preferred cleavage sites on dsDNA that are common to both the *Serratia* nuclease and DNase I on a 209 bp substrate, while several common sites on one strand of the dsDNA and on the same strand when present as a ssDNA substrate are observed upon cleavage by the *Serratia* nuclease. The most obvious difference between the two enzymes is the preference of the *Serratia* nuclease for cleavage of dsDNA in d(G)•d(C)-tracts which are not attacked preferentially by DNase I. This site preference has also been observed with the related mitochondrial endonuclease G from *Bos taurus* (Ruiz-Carrillo & Cote, 1993) and the exocytoplasmic endonuclease from *Streptomyces antibioticus* (Cal *et al*., 1995). These results suggest that DNase I and the *Serratia* nuclease interact differently with their dsDNA substrate, which is expected given the different structures of their active sites (Suck & Oefner, 1986; Lahm *et al*., 1991; Miller *et al*., 1994). A common feature of both enzymes is their relative inability to cleave dsDNA within d(A)•d(T)-tracts. It is

Table 2: Preferred Cleavage Sites by *Serratia* Nuclease and DNase I on the Fluorescently Labeled DNA Substrates[a]

Serratia nuclease				DNase I	
dsDNA		ssDNA		dsDNA	
position	cleavage site	position	cleavage site	position	cleavage site
36	GAGG↓GAGC	36	GAGG↓GAGC	43	CTAA↓CCGC
37	AGGG AGCT	37	AGGG AGCT	44	TAAC CGCT
44	TAAC CGCT	38	GGGA GCTA	71	ATCA TGTA
62	ACAT GGGG	43	CTAA CCGC	103	GAGC TGAA
63	CATG GGGG	44	TAAC CGCT	104	AGCT GAAT
64	ATGG GGGA	45	AACC GCTT	114	AGCC ATAC
65	TGGG GGAT	46	ACCG CTTT	115	GCCA TACC
66	GGGG GATC	51	TTTT TTGC	137	ACAC CACG
67	GGGG ATCA	52	TTTT TGCA	180	ATTA ACTG
69	GGAT CATG	54	TTTG CACA	192	ACTA CTTA
87	TGAT CGTT	67	GGGG ATCA	195	ACTT ACTC
93	TTGG GAAC	70	GATC ATGT		
94	TGGG AACC	72	TCAT GTAA		
99	ACCG GAGC	85	CTTG ATCG		
100	CCGG AGCT	87	TGAT CGTT		
114	AGCC ATAC	114	AGCC ATAC		
124	AACG ACGA	158	TGGC AACA		
127	GACG AGCG				
129	CGAG CGTG				
130	GAGC GTGA				
138	CACC ACGA				

[a] As preferred cleavage sites, we defined those whose cleavage intensity is 2.5 standard deviations higher than the mean intensity observed in the given experiment. The arrow (↓) indicates the hydrolyzed phosphodiester bond. The numbers refer to the position of the nucleotide 5′-adjacent to the phosphodiester bond cleaved.

known for d(A)•d(T)-tracts that they have on average a smaller than normal groove width [for a review, cf. Dickerson (1992)] and are rather inflexible because of the spine of hydration and a high propeller twist that increases base stacking, as well as the network of extra, non-Watson−Crick, bifurcated hydrogen bonds between the adenine and thymine bases on opposite strands observed in the crystal structures of DNA dodecamers (Nelson *et al.*, 1987; DiGabriele *et al.*, 1989; DiGabriele & Steitz, 1993). The oligodeoxynucleotide studied by Nelson *et al.* (1987) over a stretch of 11 bp corresponds to the most nuclease-resistant region in the 209 bp substrate analyzed here. It is tempting to speculate that the inability of both the *Serratia* nuclease and DNase I to cleave dsDNA in d(A)•d(T)-tracts reflects a common response to a global function, for example, minor groove width [near the center of the helix, the minor groove can be as narrow as 2.6 Å, as in d(CGATTAATCG), and as wide as 8.9 Å, as in d(CCGGCGCCGG), after subtracting 5.8 Å from the P−P distance to account for the van der Waals radii of the phosphates (Heinemann *et al.*, 1994)]. A corollary of this conjecture could be that the *Serratia* nuclease like DNase I approaches its dsDNA substrate via the minor groove and is reluctant to initiate phosphodiester bond hydrolysis when the minor groove is narrow. At present, this is a mere speculation which, however, is supported by modeling of d(CCGGCGCCGG) (Heinemann *et al.*, 1992) and d(CGATTAATCG) (Quintana *et al.*, 1992) which differ extremely in the width of the minor groove (Heinemann *et al.*, 1994) into the putative active site cleft of the *Serratia* nuclease, similarly as done recently by Miller *et al.* (1994) with a standard B-DNA. The results of this modeling (not shown) demonstrate that dsDNA with a narrow minor groove does not fit as well as dsDNA with a wide minor groove into the active site cleft of the *Serratia* nuclease. This may be one reason why the *Serratia* nuclease prefers to cut in d(G)•d(C)-tracts and avoids d(A)•d(T)-tracts. It is also conceivable that the *Serratia* nuclease is sensitive to the helical pitch and prefers a helical repeat of 11.1 bp as measured for d(G)•d(C)-tracts (Biburger *et al.*, 1994) and discriminates against a repeat of 9.9 bp as determined for d(A)•d(T)-tracts (Niederweis *et al.*, 1993). This conjecture is supported by the finding that addition of 20% DMSO, which partially unwinds the DNA and affects DNase I cleavage selectivity (Drew & Travers, 1984), makes the d(A)•d(T)-tract in the 209 bp fluorescently labeled substrate more accessible for the *Serratia* nuclease.

For the *Serratia* nuclease, as observed before for bovine pancreatic DNase I (Drew & Travers, 1984), there exists some correlation between rates of cleavage of opposite strands at a given position. This result could reflect the influence of global helix parameters which are by definition the same for both strands of the double helix, but it could also be explained by assuming that after cleavage of one strand the other strand can take up more easily a conformation favorable for cleavage. As a matter of fact with supercoiled pUC8 DNA and the conditions employed here, the *Serratia* nuclease mainly exhibits a diplotomic mechanism, which means that it efficiently introduces double-strand breaks. Whether the *Serratia* nuclease does this in a truly concerted reaction, i.e., within one binding event and reorientation of the enzyme between the individual phosphodiester bond cleavages, remains to be analyzed by fast mixing techniques. For other nonspecific nucleases, it has been shown that they follow a haplotomic mechanism, which means that they prefer to introduce single-strand breaks into a supercoiled DNA, which leads to the accumulation of the open circular intermediate, as is observed, for example, with the *Streptomyces antibioticus* nuclease (Cal *et al.*, 1995) or pancreatic DNase I under certain conditions (Bernardi, 1971). While DNase I preferrentially introduces double-strand breaks on supercoiled SV40 DNA in the presence of transition metal ions like Mn^{2+} or Co^{2+}, it mainly nicks the DNA in the presence of Mg^{2+} (Campbell & Jackson, 1980). For spleen acid DNase, it has been demonstrated that it degrades high molecular weight DNA via both mechanisms (Bernardi, 1971).

The preferences of the *Serratia* nuclease for cleavage at certain sites can be modulated by the reaction conditions. At higher ionic strength, the enzyme becomes more nonspecific, in contrast to bovine endoG, which becomes more specific (Ruiz-Carrillo & Cote, 1993). It could be argued that this is due to the weakening of electrostatic interactions, some of which could be responsible for sequence-dependent interactions between substrate and enzyme. When these are suppressed, the enzyme is not directed to sites preferred under standard conditions but rather attacks other accessible sites. Addition of 20% DMSO or, however not as pronounced, raising the temperature also makes the *Serratia* nuclease more nonspecific. Presumably, these changes of reaction conditions could serve to increase the flexibility of the substrate, thereby making poorer sites more accessible. A similar result was reported for DNase I (Drew & Travers, 1984) and discussed there to be due to global structural changes in the substrate. Interestingly, Mn^{2+}, when replacing Mg^{2+}, relaxes the specificity of *Eco*RI (Hsu & Berg, 1978) and several other restriction enzymes [for a review, cf. Roberts and Halford (1993)], but has hardly any effect on

the *Serratia* nuclease. With restriction enzymes, it is believed that Mg^{2+} can only be bound firmly when the enzyme interacts with its cognate substrate. A near-cognate substrate, however, cannot bind Mg^{2+} but can bind Mn^{2+} which then activates the catalytic center (Taylor & Halford, 1989; Vermote & Halford, 1992). While Mg^{2+} is essential for the catalytic function of the *Serratia* nuclease, it might be suggested that its binding is more determined by the protein than by the DNA. This would explain that Mn^{2+} does not induce the protein to change the cleavage preferences. Interestingly, in the presence of 4 M urea, the enzyme becomes more specific. We assume that urea interferes with the normal conformation of some of the amino acid side chains in the protein−DNA interface. When these amino acids are directly or indirectly responsible for binding of DNA containing average sites, then urea would suppress binding and thereby cleavage of DNA at these average sites. Only those DNA substrates would be accepted that have preferred sites which are bound by additional interactions unique for preferential sites.

The *Serratia* nuclease cleaves ssDNA as well as dsDNA with at least in part similar sequence preferences, with one significant exception, however: it accepts d(A)- and d(T)-runs in ssDNA more readily than in dsDNA. We argued above that the inability of the *Serratia* nuclease, like that of DNase I, to cleave d(A)·d(T)-tracts is due to the narrow minor groove, the smaller helical repeat, and/or the inflexibility of dsDNA at such sequences which would not allow a dsDNA to readily take up a conformation that presumably is A-DNA like, as the enzyme also accepts dsRNA as a substrate. It is not surprising, therefore, that d(A)- or d(T)-runs in ssDNA are cleaved more readily than in dsDNA, as it is not constrained by a complementary strand. The similarity in parts of the single- and double-strand cleavage pattern is attributed to localized interactions between the enzyme and individual nucleotides of the substrate, many of which can be formed as well with ssDNA and dsDNA. It will be of great interest to find out whether some of these preferences are also observed with single- and double-stranded RNA which are also cleaved by the *Serratia* nuclease.

A practical implication of our identification of cleavage site preferences is that *Serratia* nuclease, which is commercially available as BENZONASE, alone or, because of its different preferences, in conjunction with DNase I is a useful biochemical tool for footprinting analyses of dsDNA binding proteins, as shown recently (Jeltsch *et al.*, 1995) and recommended by the commercial supplier of this enzyme (Merck, 1995), as well as ssDNA binding proteins.

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