On the Advantage of Being a Dimer, a Case Study Using the Dimeric Serratia Nuclease and the Monomeric Nuclease from Anabaena sp. Strain PCC 7120*

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The extracellular endonucleases from Serratia marcescens and Anabaena sp. are members of a family of nonspecific endonucleases. In contrast to the monomeric Anabaena nuclease, the Serratia nuclease is a dimer of two identical subunits. To find out whether the two active sites of the Serratia nuclease function independently of each other and what the advantage of being a dimer for this enzyme might be, we produced (i) dimers in which the two subunits were cross-linked, (ii) heterodimers consisting of a wild type and an inactive mutant subunit which were also cross-linked, and (iii) monomeric variants which are unable to dimerize. The monomeric H184R variant and the cross-linked S140C variant exhibit the same activity as the wild type enzyme, while the cross-linked heterodimer with one inactive subunit shows only half of the activity of the wild type enzyme, demonstrating functional independence of the two subunits of the Serratia nuclease. On the other hand at low enzyme and substrate concentrations dimeric forms of the Serratia nuclease are relatively more active than monomeric forms or the monomeric Anabaena nuclease in cleaving polynucleotides, not, however, oligonucleotides, which is correlated with the ability of dimeric forms of the Serratia nuclease to form large enzyme-substrate networks with high molecular weight DNA and to cleave polynucleotides in a processive manner. We conclude that in the natural habitat of Serratia marcescens where the supply of nutrients may become growth limiting the dimeric nuclease can fulfill its nutritive function more efficiently than a monomeric enzyme.

Many enzymes are dimers of identical subunits each of which harbors one active site. Only in a few cases is it obvious that the dimeric state is essential for enzymatic activity, the paradigm being type II restriction endonucleases. These are usually dimers of identical subunits which together form one DNA-binding site, and have two catalytic centers, one per subunit, which cleave the two strands of the DNA in a concerted fashion (1, 2).

In contrast, it is not at all clear what the role of the quaternary structure of a family of sugar nonspecific nucleases is, some of which are homodimers and others monomers. Representatives of this growing group of related enzymes are Serratia nuclease which is a homodimer (3–5) and Anabaena nuclease (Anabaena sp. strain PCC 7120) which is a monomer (6). Other members of this group have been found in various other prokaryotic but also in eukaryotic organisms, viz. Streptococcus pneumoniae (7–9), cyanobacteria (10, 11), Saccharomyces cerevisiae (12, 13), Schizosaccharomyces pombe (14), Synecchocystis sp. strain PCC 7942 (15), and Bos taurus (16). In addition, open reading frames for homologous proteins have been identified in Trypanosoma brucei, Caenorhabditis elegans (accession number AF003740), Mus musculus (Ref. 17, accession number X99395), and Homo sapiens (Ref. 18, accession number X79444). Recently, the homologous nuclease from Borrelia burgdorferi was identified through a genome sequencing project (19).

Although the nucleases of this family occur ubiquitously their cellular roles are often unknown or poorly understood. The bovine EndoG nuclease is known to be involved in generating primers for the mitochondrial DNA replication by cleaving GC-rich sequences in the displacement loop of mitochondrial DNA and in the region upstream of the mitochondrial tRNA (Ref. 66) gene (16), and has been discussed to be involved in the maintenance of mitochondrial DNA by eliminating defective genomes from the multicopy pool (20). NUC1 from S. cerevisiae seems to be involved in DNA recombination and DNA repair. However, null mutants which lack the NUC1 gene show the wild type phenotype (21). The secreted nucleases from prokaryotic organisms, namely Serratia nuclease, Anabaena nuclease, and EndA from S. pneumoniae most likely fulfill a nutritive function by providing the bacterial cell in its natural habitat with precursors for nucleic acid synthesis. They also may act as bactericides to inhibit the growth of competing organisms. This was shown to be important for efficient transformation of S. pneumoniae (9). They may also constitute virulence factors.

Among this family of nucleases the Serratia (for review, see Ref. 22) and the Anabaena nuclease are by far the best studied enzymes. Both enzymes, Serratia nuclease secreted by the Gram-negative bacterium Serratia marcescens (23–25) and Anabaena nuclease produced by members of the cyanobacterial genus Anabaena (10) are largely nonspecific endonucleases which cleave double- and single-stranded DNA and RNA and produce 5’-phosphorylated oligonucleotides. For the Serratia nuclease the three-dimensional structure is known (5, 26, 27), and due to a detailed mutational (28, 29) as well as a kinetic analysis using natural and synthetic, in part chemically modified, substrates (30–32) its mechanism of action is well understood. Anabaena nuclease recently has been cloned, sequenced,

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1 T. Nozaki and G. A. M. Cross, unpublished results.
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and expressed in *Escherichia coli* (10, 33) and biochemically characterized in detail (6). It is the only representative of this group whose intracellular activity is regulated by the polypeptide inhibitor NuiA which is specific for the *Anabaena* nuclease, as it does not inhibit the activity of the related *Serratia* nuclease (6, 33). *Serratia* nuclease is not active intracellularly because two disulfide bonds are essential for its nucleolytic function (34), which are not formed prior to secretion into the oxidizing milieu of the periplasm.

The active site of these nucleases is characterized by the DRCH motif typical for this family of nucleases. It contains the essential histidine, His89 in *Serratia* nuclease, which presumably acts as the general base to generate the active nucleophile for phosphodiester bond cleavage. The nucleolytic activity requires divalent metal ions like Mg2⁺ or Mn2⁺ which in *Serratia* nuclease are bound by Asn119. The metal ion cofactor could be involved in polarization of the P-O bond to make the phosphorus more accessible for a nucleophilic attack and together with Arg57 stabilize the phosphorane intermediate in the transition state. Glu127 seems to be involved in protonation of the leaving group. These residues are conserved in the *Serratia* family of nucleases and their essential function has been demonstrated for *Serratia* nuclease (29, 31, 32) as well as for *Anabaena* nuclease.³

While it appears from a comparison of the properties of these two nucleases that they have a similar mechanism of action (6), they differ as mentioned above in their quaternary structure. The question, of course, arises, whether the dimeric structure of *Serratia* nuclease leads to a functional dependence of the two active sites, a question that cannot easily be resolved by kinetic studies, as the enzyme is subjected to a monomer/dimer equilibrium and attacks nucleic acids at many sites with different rates which obscures any analysis regarding cooperative effects (31). We have, therefore, taken another approach, namely to produce monomeric and cross-linked homo- and heterodimeric variants of the *Serratia* nuclease and to compare their nucleolytic activity and mechanism of action toward different nucleic acid substrates. Our results demonstrate that the subunits of *Serratia* nuclease are functionally independent, but that the dimeric state of this enzyme is of advantage at very low enzyme and substrate concentration, because it allows the *Serratia* nuclease to stay associated with high molecular weight nucleic acids and to cleave them in a processive manner.

**EXPERIMENTAL PROCEDURES**

**Strains and Vectors**—For the cloning and expression of the wild type and the mutant genes of *Serratia* nuclease the vector pHisNuc (4) was used. *Anabaena* nuclease was cloned and overexpressed using the pHuINuAc expression vector (6). Cloning of *pHuINuAc* and pHuINuAc was performed in *E. coli* K12 LK111(λ) (35). For the overexpression of *Serratia* nuclease *E. coli* K12 TGE900 and for *Anabaena* nuclease *E. coli* BL21(DE3)LysS (36) were used. *E. coli* DE3 (37) was used for the propagation of the plasmid pBSK+ (Stratagene) to achieve a maximum yield of supercoiled plasmid DNA. The pBSK+ plasmid was isolated using alkaline lysis (38, 39) followed by differential nucleic acid precipitation using a modified protocol from (40).

**In Vitro Mutagenesis and Sequencing**—The double mutant H89A,S140C variant of *Serratia* nuclease was generated by ligating the BamHI/BsaI fragment of the gene of the H89A variant (28) into pHisNuc (SI40C) (41) which had been digested with the same restriction endonucleases.

The double mutant S140C,H184R and H89A,H184R variants were generated applying an inverse polymerase chain reaction strategy (6). Mutations were verified by sequencing the whole gene using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS, on an ABI 373A DNA sequencer (Applied Biosystems) according to the supplier’s protocol.

**Cloning of pStrepINuc—**pStrepINuc is a derivative of pHisNuc in which the codons for the N-terminal His6-tag were replaced by codons for the Strep-tag II. Proteins with the Strep-tag II bind to StrepTactin, an alternatively used streptavidine protein. In contrast to the His6-tag the Strep-tag II can be employed not only as a C-terminal fusion peptide but also as a linker between protein domains or as an N-terminal fusion peptide. The original sequence, CATCATCATCACCACCATC, coding for the His6-tag was excised as a HindIII/BamHI fragment from pHisNuc and replaced by a cassette containing the coding sequence, TCTGCTTACGCCAGTCGAAA, for the Strep-tag II (Ser-Ala-Trp-Strep-His-Pro-Gln-Phe-Glu-Lys). Codons most frequently used in *E. coli* were employed. Positive clones were identified by polymerase chain reaction screening using a forward primer specific for the codons of the Strep-tag II and a reverse primer binding to the 3’-region of the *Serratia* nuclease gene. Positive clones were verified by sequencing as described above. The newly generated expression vector containing the gene of *Serratia* nuclease was named pStrepINuc.

**Overexpression and Purification of *Serratia* Nuclease Variants**—As in *E. coli* all overexpressed variants of *Serratia* and *Anabaena* nuclease are produced as inclusion bodies, in all cases a denaturing-renaturing purification procedure was used (28). *Anabaena* nuclease was overexpressed as the His6-tagged protein and purified as described previously (6). The highly active nuclease can be expressed in *E. coli*, as they are inactive intracellularly, either because they require for activity disulfide bonds to be formed (*Serratia* nuclease) or because they are kept in an inactive state due to the presence of a specific polypeptide inhibitor (*Anabaena* nuclease). Wild type *Serratia* nuclease and the variant enzymes H89A, S140C, H184R, H89A,S140C, and H89A,H184R were produced as His6-tagged proteins and purified as described (4, 29).

In a modification of the previously described purification procedure we employed for some enzyme preparations an additional gel filtration purification step using a Beckmann Biosys 2000 HPLC system and a Merck Superperformance 600-16 Fractogel EMD BioSEC (S) which removes inactive aggregates from the preparation of the His6-tagged proteins. With this extra step the specific activity of the nuclease increases from 1 × 10⁶ units mg⁻¹ to 3–4 × 10⁶ units mg⁻¹.

The SI40C variant was also produced with the Strep-tag II as an N-terminal affinity peptide. Overexpression of the nuclease was carried out in the same way as described for the His6-tagged variants (4). The inclusion bodies formed after induction of a 1-liter culture were first solubilized using 6 M urea and then dialyzed against 10 mM Tris-HCl, pH 8.2, 0.1 M EDTA, and 20% glycerol. The nuclease was overexpressed and purified separately. A high molecular weight nucleic acid substrate was added to both samples to allow the nuclease to refold. The solution containing the native nuclease was applied to the column was 40 mM Tris-HCl, pH 8.2, 150 mM NaCl, 0.1 M EDTA, and 20% glycerol. The nuclease was eluted with a linear gradient of 0–1 M NaCl.

**Production of Obligatory Dimers and Obligatory Monomers of the *Serratia* Nuclease**—Obligatory dimers of *Serratia* nuclease were generated by chemical cross-linking of the SI40C variant (and other variants carrying the Ser⁴⁰ → Cys substitution) across the dimer interface at Cys⁴⁰ using the SH-specific bismaleimidoisothiocyanate as cross-linking reagent (41). Obligatory monomers were obtained by replacing His⁸⁹ and Arg¹⁸⁴ by Ala, Thr, Asn, and Arg (45).

**Analysis of the Quaternary Structure of *Serratia* and *Anabaena* Nuclease**—The quaternary structure analysis of *Serratia* nuclease variants and of *Anabaena* nuclease was carried out using gel filtration and analytical ultracentrifugation (6, 28, 45).

Analytical gel filtration experiments were performed at 25 °C on a Beckmann Biosys 2000 HPLC system using a Pharmacia Superdex 75 HR 10/30 column. The protein concentration of the 100-μl sample applied to the column was 40 μg.

Analytical ultracentrifugation experiments were carried out at 20 °C and 45,000 min⁻¹ in a Beckmann Optima XL-A centrifuge equipped with absorption optics and an An-50 8-place rotor. The protein concentration was 6 μg. Sedimentation velocity data were evaluated with the program package AKKUprog (46).

**Generation of an Obligatory Heterodimer of *Serratia* Nuclease**—An obligatory heterodimer of *Serratia* nuclease consisting of a wild type subunit and one subunit with an active site mutation was generated using the His6-tagged H89A,S140C variant which is catalytically inactive and the StrepII-tagged variant SI40C which has wild type activity (Fig. 2). Both variants were overexpressed and purified separately. A

² K. Krause, personal communication.
³ G. Meiss, O. Gimadutdinow, and A. Pingoud, manuscript in preparation.

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stoichiometric mixture of both variants was then dialyzed against 10 mM Tris-HCl, pH 8.2, 6 M urea to denature the protein. The refolding and stochastic formation of His6-tagged/His6-tagged (denoting: subunit 1/subunit 2), His6-tagged/StrepII-tagged and StrepII-tagged/StrepII-tagged dimers were achieved by dialysis against 10 mM Tris-HCl, pH 8.2. The dimers obtained were cross-linked using the same procedure that was used to generate the cross-linked S140C homodimer (41). To separate the heterodimer from the two homodimeric forms the solution containing the mixture of dimers was first applied to a Ni²⁺-NTA column which binds only the His6-tagged homodimers and the heterodimer. After the StrepII-tagged homodimer was removed by washing the column with 10 mM Tris-HCl, pH 8.2, 200 mM imidazole and after dialysis against 10 mM Tris-HCl, pH 8.2, applied to a StreptTactin column. The His6-tagged homodimers appeared in the flow-through and in the wash with 10 mM Tris-HCl, pH 8.2. The heterodimer was eluted with 10 mM Tris-HCl, pH 8.2, 2.5 mM desthiobiotin.

As the His6-tagged and the StrepII-tagged variant have almost the same molecular weight, they cannot be distinguished by SDS-polyacrylamide gel electrophoresis. To ensure that we indeed purified the heterodimer described above we applied a His6-tagged variant to a StreptTactin column and a StrepII-tagged variant to a Ni²⁺-NTA column. After washing with the same amount of buffer that was used in the procedure to prepare the heterodimer, all nucleolytic activity appeared in the flow-through and the wash.

Circular Dichroism (CD)—CD spectra were recorded with a Jasco J-710 circular dichrograph in 0.05-cm cells at protein concentrations of 5 μM as described previously (28).

Hyperchromicity Assay—The DNase activity of Anabaena nuclease and the different variants of Serratia nuclease were determined using a hyperchromicity assay (47) with high molecular weight DNA from herring sperm as described previously (31).

Plasmid Cleavage Assay—Plasmid cleavage assays were performed at 37 °C (steady state experiments) or 25 °C (pre-steady state experiments which were carried out in a quenched-flow apparatus (Biologic)) in 50 mM Tris-HCl, pH 8.2, 5 mM MgCl₂ with Serratia nuclease variants and in 50 mM Tris-HCl, pH 7.5, 5 mM MnCl₂, 0.01% (w/v) Lubrol with Anabaena nuclease. Protein concentrations varied between 5 × 10⁴ and 2.5 × 10⁶ units liter⁻¹. Cleavage products were analyzed using native 0.8% Tris borate-agarose gels. The gels were quantified by densitometry using a video documentation system (Intas).

Precipitation Assay—For the coprecipitation of the Serratia nuclease in the presence of DNA due to the formation of enzyme-DNA networks the nuclease was incubated at a concentration of 10 μM with 0.05 mg ml⁻¹ high molecular weight DNA from herring sperm in 50 mM Tris-HCl, pH 7.5, 5 mM MnCl₂, and 50 mM Tris-HCl, pH 8.2, 5 mM MgCl₂, respectively. Visually apparent precipitation was taken as an indicator for the formation of very high molecular weight enzyme-DNA complexes. To demonstrate that these complexes consist of DNA and enzyme the precipitate after centrifugation was analyzed by SDS-polyacrylamide gel electrophoresis for the presence of nuclease. The supernatant was analyzed for the decrease in DNA content by measuring the UV absorbance at 260 nm.

RESULTS

It has been the aim of this study to compare the properties and activities of obligatory monomeric and obligatory dimeric

**FIG. 1.** Ribbon drawing of the three-dimensional structure of the Serratia endonuclease dimer (5). The positions of His⁹⁹, Cys¹⁴⁰, and His¹⁸⁴ are indicated. His⁹⁹ acts as general base in the cleavage of phosphodiester bonds; its substitution by Ala inactivates the enzyme. The S140C variant was used to cross-link the nuclease at the dimer interface with bismaleimidohexane (41). His¹⁸⁴ makes specific contacts to a loop region in the second subunit. Replacement by Ala, Thr, Asn, and Arg leads to monomeric proteins (45).

**FIG. 2.** Scheme to illustrate the procedure used for the generation of cross-linked heterodimeric variants of Serratia nuclease with two different affinity tags.
variants of Serratia nuclease and for reference, of Anabaena nuclease which is a monomer. For this purpose we had prepared obligatory monomeric variants of Serratia nuclease by substituting His residues by other amino acid residues that interfere with subunit-subunit interaction, e.g. H184R (Fig. 1 (45)). We also had prepared obligatory dimers by substituting Ser by Cys and cross-linking the two subunits of this Serratia nuclease variant via the Cys residues by specifically designed bis-maleimides, e.g. S140C_X (Fig. 1 (41)). We have now prepared also a heterodimer of the Serratia nuclease consisting of an active subunit (S140C) and an inactive subunit (H89A,S140C) in which the catalytically essential His is substituted by Ala (Fig. 1). For this purpose, two variants of Serratia nuclease were produced, the S140C variant carrying at its N terminus the Strep-tag II and the H89A,S140C variant carrying at its N terminus the His6-tag. Upon mixing, denaturation and renaturation of the two variants homo- and heterodimers are formed. To prevent subunit exchange the mixture of homo- and heterodimers was treated with bismaleimidohexane as described before for the S140C variant to cross-link the two subunits (41). The cross-linking reaction had a yield of >95%. Cross-linked dimers were subjected to a series of two affinity purification steps involving a Ni2+-NTA and a StrepTactin column, after which a pure preparation of cross-linked heterodimer was obtained with an overall yield of 30% (Fig. 2).

The heterodimer and its “parent” homodimers, whose dimeric state was verified by gel filtration and/or analytical ultracentrifugation (data not shown) have the same circular dichroism (CD) spectrum as the wild type Serratia nuclease (Fig. 3). It had been demonstrated before that the H89A, the H184A, N, T, or R, and the cross-linked S140C variants all have the same CD spectrum as the wild type enzyme indicating that the secondary structure composition and presumably also the tertiary structure is unaltered by the amino acid substitution(s) and the cross-linking (41, 45).

The suggestion that the tertiary structure is unaffected by the amino acid substitution and the cross-linking is supported by functional assays. The specific activities of the monomeric variants H184A, N, T, or R, the cross-linked S140C, and the wild type enzyme toward high molecular weight DNA as determined by the Kunitz assay are very similar (Table I). This finding demonstrates that the subunits of Serratia nuclease are functionally independent; they can be irreversibly joined or irreversibly separated without effect on their activity. It is not unexpected, therefore, that the cross-linked heterodimeric variant with one active and one inactive subunit (H89A,S140C/S140C_X) has approximately half of the specific activity of the wild type enzyme (for this comparison enzyme preparations were used that had not been passed over a gel filtration column, cf. Table I). The steady state kinetic analysis of the cleavage of herring sperm DNA by cross-linked S140C and the cross-linked H89A,S140C/S140C heterodimer shows that the difference in activity is mainly due to a difference in $k_{cat}$ (S140C_X: $k_{cat} = 245 \text{ s}^{-1}$, $K_m = 39 \mu\text{M (nucleotide)}^{-1}$, $K_m/k_{cat} = 6 \mu\text{M}^{-1} \text{ s}^{-1}$; H89A,S140C/S140C: $k_{cat} = 135 \text{ s}^{-1}$, $K_m = 51 \mu\text{M (nucleotide)}^{-1}$, $K_m/k_{cat} = 3 \mu\text{M}^{-1} \text{ s}^{-1}$), demonstrating that in the heterodimer only one active site is present which is unaffected by the presence of the inactive subunit.

The Kunitz assay allows determination of specific activities, but gives little information regarding the course of the reaction, in particular whether the nuclease stays associated with a macromolecular substrate after cleavage and cleaves adjacent phosphodiester bonds in a concerted manner or whether it dissociates after each cleavage event and cleaves phosphodiester bonds in a sequential manner. To distinguish between these possibilities we have carried out cleavage assays with sc plasmid DNA. An enzyme which works in a processive manner will leave more sc plasmid DNA unattacked in the initial phase of the reaction than an enzyme of the same specific activity, which follows a distributive mechanism. As shown in Fig. 4 there is a clear difference in the time course of sc plasmid DNA cleavage between the monomeric and the dimeric variants of Serratia nuclease: the monomeric H184R variant works in a more distributive manner, as the sc plasmid DNA is degraded rapidly, while the obligatory dimer S140C_X follows a more processive mechanism which leaves the sc plasmid DNA intact for a longer period of time and preferentially produces fragments of the linear DNA (which appear as an unresolved

### Table I

| Variant | Quaternary structure | Specific activity $10^6$ units mg$^{-1}$
|---------|---------------------|-------------------------------|
| wt      | Dimeric             | 3.7                           |
| S140C_X | Obligatory dimeric  | 3.4 ($^a$)                    |
| H89A,S140C/S140C_X | Obligatory heterodimer | 0.4                           |
| H184A   | Monomeric           | 4.2                           |
| H184N   | Monomeric           | 4.0                           |
| H184T   | Monomeric           | 5.7                           |
| H184R   | Monomeric           | 5.8                           |
| S140C,H184R_X | Obligatory dimeric | 1.1 ($^b$)                    |
| Anabaena nuclease | Monomeric         | 1.7 ($^b$)                    |

$^a$ In these preparations inactive aggregates were not removed by gel filtration, such that the specific activity is lower due to the presence of about 70% aggregates.

$^b$ Mn$^{2+}$ as cofactor.

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* $^a$S140C_X denotes the cross-linked S140C dimer. An analogous abbreviation is used for the other cross-linked Serratia nuclease variants.
“smear” on the gel). The heterodimer produces an intermediate pattern. It is important to note that the monomeric H184R when forced into a dimer structure by cross-linking produces a cleavage pattern typical of the dimer. This clearly demonstrates that the mode of cleavage (more processive or more distributive) is a consequence of the quaternary structure.

It is noteworthy that with Mg$^{2+}$ as cofactor all Serratia nuclease variants cleave sc plasmid DNA without accumulation of the oc intermediate, indicating that both strands of the double-stranded DNA are cleaved in a correlated manner at sites in close proximity. This mode of cleavage was termed diplotomic and is characteristic of many deoxyribonucleases, e.g. spleen acid DNase (for review, see Ref. 48). Only in the pre-steady state phase of the reaction, i.e. in a quenched-flow experiment, can one observe under these conditions the formation of the oc intermediate. Its rate of appearance is very similar for the monomeric H184R and the dimeric S140C variant at the same specific activity and in the presence of Mn$^{2+}$. Fig. 6 also shows that in the presence of Mn$^{2+}$ the monomeric and dimeric variants of Serratia nuclease accumulate more oc DNA than in the presence of Mg$^{2+}$. This suggests that with Mn$^{2+}$ as the cofactor monomeric and dimeric variants of Serratia nuclease prefer to nick DNA and do not cleave the two strands in a correlated manner, i.e. to follow a haplotomic mode of action. Nevertheless, the dimeric variants cleave DNA processively also in the presence of Mn$^{2+}$ as can be deduced from the relatively slow decline of sc plasmid DNA.

The finding that dimeric forms of Serratia nuclease cleave high molecular weight DNA in a more processive manner than monomeric forms can be explained by the simultaneous binding of the substrate to two binding sites on the enzyme and...
thus critically depends on the affinity of the enzyme for the substrate. If binding is weak or dissociation fast compared with cleavage, the advantage of the dimer for a processive action will be diminished. This is indeed observed, when the binding of the nucleic acid to the nuclease is weakened by the addition of salt. Fig. 7 shows that with 125 mM NaCl in the reaction buffer the time course of cleavage of sc plasmid DNA by the cross-linked dimeric S140C variant is similar to that of the monomeric H184R variant. Furthermore, under these conditions both follow a haplotomic mode of cleavage.

The Kunitz assay had shown that wild type Serratia nuclease, the cross-linked S140C variant and H184A, T, N, or R variants have the same specific activity, despite the fact that they are in a monomer/dimer equilibrium (wild type enzyme), are obligatory dimers (S140C_X) or obligatory monomers (H184A, T, N, or R), respectively. On the other hand the course of the reaction is different with these variants, dimeric forms working more processively, monomeric forms more distributively on plasmid substrates. It must be expected, therefore, that at very low concentrations of enzyme and high molecular weight DNA (much lower than used in the Kunitz assay), where substrate binding becomes rate-limiting, dimeric variants that tend to stay associated with their substrate after cleavage should appear to be more active than monomeric variants which dissociate from the DNA after cleavage. This should only be the case with high molecular weight DNA, not, however, with oligonucleotides which cannot bind to two subunits simultaneously. Fig. 8 shows that the specific activity of dimeric S140C_X increases with decreasing protein concentration, but only when the substrate is high molecular weight DNA, not when it is an oligonucleotide. The monomeric H184R variant and Anabaena nuclease do not show this effect. The specific activity of the heterodimer displays an intermediate concentration dependence, presumably, because the H89A subunit of the heterodimer has a lower affinity to DNA than the wild type subunit.

The fact that the increase in specific activity is only observed with high molecular weight DNA but not with an oligonucleotide substrate demonstrates that this effect is not a consequence of a greater stability of the dimer compared with the monomer at low protein concentration but rather an effect mediated by the size of the substrate. The simultaneous binding of a high molecular weight DNA to the two subunits of Serratia nuclease should lead to a “network” of enzyme and DNA, which in the absence of cleavage, i.e. when an inactive variant like the H89A variant is used, should lead to precipitation of these aggregates. This is observed with the dimeric H89A and the H89A,S140C variants, not, however, with the monomeric H89A,H184R and the inactive H124A variant of monomeric Anabaena nuclease (data not shown). The aggregates are “dissolved” by addition of catalytic amounts of active Serratia or Anabaena nuclease, demonstrating that the aggregation is mediated by high molecular weight DNA. Serratia nuclease-DNA aggregates are formed at low and high concentration, but only at low concentrations, when association becomes rate-limiting, does the formation of aggregates lead to an increase in specific activity.

**DISCUSSION**

We have been concerned in the present paper with the importance of the dimeric state for the activity of the Serratia nuclease, the prototypic member of a family of nonspecific nucleases, some of which are dimers of identical subunits and others monomers. We have for this purpose analyzed the DNA cleavage activity of obligatory monomeric and dimeric variants of the Serratia nuclease and for comparison of Anabaena nuclease, a monomeric enzyme related to Serratia nuclease. Our results demonstrate that the two subunits of Serratia nuclease
function independently of each other. Nevertheless, the dimeric state has several consequences for the activity of the enzyme, due to the fact that the two subunits can bind simultaneously to one nucleic acid molecule if it is macromolecular and, therefore, provides many binding sites for the nuclease. In the absence of cleavage, as shown here for an inactive dimeric variant of Serratia nuclease, huge networks are formed between the enzyme and the macromolecular substrate that lead to precipitation, similarly as observed with antibodies and multivalent antigens (immunoprecipitation). With active dimeric variants such networks are not formed as substrate binding is immediately followed by cleavage. However, nucleic acids cleaved at one site are likely to stay associated with the enzyme after cleavage if the nucleic acid is bound also by the other subunit which then may cleave the nucleic acid at another site. This leads to a processive degradation of the nucleic acid substrate. At low concentrations of enzyme a dimer following a processive mechanism will be more effective than a monomer following a distributive mechanism in degrading nucleic acids, because finding a new site for cleavage under these conditions is not rate-limiting for the dimeric enzyme. It is conceivable that S. marcescens which secretes several enzymes, among them several proteases (49, 50), lipases (51–53), and chitinases (54, 55), to make available nutrients from the environment, has evolved to produce such a dimeric nuclease to make efficient use also of nucleic acids. This evolutionary achievement is not unique to Serratia nuclease, as other members of the Serratia family of nucleases, for example, EndoG (16), are dimeric. However, it seems, as if S. marcescens has found an independent solution, because amino acid residues forming the dimer interface are not conserved among the dimeric nucleases, in contrast to amino acid residues that make up the catalytic core.

Another feature of the mode of cleavage observed with Serratia nuclease, namely like several other nuclease-DNA complexes that cleave double-stranded DNA in a diprotic fashion (for review, see Ref. 48), i.e., in both strands “simultaneously,” is not dependent on the dimer state but also may help to efficiently degrade high molecular weight DNA to small oligodeoxynucleotides.

In conclusion, we have analyzed the role of the dimer state for the Serratia nuclease and shown that it is of importance for the processive and, therefore, efficient degradation of nucleic acids, even at low concentrations. The approach we have taken, namely to compare the enzymatic activity of obligatory monomeric and dimeric variants is sufficiently general to be of use also for other systems.

It remains to be explained why the Serratia nuclease is a dimer and the Anabaena nuclease a monomer, despite the fact that they apparently perform the same function. One could argue that nature just has not gotten around to dimerizing the Anabaena enzyme, because the evolutionary pressure on Anabaena sp., an organism capable of making use of light energy, has not been as strong as on S. marcescens which is absolutely dependent on nutrients from the environment to cover its energy requirements.

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