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Analysis of the mechanism of the *Serratia* nuclease using site-directed mutagenesis

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ABSTRACT

Based on crystal structure analysis of the *Serratia* nuclease and a sequence alignment of six related nucleases, conserved amino acid residues that are located in proximity to the previously identified catalytic site residue His89 were selected for a mutagenesis study. Five out of 12 amino acid residues analyzed turned out to be of particular importance for the catalytic activity of the enzyme: Arg57, Arg87, His89, Asn119 and Glu127. Their replacement by alanine, for example, resulted in mutant proteins of very low activity, <1% of the activity of the wild-type enzyme. Steady-state kinetic analysis of the mutant proteins demonstrates that some of these mutants are predominantly affected in their k_{cat}, others in their K_m. These results and the determination of the pH and metal ion dependence of selected mutant proteins were used for a tentative assignment for the function of these amino acid residues in the mechanism of phosphodiester bond cleavage by the *Serratia* nuclease.

INTRODUCTION

Serratia nuclease (EC 3.1.30.2) catalyzes the hydrolytic cleavage of DNA and RNA between the 5′-phosphate and the 3′-oxygen of the sugar moiety in the presence of Mg^{2+} or several other divalent metal ions (1,2). The enzyme is able to cleave both single- and double-stranded substrates with similar efficiency, but like other non-specific nucleases shows certain sequence preferences (3,4). The gene of the nuclease has been cloned (5) and protein sequence alignments (6,7) have shown that this enzyme belongs to a new class of non-specific nucleases from different species, namely *Saccharomyces cerevisiae* (8), *Anabaena* sp. 7120 (9), *Syncephalastrum racemosum* (10), *Bos taurus* (11) and probably also *Streptococcus pneumoniae* (12). Although significant sequence homology exists among these proteins, the biological functions of these nucleases are quite distinct, ranging from an apparently simple nutritional function for the extracellular *Serratia* and *Anabaena* nucleases, involvement in DNA repair or recombination in the case of the endo-exonuclease from yeast (6), to generation of primers for mitochondrial DNA replication (11). For this group of proteins the *Serratia* nuclease has become the paradigm, as many biochemical and biophysical studies have been performed on this enzyme, in particular the recently published 2.1 Å X-ray crystal structure analysis (13,14). Although the structure was solved for the free enzyme, i.e. in the absence of a metal ion cofactor and substrate, several lines of evidence led to the proposal that the active site is located around His89 and Glu127, residues which are conserved among the homologous nucleases (13). The independently carried out mutational analysis of this enzyme in which several conserved amino acid residues were substituted by Ala confirmed this proposition (7). The two mutants with the lowest residual activity were H89A and E127A, showing <0.001 and 0.1% respectively of the activity of the wild-type enzyme. However, based on these data it was neither possible to propose a mechanism of nucleic acid cleavage nor to assign a role for these two residues in catalysis. Moreover, as the unique tertiary fold of the *Serratia* nuclease and the structure of the presumptive active site suggests that the mechanism of nucleic acid cleavage is different from other nucleases of known structure, arguments by analogy for a likely mechanism of action could not be presented.

In this paper we report the results of biochemical experiments designed to identify the catalytic function of several residues within the presumptive active site of the *Serratia* nuclease. To this end we have extended our previously published mutational analysis to all residues conserved among the six homologous nucleases and located those that can be considered candidates for involvment in catalysis. The mutational analysis presented here was guided both by the X-ray structure and by an improved and expanded sequence alignment. It includes an investigation of the pH dependence and of the metal ion cofactor requirement of selected mutants. Based on the results presented here we put forward a suggestion for a likely mechanism of action of this enzyme.

MATERIALS AND METHODS

Chemicals, enzymes and DNA

Chemicals for electrophoresis were purchased from Gibco BRL or Baker, chemicals for oligonucleotide synthesis from Millipore. All other chemicals were supplied by Merck. Restriction enzymes were

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obtained from Amersham-Buchler or Boehringer-Mannheim. T4 polynucleotide kinase, T4 DNA ligase and *Taq* DNA polymerase were from Amersham-Buchler, *Pfu* DNA polymerase from Stratagene. All enzymes were used according to the respective manufacturer's recommendations. Salmon testis DNA and herring sperm DNA were from Sigma and Pharmacia respectively.

Oligodeoxyribonucleotides

Oligodeoxyribonucleotides were synthesized on a Millipore Cyclone Plus DNA synthesizer using standard β-cyano-ethylphosphoramidite chemistry (15) on the 0.2 µmol scale. Synthesis was carried out in the trityl-off mode and deprotection was performed using established protocols. Purification was performed by standard denaturing gel electrophoresis and the purified oligodeoxyribonucleotides were desalted by gel filtration using NAP-10 columns.

Multiple sequence alignment

Protein sequences of *Serratia* nuclease (NUC_SERMA from the SWISS-PROT Protein Sequence Database), *Saccharomyces cerevisiae* mitochondrial endo-exonuclease (NUC1_YEAST), *Anabaena* sp. nuclease (NUCA_ANASP), *Bos taurus* endonuclease G (NUCG_BOVIN), DNA entry nuclease from *Streptococcus pneumoniae* (NUCE_STRPN) and a partial sequence of the deoxyribonuclease from *Syncephalastrum racemosum* (PIR S33276 from PIR Protein; 10) were aligned using CLUSTAL W 1.5 (16) with the standard parameters (for details see 7). The alignment was refined after comparison with the X-ray structure of the *Serratia* nuclease (13).

Site-directed mutagenesis

Site-directed mutagenesis was performed using a two step PCR mutagenesis protocol essentially as described in detail before (7). After PCR, the mutated gene was cloned into the *Bam*HI and *Sal*I-cleaved plasmid pHisNuc and transformed into *Escherichia coli* LK111(λ) (17). Mutant clones were identified after PCR amplification of the *Serratia* gene starting with DNA from single colonies, followed by digestion of the PCR product by restriction enzymes specific for silent marker sites introduced together with the codon exchange. After plasmid preparation (QIAGEN Plasmid Mini Kit), the mutation was verified by DNA sequencing of the whole *Serratia* nuclease gene.

Preparation of *Serratia* nuclease

Plasmids containing the gene for the His_6-tagged *Serratia* nuclease (wild-type or mutant) were transformed into *E.coli* TGE900 (18). Cells were grown at 28°C, heat induced at 42°C and the nuclease extracted from inclusion bodies by urea treatment and purified to homogeneity by Ni–NTA affinity chromatography essentially as described before (19). Protein concentrations were determined using the extinction coefficient for the isoenzyme SM2 of $\varepsilon_{280} = 47\,292\ M^{-1}cm^{-1}$ (20). For Y76F a theoretical extinction coefficient of $\varepsilon_{280} = 43\,100\ M^{-1}cm^{-1}$, based on the content of Trp, Tyr and Phe, was used (21). The enzyme with the same amino acid sequence as isoenzyme SM2 (22) except for the N-terminal Met-$(His)_6$-Gly-Ser affinity tag is referred to as wild-type throughout the following text.

Circular dichroism(CD)

CD spectra of the *Serratia* nuclease at a concentration of 5 µM were recorded in a Jasco J-710 model dichrograph from 185 to 250 nm at 16°C in a cylindrical cuvette of 0.05 cm path length in 10 mM Tris–HCl, pH 8.2. After recording the CD spectrum of the protein, the baseline given by the CD spectrum of 10 mM Tris–HCl, pH 8.2, was recorded and subtracted from the protein CD spectrum.

Kinetic measurements with high molecular weight DNA as substrate

The activity of wild-type and mutant *Serratia* nuclease preparations was determined using high molecular weight DNA from salmon testis (Sigma D-1626) or herring sperm DNA (Pharmacia 27-4564-01) as substrate. Experiments were carried out in 10 or 1 mm (for DNA concentrations >0.1 mg/ml) cuvettes at 25°C with a Hitachi U-3000 spectrophotometer set at 260 nm. Substrate DNA concentrations were varied between 0.005 and 1 mg/ml (~15 µM to 3 mM nucleotides). Concentration of substrates (in nucleotides) were calculated using e_{260} of 6600 $M^{-1}cm^{-1}$ for double-stranded DNA. As for DNase I, the specific activity of the *Serratia* nuclease can be expressed in terms of Kunitz units (KU) per mg protein (23). 1 KU is defined as the amount of enzyme needed for an increase of 0.001 $A_{260\ nm}$/min at 25°C in a 1 ml volume at 1 cm path length. For determination of the apparent rates of reaction, full progress curves were recorded and the maximum rate expressed as ΔA_{260}/min was determined. Measurements were performed in a volume of 120 µl. After pre-warming the solution containing all components but the nuclease for 1 min, the reaction was started by addition of 1–10 µl appropriately diluted wild-type or mutant enzyme to obtain maximum velocities of ≤10%/min of the total hyperchromic effect.

For the Mg^{2+} ion dependence of DNA cleavage by wild-type and mutant proteins, the substrate DNA was dialyzed extensively against 10 mM Tris, 1 mM EDTA, pH 8.0 to remove all traces of divalent metal ions and thereafter against 10 mM Tris–HCl, pH 8.0. Aliquots of 180 µl nuclease at appropriate concentration (for example wild-type 0.5 nM, D86A 70 nM and E127A 600 nM) and DNA (0.075 mg/ml) were pre-incubated at 25°C in 40 mM Tris–HCl, pH 8.0, and NaCl at concentrations to give a constant ionic strength of 0.1 M. The reaction was started by addition of 20 µl $MgCl_2$ solution of appropriate concentration.

The change in absorbance accompanying DNA hydrolysis was used to convert velocities measured as ΔA_{260}/min to k_{cat} units (s^{-1}). The hyperchromic effect occurs when double-stranded DNA is separated into individual strands and/or cut to small single-stranded oligonucleotides ≤10 nt in length (24). For example, the increase in absorbance at 260 nm for salmon testis DNA after complete hydrolysis with *Serratia* nuclease is 33%. This increase in absorbance is proportional to the decrease in chain length for a substantial part of the reaction. The change in hyperchromicity can, therefore, be converted to the rate of phosphodiester bond cleavage, similarly to as in the report of Hale *et al.* (25):

$$V[s^{-1}] = \left(\frac{\Delta A^{260}}{\Delta t}\right)_{max} \cdot \frac{A^{260}_{t=0}}{A^{260}_{t=\infty} - A^{260}_{t=0}} \cdot \frac{1}{\epsilon^{260}} \cdot \frac{1}{n} \cdot \frac{1}{c_{Nuc}}, \qquad \mathbf{1}$$

where $(\Delta A_{260}/\Delta t)_{max}$ is the maximum slope in the A_{260} versus t plot, $A^{260}_{t=0}$ and $A^{260}_{t=\infty}$ are the absorbances at the beginning and end of the reaction respectively, ε_{260} ($M^{-1}cm^{-1}$) is the molar extinction coefficient of the substrate (in nt), n is the average size

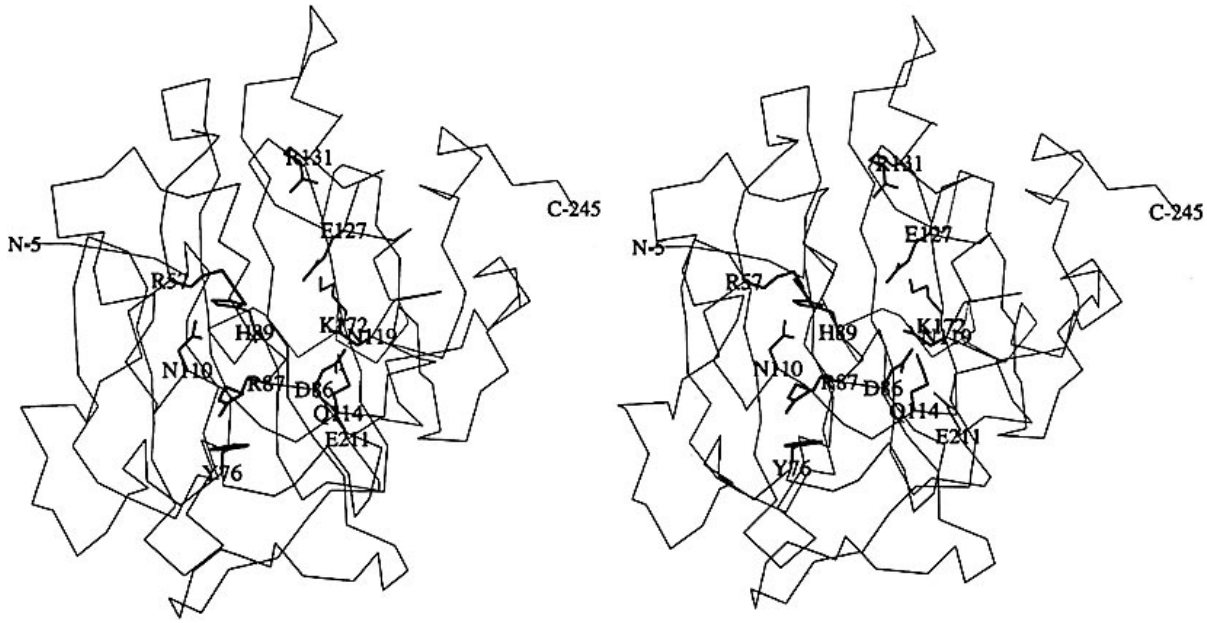

Figure 1. Stereo view of the structure of the *Serratia* nuclease (13). Amino acid residues which might play a role in catalysis are highlighted.

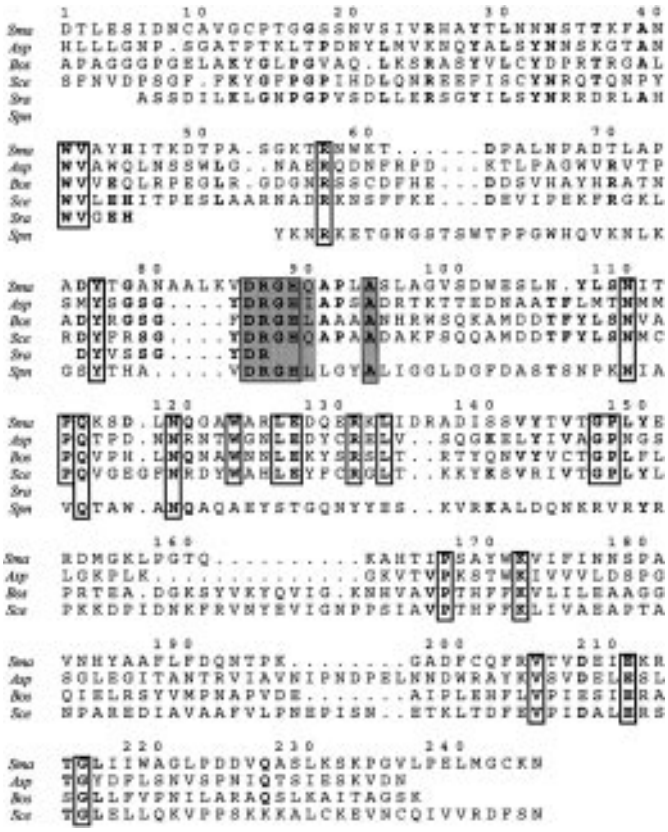

Figure 2. Alignment of DNA/RNA non-specific nucleases. Sma, *S.marcescens*; Asp, *Anabaena* sp.; Bos, *B.taurus*; Sce, *S.cerevisiae*; Sra, *S.racemosum*; Spn, *S.pneumoniae*. The numbering above the sequences refers to the isoenzyme SM2 of the *Serratia* nuclease. Identical amino acid residues in >60% or >80% of the sequences are in bold face and boxed respectively. The PROSITE motif PDOC00821 is shaded in gray. It should be mentioned that these sequences are the only ones picked up by PROSITE from the SWISS-PROT Protein Sequence Database using this motif.

(in nt) of the products obtained and c_{Nuc} is the concentration of *Serratia* nuclease. The average final product of DNA cleavage by the *Serratia* nuclease is 2.5 nt in length (2,26), which means that not all phosphodiester bonds are cleaved. This approach of deducing Michaelis–Menten parameters from Kunitz-type assays is similar to that used for staphylococcal nuclease (27). For evaluation of k_{cat} and K_m the data were fitted to the Michaelis–Menten equation using the program ENZFITTER 1.05 (28).

Activity versus pH profile

For evaluation of the pH dependence of the *Serratia* nuclease-catalyzed cleavage of nucleic acids we used the following buffer substances (pK_a, pH range): NaAc (4.75, 3.5–5.8); HEPES–NaOH (7.55, 6.5–8.6); Tris–HCl (8.30, 7.2–9.6); CAPS–NaOH (10.4, 9.0–10.9). All buffers were made from stocks containing 180 mM NaCl, 10 mM $MgCl_2$, 20 mM buffer substance by mixing the base and the acid form of the buffer substance, e.g. sodium acetate with acetic acid. pH was measured at 20°C using a pH meter 761 (Knick) equipped with a combination electrode type N6280 (Schott) calibrated with standard buffer pH set 1 (Merck). Variation in ionic strength was <10% and controlled by measuring the conductivity with a Schott conductometer CG 855 equipped with an LF1100 electrode. Cleavage kinetics were recorded using a microtiter dish assay by measuring the disappearance of ethidium stained DNA fluorescence upon cleavage of DNA. The method will be described in more detail elsewhere. Briefly, 0.025 mg/ml salmon testis DNA in 10 mM buffer, 90 mM NaCl, 5 mM $MgCl_2$ and 5 µM ethidium bromide were incubated in a 100 µl volume at room temperature with the nuclease. The ethidium stained DNA fluorescence was monitored after excitation at 312 nm on a transilluminator with a video documentation system (INTAS, Göttingen, Germany).

RESULTS

The active site of the *Serratia* nuclease has been located by crystallographic analysis of the enzyme (14) and by an alignment-guided mutagenesis approach (7). According to these studies His89 is part of the active site and essential for catalysis. It is surrounded by several amino acid residues conserved among a group of non-specific nucleases (Figs 1 and 2). Mutagenesis of some of these residues to Ala had shown that Asp86, Arg87 and Glu127 are also strong candidates for being involved in catalysis; their specific functions, however, have not yet been analyzed. Based on a new multiple sequence alignment containing six nucleases and, more importantly, by using the detailed information of the 3-dimensional structure of the *Serratia* nuclease (14) we have extended our previous mutational analysis (7) to characterize amino acid residues likely to be involved in catalysis and to analyze their role. The new alignment shows that a substantial number of residues are conserved in all five nucleases (for the sixth nuclease only partial sequence information is available). We therefore included in our mutational analysis all conserved functional amino acid residues located in the direct spatial neighborhood of His89 (Figs 1 and 2 and Table 1). All amino acid residues but Tyr76, which was substituted by Phe, were changed to Ala and several of them also for other, usually similar, amino acid residues. Of 27 mutants analyzed here, 21 had to be produced and six were already available from a previous study (7). Mutant proteins were purified to homogeneity and analyzed for the integrity of their (secondary) structure by CD spectroscopy. The nucleolytic activity of the mutants was determined and, where possible, steady-state parameters were evaluated for the cleavage reaction. Mutants with substitution of amino acid residues considered to be involved in acid–base catalysis and/or Mg^{2+} binding were also analyzed with respect to their pH dependence and metal ion requirement.

Homology of six non-specific nucleases

The homology between the *S.marcescens*, *S.cerevisiae*, *Anabaena* sp. and *B.taurus* nucleases has been reported before (6,7,9). The nuclease of *S.racemosum* was considered to be homologous to DNase I, although it has only 12 of 40 N-terminal amino acid residues in common with DNase I (10). However, as can be seen from Figure 2, this nuclease shares 16 identical residues with the nuclease of *S.cerevisiae* and, more importantly, an internal tryptic fragment comprising nine amino acids has seven amino acids in common with this nuclease but no homology to DNase I. We therefore believe that this nuclease fits well into the class of DNA/RNA non-specific endonucleases. The extracellular nuclease of *S.pneumoniae* was included in this alignment, although the overall similarity to the other nucleases is low, because most of the conserved amino acid residues located around the active site of the *Serratia* nuclease are conserved in this nuclease, including all residues of the PROSITE motif D-R-G-H-[QIL]-X_3-A (accession no. PDOC00821), characteristic of the family of DNA/RNA non-specific endonucleases. Incident- ally, no other proteins homologous to the *S.pneumoniae* nuclease were found in the SWISS-PROT databank.

Purification and secondary structure analysis

We have produced 27 mutants of the *Serratia* nuclease at 12 different positions by site-directed mutagenesis and overexpression in *E.coli* (see Table 1 for an overview). All mutant proteins were purified to homogeneity. They were obtained as soluble proteins, although some of them, upon incubation with DNA, showed a tendency to aggregate. As judged from their CD spectra all mutants are likely to have a similar structure to the wild-type enzyme. The CD spectrum of K172A was the only one to show a minor deviation from the CD spectrum of the wild-type nuclease (data not shown), which could be due to the fact that this residue is part of the central β-sheet.

Steady-state kinetic analysis

The cleavage activity of the mutants was measured with the hyperchromicity assay using salmon testis DNA as substrate (Table 1). The activity of the mutants H89D and H89Q could not be determined accurately, as they aggregate rapidly in the presence of DNA.

Mutations at two positions, His89 and Asn119, lead to almost inactive enzymes, suggesting that these amino acid residues have a very important function in catalysis. At position 89 only H89N shows measurable activity (0.13%). It is mainly affected in its k_{cat}. All other mutants (His→Ala, Asp, Gln, Glu or Lys) with amino acid substitutions introduced at position 89 show drastically reduced activity (<0.001%). At position 119 all amino acid substitutions tested (Asn→Ala, Asp, Gln or His) result in mutant proteins with very little nuclease activity (0.001–0.1%), N119D and N119Q being the most active ones (0.05 and 0.1% respectively). N119D and N119Q, like H89N, are also much more affected in their k_{cat} than K_m.

The next group of mutants with markedly reduced activity contains the amino acid residues Arg57, Arg87 and Glu127. The large decrease in activity observed upon substitution by Ala (0.1–0.6%) indicates that these amino acids could be directly involved in catalysis. Arg57 and Arg87 are thought to be near the phosphate backbone of the nucleic acid substrate and alterations in these positions could change the binding and orientation of the substrate (13). A similar argument would hold for Glu127, which, via Mg^{2+}, could also serve to bind and position the substrate. In these mutants the substitution of Arg by Ala is more deleterious than by Lys and the substitution of Glu by Ala or Gln is more deleterious than by Asp. Thus, the conservation of charge helps to retain some activity, but only in the case of R87K is the wild-type activity almost reached. This result is interpreted to mean that Arg57 and Glu127 are, in contrast to Arg87, needed for very precise interactions and therefore most likely are directly involved in catalysis. Mutants of Arg57 are more affected in their k_{cat} than K_m, while the opposite is true for the R87A mutant. With Glu127 amino acid substitutions lead to mutant enzymes impaired more or less in k_{cat} and K_m, depending on the substitution.

Less dramatic effects are observed when the conserved amino acid residues Asp86, Asn110 and Gln114 are substituted by Ala (~1%). For Asn110 and Gln114 this reduction can be explained by indirect effects on the catalytic center. These residues, as shown by the X-ray structure, have important structural functions, which include making hydrogen bonds to either the main chain NH and O at position Ala91 (by Asn110) of the active site loop containing the D-R-G-H-motif or to the side chain of Asn119 (by Gln114) respectively. The effects of substitutions for Asp86 (Asp→Ala or Glu) cannot be rationalized in a straightforward manner by structural effects. While D86A is a K_m mutant, D86E exhibits a decrease in both k_{cat} and K_m.

Table 1. Steady-state kinetic data for *Serratia* nuclease mutants

Position	Amino acid exchange	k_{cat}[a]	$1/K_m$[a]	k_{cat}/K_m[a]
Wild-type		1 ± 0.09	1 ± 0.25	1 ± 0.33
Arg57	Ala	$(1.7 \pm 0.1) \times 10^{-2}$	$(3.7 \pm 0.7) \times 10^{-1}$	$(6.2 \pm 2) \times 10^{-3}$
	Lys	$(5 \pm 0.3) \times 10^{-3}$	12.5 ± 1.4	$(6 \pm 0.7) \times 10^{-2}$
Tyr76	Phe	$(3.7 \pm 0.1) \times 10^{-1}$	4 ± 0.5	1.1 ± 0.2
Asp86	Ala	$(7.5 \pm 0.5) \times 10^{-1}$	$(2 \pm 0.2) \times 10^{-2}$	$(1 \pm 0.2) \times 10^{-2}$
	Glu	$(1 \pm 0.1) \times 10^{-2}$	11.1 ± 2.8	$(1 \pm 0.08) \times 10^{-1}$
Arg87	Ala	$(3.4 \pm 1) \times 10^{-1}$	$(9 \pm 4) \times 10^{-3}$	$(2.9 \pm 2) \times 10^{-3}$
	Lys	$(1.4 \pm 0.07) \times 10^{-1}$	3.8 ± 0.4	$(5 \pm 0.5) \times 10^{-1}$
His89	Ala	n.d.	n.d.	$<10^{-5}$
	Asn	$(3 \pm 0.4) \times 10^{-4}$	4 ± 1.5	$(1.3 \pm 0.6) \times 10^{-3}$
	Asp	n.d.	n.d.	$<10^{-5}$
	Gln	n.d.	n.d.	$<10^{-5}$
	Glu	n.d.	n.d.	$<10^{-5}$
	Lys	n.d.	n.d.	$<10^{-5}$
Asn110	Ala	n.d.	n.d.	$(2 \pm 0.5) \times 10^{-2}$
Gln114	Ala	n.d.	n.d.	$(1.8 \pm 0.5) \times 10^{-2}$
Asn119	Ala	n.d.	n.d.	$< 10^{-5}$
	Asp	$(4.8 \pm 0.4) \times 10^{-3}$	$(1 \pm 0.2) \times 10^{-1}$	$(5 \pm 1) \times 10^{-4}$
	Gln	$(2 \pm 0.8) \times 10^{-3}$	$(6.3 \pm 1.8) \times 10^{-1}$	$(1 \pm 0.3) \times 10^{-3}$
	His	n.d.	n.d.	$< 10^{-5}$
Glu127	Ala	$(3.9 \pm 0.5) \times 10^{-2}$	$(2.8 \pm 0.6) \times 10^{-2}$	$(1.1 \pm 0.4) \times 10^{-3}$
	Asp	$(8.3 \pm 2) \times 10^{-1}$	$(9.9 \pm 3) \times 10^{-2}$	$(8.2 \pm 4) \times 10^{-2}$
	Gln	$(1.4 \pm 0.3) \times 10^{-2}$	$(2.8 \pm 0.9) \times 10^{-1}$	$(4 \pm 2) \times 10^{-3}$
Arg131	Ala	$(2.4 \pm 0.6) \times 10^{-1}$	$(2.9 \pm 2) \times 10^{-2}$	$(6.9 \pm 7) \times 10^{-2}$
Lys172	Ala	$(9.3 \pm 0.8) \times 10^{-2}$	$(1.3 \pm 0.2) \times 10^{-1}$	$(1.2 \pm 0.3) \times 10^{-2}$
Glu211	Ala	$(4.1 \pm 0.2) \times 10^{-3}$	2.3 ± 1	$(9.4 \pm 5) \times 10^{-3}$
	Asp	n.d.	n.d.	1 ± 0.33
	Gln	n.d.	n.d.	1 ± 0.33

[a]For wild-type (HisNuc) nuclease the following kinetic parameter for salmon testis DNA cleavage were obtained with the hyperchromicity assay: $k_{cat} = 980\ s^{-1}$ ($V_{max} = 4.9 \times 10^6$ KU/mg); $K_m = 60\ \mu M$ (nt) (0.02 mg/ml); $k_{cat}/K_m = 16\ s^{-1}\mu M^{-1}$ (nt). >, substrate saturation was not obtained and the values given for k_{cat} and K_m are lower limits. Errors were calculated with the program ENZFITTER.
n.d., not determined.

Although E211A shows reduced activity (7), the X-ray-structure and the results with the two mutants E211D and E211Q, both having wild-type activity, show that Glu211 is not likely to be directly involved in catalysis. The structural analysis (13) suggests that it has an important structural function by forming a salt link with Lys231. This electrostatic interaction also includes the participation of Lys212 via an intervening water molecule. Glu211 is also hydrogen bonded to the main chain at position Ile218 of β-sheet 2′. This contact presumably can also be made by Asp or Gln, but not by Ala.

Mutation of the conserved Tyr76 to Phe results in a mutant protein with only slightly reduced activity. Therefore, the hydroxyl group of Tyr76 is unlikely to participate in the catalytic action of the *Serratia* nuclease. In particular, it cannot be involved in a covalent intermediate as observed, for example, with topoisomerases (reviewed in 29).

Activity versus pH profiles

Activity versus pH profiles were determined for H89N, N119D, E127Q and E127D. For the activity of the wild-type enzyme a bell-shaped curve was obtained with an optimum around pH 8. The shapes of the activity versus pH profiles of E127Q and E127D are similar to that of the wild-type enzyme (data not shown), while those of H89N and of N119D are significantly different (Fig. 3A). In contrast to the pH dependence of the activity of the wild-type enzyme, the activity of the H89N mutant rises almost steadily over the entire pH range. Comparison of the two profiles suggests that His89 is engaged in a protonation/deprotonation equilibrium around pH 5.5–6.5 and that protonation interferes with activity of the enzyme. For the N119D mutant, the profile has a maximum at ~pH 5 which can be rationalized by assuming that deprotonation of Asp119 introduces a negative

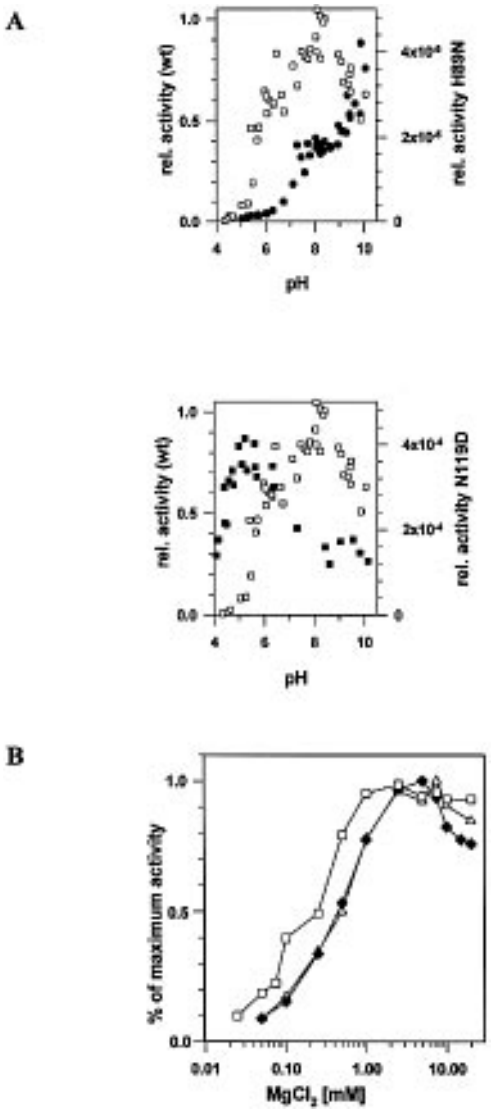

Figure 3. pH and Mg^{2+} concentration dependence of the activity of the wild-type *Serratia* nuclease and selected mutants. (**A**) Activity versus pH profiles for the wild-type *Serratia* nuclease (□) as well as the H89N (●) and N119D mutants (■) are shown. Activities are given as relative values with respect to the wild-type enzyme activity at pH 8.0. (**B**) Mg^{2+} concentration dependences of DNA cleavage for the wild-type *Serratia* nuclease (□) and the D86A (Δ) and E127A (◆) mutants are shown. Activities are given as relative values with respect to the maximum activity for each enzyme.

charge at the active site of the nuclease which is deleterious for nuclease activity. At low pH Asp119 is protonated and hence could function in a similar way to Asn119. Indeed, the activity of the N119D mutant approaches the wild-type activity at very low pH.

Dependence of nuclease activity on metal ions

We investigated the divalent metal ion preference of the nuclease activity of the wild-type enzyme and selected mutant proteins (D86A, H89N, N119D, E127A, E127D and E127Q). Only H89N shows a similar divalent metal ion preference to the wild-type nuclease, which is half as active with Mn^{2+} as with Mg^{2+}. The other mutant proteins are at least twice as active with Mn^{2+} than with Mg^{2+}, E127A being up to five times more active. Furthermore, the activity of N119D is dramatically increased with either Co^{2+} (20 times) or Zn^{2+} (40 times) in comparison to Mg^{2+}, while the wild-type enzyme shows equal or reduced activity with Co^{2+} and Zn^{2+} compared to Mg^{2+}. Of particular interest were Asp86 and Glu127, as in proteins Asp and Glu residues are very often observed as ligands of Mg^{2+}. For the mutants D86A and E127A the Mg^{2+} concentration dependence is given in Figure 3B. Both mutants show similar profiles which are offset by a factor of 2–3 towards higher Mg^{2+} concentrations compared to the profile of the wild-type nuclease, indicating that these mutants have a slightly lower affinity for the metal ion cofactor than the wild-type enzyme. A similar result has also been obtained for N119D (data not shown).

DISCUSSION

One of the principal goals of an enzymological study is the elucidation of the mechanism of action for the enzyme under investigation. For a hydrolase like the *Serratia* nuclease this means in particular identifying those amino acid residues that are responsible for activation of the attacking water molecule, stabilization of the transition state and protonation of the leaving group and, based on this assignment, proposing a reaction mechanism. As for many nucleases, as well as hydrolases in general, propositions have been published regarding a likely mechanism of action and reference data and models exist which can be used for a comparison with the *Serratia* nuclease.

Based on a detailed sequence comparison among related nucleases and a mutational analysis of conserved amino acid residues and the X-ray structure of the *Serratia* nuclease, we suggest that the active site comprises at least four residues, namely Arg57, His89, Asn119 and Glu127. These residues are not only located in the immediate neighborhood of each other (Fig. 1), but mutagenesis of any of these residues results in proteins with drastically reduced activity.

Water activation

The most important residue of the active site seems to be His89. Among the six H89 mutants only the H89N mutant nuclease shows measurable residual activity. This mutant is mainly affected in k_{cat}, arguing for a defect in a catalytic function and not in substrate binding. That His89 is not involved in substrate binding is supported by the finding that the wild-type protein and H89A bind with similar affinity to a non-cleavable modified dodecamer in which all phosphate residues were substituted by phosphorothioates (Friedhoff, unpublished results). H89N has a different pH profile compared to the wild-type enzyme: the pK_a of ~5.5–6.5 in the ascending branch of the wild-type activity versus pH profile (Fig. 3A), which is missing in that of H89N, may therefore be assigned to His89. The interpretation most consistent with our data is that His89 must be deprotonated to support catalysis, i.e. to function as the general base. For the H89N mutant specific base catalysis (OH^-) would then replace general base catalysis in activation of the water molecule.

A role for His89 as a general acid is unlikely. In this respect *Serratia* nuclease differs from DNase I. For DNase I, which has a pH optimum of 7.5, a His residue (His134) is considered to be the general acid (30). If His89 in the *Serratia* nuclease were the general acid, the question would arise as to which is the general base. As discussed in Miller *et al.* (13), Glu127 could be the

general base (rather than His89), however, the relatively high residual activity of E127Q and the similarities of the pH profiles of the wild-type enzyme and the E127Q mutant argue against this alternative. RNase T_1, which uses Glu58 as the general base and His92 as the general acid, has an optimum around pH 5–6 (31).

Transition state stabilization

The substitution of Asn119 by other amino acids is as deleterious for enzyme activity as substitutions at position His89. Comparison with other hydrolases of known structure and mechanism offers several possible roles for Asn119. In RNase A, Gln11 forms a direct contact with the scissile phosphate, which may be used for transition state stabilization (32). However, recent mutagenesis results have shown that the RNase A mutants Q11A, Q11D and Q11H did not have a markedly reduced activity towards natural substrates (33). In DNase I, Asn170 is also seen at hydrogen bond distance from the phosphate in some structures of this enzyme (30) and is also found (Asn153) in the structurally related exonuclease III (34). However, no mutagenesis data are available for these residues in the respective proteins and, as has been shown for RNase A, without such data the importance of this possible phosphate contact for transition state stabilization remains speculative. On the other hand, Asn119 in the *Serratia* nuclease might have a similar function to Gln residues (Gln61, Gln200 or Gln204 respectively) in the GTPases $p21^{ras}$ (35), transducin (36) or $G_{i\alpha}$ (37) respectively. These residues are involved in positioning the attacking water and later in stabilization of the transition state. Such a function for Gln or Asn is also well documented for several proteases, e.g. papain and subtilisin, were they stabilize the oxyanion in the transition state (38–40). A plausible interpretation of our data could be that in the *Serratia* nuclease Asn119 is involved in transition state stabilization.

The Ala mutant at position Arg57 is very much impaired in its activity, mainly due to a large decrease in k_{cat}. This suggests that Arg57, similarly to in other nucleases [Arg77 in RNase T_1 (41) and Arg48 in nuclease P1 (42)], is likely to be involved in positioning and polarizing the phosphate of the scissile phosphodiester bond and/or stabilization of the transition state.

Mg^{2+} binding

The *Serratia* nuclease requires Mg^{2+} for phosphodiester bond cleavage. In the presumptive active site only two acidic amino acid residues are present, Asp86 and Glu127, which are often found as ligands of Mg^{2+}. The cleavage activities of mutants at positions 86 and 127 argue for Glu127 as the principal ligand of Mg^{2+}, as the effects of substitutions by Ala are more severe for Glu127 than for Asp86. This is supported by the fact that the E127A mutant is stimulated more by Mn^{2+} compared to the D86A mutant. This divalent cation binds more strongly to multidentate ligands than Mg^{2+} and, thus, may compensate for the loss of one ligand as in the Glu127 mutants. It must be emphasized, however, that N119D and N119Q are also activated by Mn^{2+}. It might well be that Mn^{2+} can better support cooperative formation of the transition state complex than Mg^{2+} when critical residues needed for transition state stabilization are missing. Similar observations have been made with the restriction endonucleases *Eco*RI and *Eco*RV (43). It is remarkable that N119D, similarly to D86A and E127A, needs a 2-fold higher Mg^{2+} concentration for maximum activity than the wild-type enzyme and that its activity is increased 20-fold by Co^{2+} and 40-fold by Zn^{2+}, while the wild-type enzyme is only stimulated 2- to 3-fold. These results may be considered as an indication that Asn119 is also involved in metal ion binding.

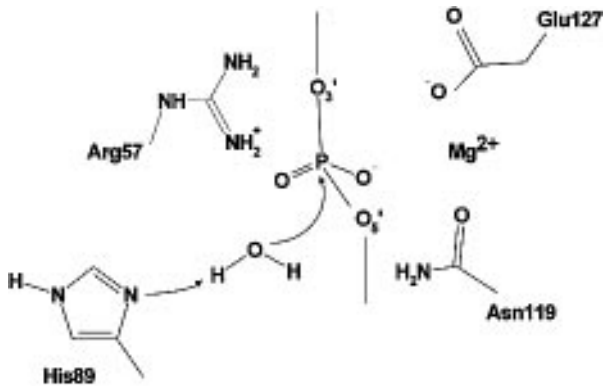

Figure 4. A model for the mechanism of phosphodiester bond hydrolysis by the *Serratia* nuclease.

Leaving group protonation

As we have not been able to identify the general acid needed to protonate the leaving group, it is tempting to speculate that Glu127 is indirectly involved in this function by binding the Mg^{2+} ion which could associate itself with the leaving group as discussed for the 3′→5′ exonuclease of the Klenow polymerase (44). Alternatively, leaving group protonation could occur at the expense of a Mg^{2+}-bound water molecule, as discussed for the restriction endonucleases *Eco*RI and *Eco*RV (45).

Substrate binding

Three other residues conserved among six related nucleases and located within or close to the presumptive active site are thought to be involved in substrate binding, namely Asp86, possibly via Mg^{2+}, as indicated by the altered metal ion concentration dependence of the D86A mutant, Arg87 and Arg131. This conjecture is based on the finding that exchange to Ala leads to proteins which are mainly affected in catalytic activity by an increase in K_m (between 50- and 100-fold increased K_m values compared to between 1.3- and 2.9-fold decreased k_{cat} values).

CONCLUSIONS

Starting with the crystal structure of the *Serratia* nuclease and a detailed sequence comparison among the *Serratia* nuclease family of non-specific nucleases, we have carried out a mutational analysis designed to identify the amino acid residues directly involved in catalysis and to propose a mechanism of action for this enzyme. Based on the results obtained, the following mechanism for the hydrolysis of DNA by the *Serratia* nuclease is proposed (Fig. 4): His89 is likely to function as the general base, which abstracts a proton from a water molecule which then serves as the attacking nucleophile. By analogy with many nucleases (46), we assume that the attack is in-line with the phosphodiester bond to be cleaved and that the reaction proceeds without a covalent intermediate. This assumption rests on our finding that the only reasonable candidate amino acid residue to

form a covalent intermediate, the conserved Tyr76, could be exchanged for Phe without effect on cleavage activity. The transition state, characterized by a pentacoordinated phosphorous, could be stabilized by Arg57 and/or Asn119. Glu127 and/or Asn119 could be ligands of the essential cofactor Mg^{2+}, which may have two functions, namely to help stabilize the transition state and to facilitate leaving of the group that carries an extra negative charge after cleavage of the phosphodiester bond. Leaving group stabilization could occur by association of Mg^{2+} with the terminal phosphate group or by protonation at the expense of a water molecule from the coordination sphere of the Mg^{2+} ion. Several amino acid residues could be indirectly involved in catalysis by binding to the substrate and positioning the protein backbone for nucleophilic attack: Arg87 and Arg131 and possibly also Asp86. Asp86 might be engaged in an attractive interaction via a divalent metal ion or a repulsive interaction, both types of interaction being in principle useful for a proper orientation of the phosphodiester backbone. It must be emphasized that the *Serratia* nuclease accepts both DNA and RNA in double- and single-stranded form as substrates; this makes it necessary that these different substrates are 'forced' into a similar conformation by the enzyme to be acceptable. For this purpose contacts to the sugar–phosphodiester backbone not too far from the phosphodiester bond to be cleaved are indispensable for the catalytic machinery.

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