Cleavage experiments with deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) suggest that the homing endonuclease I-*Ppo*I follows the same mechanism of phosphodiester bond hydrolysis as the non-specific *Serratia* nuclease

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Abstract We show here that two nucleases, *Serratia* nuclease and I-*Ppo*I, with contrasting specificities, i.e. non-specific vs. highly sequence specific, share a structurally similar active site region with conservation of the catalytically relevant histidine and asparagine residues. On the basis of a comparison of the available structures and biochemical data for wild type and mutant variants of *Serratia* nuclease and I-*Ppo*I we propose that both enzymes have a common catalytic mechanism, a proposition that is supported by our finding that both enzymes accept deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) as a substrate and cleave it in an identical manner. According to this mechanism a histidine residue functions as a general base and Mg^{2+} bound to an asparagine residue as a Lewis acid in phosphodiester bond cleavage.

Key words: DNase; Active site; Acid-base catalysis; Sequence alignment; Homology; DNA-entry nuclease

1. Introduction

Nucleases are ubiquitous in living organisms and have very diverse functions. Regarding the substrate specificities two extremes exist. On one side there are non-specific nucleases which are able to cleave any kind of nucleic acid, i.e. single or double stranded RNA or DNA, with no or only weak sequence or structure specificity, a typical example being *Serratia* nuclease (review: [1]); on the other side there are highly sequence specific nucleases, the most specific ones represented by the homing endonucleases (review: [2]), which cleave only double stranded DNA and have long, often palindromic recognition sites.

Here we discuss the structural similarity of the active site region of a non-specific and a highly sequence specific nuclease, namely the secretory endonuclease of *Serratia marcescens* [3] and the intron-encoded homing endonuclease of *Physarum polycephalum* I-*Ppo*I [4]. The *Serratia* nuclease shares sequence homology with a number of non-specific nucleases [5]. The active site of these nucleases is characterized by several conserved residues (*Serratia* nuclease: Arg-57, Arg-87, His-89, Asn-119, Glu-127, Arg-131). The role of these residues, in particular His-89 and Asn-119, in the mechanism of cleavage has been analyzed by a detailed mutational and structural analysis [3,5–9]. However, since there are no structural data available for an enzyme-substrate complex, it has been difficult to asses the precise catalytic mechanism for this enzyme. I-*Ppo*I shares sequence homology with two other homing endonucleases of the His-Cys box family [10]. Two residues (I-*Ppo*I: His-98 and Asn-119) are conserved among these enzymes and based on the structural data it has been suggested that His-89 serves as a Lewis acid to stabilize the negative charge on the pentacoordinate phosphate in the transition state [4]. The divalent metal ion cofactor is bound by the side chain of Asn-119 and it has been proposed that a water molecule from the hydration sphere of the metal ion is deprotonated by a general base and serves as the attacking nucleophile [4].

Both enzymes, I-*Ppo*I and *Serratia* nuclease, have similar biochemical properties: the pH optimum for phosphodiester bond cleavage is around pH 8.5–9; they are dependent on divalent metal ions with a similar preference for Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} [5,11], and their reaction products have 5′-phosphate and 3′-OH ends. On the basis of published structural data and the results of a mutational analysis as well as new biochemical data, we propose a common cleavage mechanism characteristic for the active site of *Serratia* nuclease and I-*Ppo*I. A sequence database search revealed at least one other family of nucleases which probably have a similar mechanism of action.

2. Materials and methods

2.1. Structural comparison of Serratia nuclease and I-PpoI

Structures of *Serratia* nuclease (PDB code: 1smn) and I-*Ppo*I (PDB codes: 1a73, 1a74 and 1ipp) were inspected using the Swiss-PdbViewer [12]. Ribbon diagrams were drawn with POV-Ray v.3.1.

2.2. Protein sequences and sequence alignments

Homologous protein sequences were searched though the BLAST interface at the NCBI searching the non-redundant database and the databases of unfinished bacterial genomes available through the NCBI using the BLAST interface (blastp or tblastn) at NCBI [13] and the genome databases available through WIT. Protein sequences of individual families were aligned using the ClustalW program at EBI [14]. Alignment files were further manually refined using GeneDoc [15] based on the structural information available for *Serratia* nuclease and I-*Ppo*I.

2.3. Enzymes and substrates

The *Serratia* nuclease was prepared as described in [7]. I-*Ppo*I was purchased from Promega. Deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) was synthesized following a protocol kindly provided by

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Dedicated to Prof. Dr. Günter Maass on the occasion of his 65th birthday.

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Table 1
Sequence accession numbers of nucleases displayed in Fig. 4

Name[a]	Organism	Predicted size[b] (aa)	Accession (GenBank)
NUCG_HUMAN	*Homo sapiens*	297	X79444
NUCG_BOVIN	*Bos taurus*	299	X72802
NUCG_MOUSE	*Mus musculus*	294	X99395
Nuc Caeel	*Caenorhabditis elegans*	(760)	AF003740
Nuc Drome	*Drosophila melanogaster*	(247)	AC004340
NucC1 Cunec	*Cunninghamella echinulata*	252	AF043517
NUC1_YEAST	*Saccharomyces cerevisiae*	329	X06670
NUC1_SCHPO	*Schizosaccharomyces pombe*	(335)	Z73099
Nuc Trybr	*Trypanosoma brucei*	(506)	U43702
Y4FB_RHISN	*Rhizobium* sp.	(664)	AE000072
Nuc Pseae	*Pseudomonas aeruginosa*	(892)	RPA01594[c]
K123 Chick	*Gallus gallus*	(276)	O73911[d]
Nuc Borbu	*Borrelia burgdorferi*	(195)	RBB00337[c]
Nuc Camje	*Campylobacter jejuni*	(150)	RCJ01399[c]
NUCA_ANASP	*Anabaena* sp.	276	X64706
NUC_SERMA	*Serratia marcescens*	245	M19495
Nuc3 Strpy	*Streptococcus pyogenes*	(268)	RST00226[c]
Nuc2 Strpy	*Streptococcus pyogenes*	(252)	RST00413[c]
Nuc Entfa	*Enterococcus faecalis*	(205)	REF02204[c]
DRN1_STREQ	*Streptococcus equisimilis*	327	X17241
Nuc1 Strpy	*Streptococcus pyogenes*	(385)	O33735[d]
MF25 Strpy	*Streptococcus pyogenes*	271	Q54969[d]
Nuc4 Strpy	*Streptococcus pyogenes*	(121)	X54225
NUCE_STRPN	*Streptococcus pneumoniae*	274	X54225
Nuc Porsp	*Porphyrea spiralis*	(137)	L26177
Nuc Banat	*Bangia atropurpurea*	(113)	L36066
Nuc Naesp	*Naegleria* sp.	(177)	AJ001315
Nuc1 Porte	*Porphyra tenera*	(162)	AB013176
Nuc2 Porte	*Porphyra tenera*	(109)	AB013176
Nuc Necga	*Nectria galligena*	(113)	Y16424
I-DirI	*Didymium iridis*	261	X71792
I-NanI	*Naegleria andersoni*	245	X78280
I-PpoI	*Physarum polycephalum*	163	M38131

[a]Names as given in Fig. 4. Names in uppercase are Swiss-Prot IDs.
[b]Numbers in parentheses for nucleases which have not be proven experimentally.
[c]Sequences obtained through WIT database.
[d]Sequences obtained from non-redundant database.

Dr. B.A. Connolly (The University of Newcastle, UK). The purity of the HPLC purified product, especially the absence of nitrophenol, was monitored by UV-VIS spectroscopy in a wavelength range from 250 to 450 nm. Oligonucleotides were purchased from Interactiva.

2.4. Cleavage of deoxythymidine 3′,5′-bis-(p-nitrophenyl phosphate)

Cleavage of the synthetic substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) by the *Serratia* and the I-*Ppo*I nuclease was carried in 50 mM Tris-HCl, pH 8.2, 5 mM $MgCl_2$ (*Serratia* nuclease) or 25 mM CHES, 25 mM CAPS, pH 10, 2 mM $MgCl_2$ and 1 mM DTT (I-*Ppo*I) in a 1 cm cuvette at 25°C. The rate of cleavage of deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) by the *Serratia* nuclease and I-*Ppo*I was determined via the absorbance increase at 400 nm which is typical for the cleavage product *p*-nitrophenol in its deprotonated form (ε_{400}=17 200 M^{-1} cm^{-1}). The position of cleavage by I-*Ppo*I was determined as described for *Serratia* nuclease [8].

3. Results and discussion

3.1. Structural comparison of the active sites of Serratia nuclease and I-PpoI

Although the overall structures of *Serratia* nuclease and I-*Ppo*I are different (Fig. 1), superposition of the active site residues His-89 and Asn-119 of *Serratia* nuclease and His-98 and Asn-119 of I-*Ppo*I revealed a remarkable structural similarity (rmsd of 0.52 Å for the side chain atoms). Moreover, the architecture of the active site region involving residues Thr-93–Asn-119 in I-*Ppo*I and Lys-84–Asn-119 in the *Serratia* nuclease is also very similar with an rmsd of 1.14 Å for the main chain atoms of 23 residues. Furthermore, the position of the guanidinium group from the catalytically important Arg (Arg-61 shown in the co-crystal structure of I-*Ppo*I with its reaction products to make contact with the 5′-phosphate and Arg-57 shown by mutational analysis of the *Serratia* nuclease to be essential for catalysis) are in similar spatial positions with respect to the histidine and asparagine residues. Moreover, the position of the catalytically essential cofactor Mg^{2+} ion is the same in both the product complex of I-*Ppo*I (1ipp) and the *Serratia* nuclease (M.D. Miller and K.L. Krause, unpublished), with almost identical geometry of the oxygen ligands.

Importantly, in the superimposed structures, the DNA of the I-*Ppo*I•DNA complex does not clash into the structure of the *Serratia* nuclease (not shown), suggesting that the DNA around the scissile bond might be bound in a similar way by *Serratia* nuclease as it is bound by I-*Ppo*I. This suggestion is supported by the fact that the 5′ and 3′ neighboring phosphate groups of the scissile phosphodiester bond in the DNA as seen in the superimposed structures are in close proximity to the conserved Arg-131 and Arg-87 of the *Serratia* nuclease. These residues have been shown to be important for the activity of *Serratia* nuclease [7].

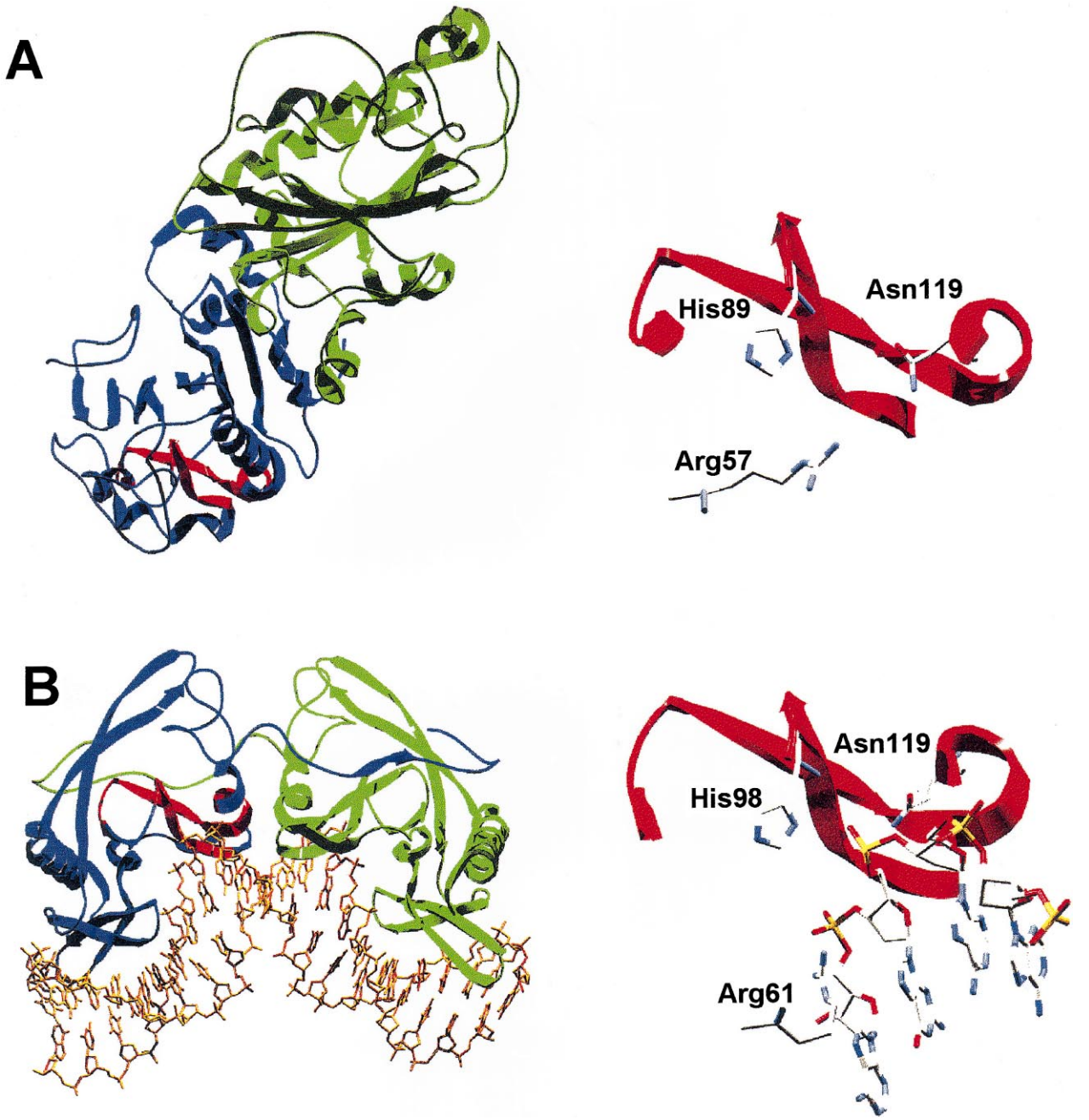

Fig. 1. Comparison of the active site structure of *Serratia* nuclease and I-*Ppo*I. A: Ribbon diagram of (top) *Serratia* nuclease dimer and (bottom) I-*Ppo*I dimer with DNA substrate. The two subunits are colored green and blue. The structurally similar region of the active site is shown in red. The DNA substrate of I-*Ppo*I is colored brown. B: A blow up of the active site region of *Serratia* nuclease and I-*Ppo*I with the catalytically essential histidine, asparagine and arginine residues highlighted.

3.2. *Cleavage of the minimal substrate deoxythymidine 3′,5′-bis-(p-nitrophenyl phosphate) by Serratia nuclease and I-PpoI*

Since the active sites of *Serratia* nuclease and I-*Ppo*I are so similar it was tempting to find out whether the minimal substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) of the *Serratia* nuclease [9] is also cleaved by I-*Ppo*I. As demonstrated in Fig. 2, I-*Ppo*I is indeed capable of cleaving this minimal substrate in a concentration dependent manner yielding the same products as obtained with *Serratia* nuclease as indicated by the absorption spectrum of the products (data not shown). Moreover, both enzymes exhibit a similar catalytic activity in cleaving this substrate with rate constants of approximately 0.05–0.1 min^{-1} at a substrate concentration of 75 μM. In order to show that this activity is due to I-*Ppo*I and not to a contaminating activity, the assay was also performed in the presence of a specific substrate for I-*Ppo*I and a non-specific DNA. Only in the presence of the specific oligonucleotide substrate cleavage of the minimal substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) was inhibited (Fig. 2a) and cleavage of the specific oligonucleotide occurred instead (data not shown). These results demonstrate that I-*Ppo*I

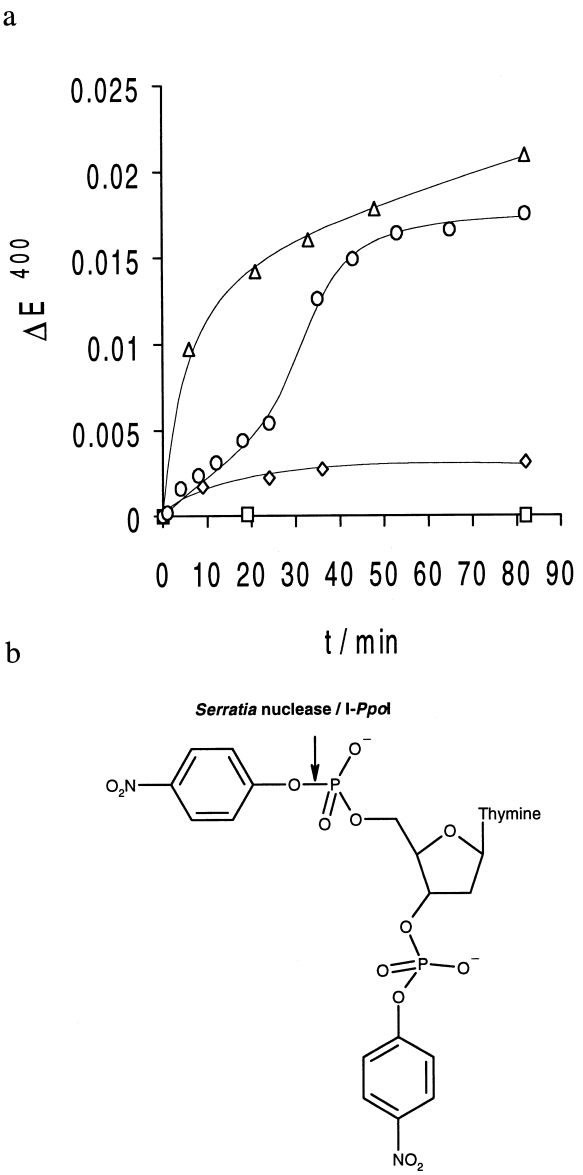

Fig. 2. Kinetics of cleavage of the minimal substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) by I-*Ppo*I. a: Time course of the hydrolysis of 75 μM substrate catalyzed by different I-*Ppo*I concentrations (◇, 1000 U; △, 10000 U) as well as the inhibition of hydrolysis by 35 μM of a specific oligonucleotide (○), which is relieved after the specific oligonucleotide is hydrolyzed. □ denotes the control experiment without added nuclease. b: Cleavage positions for *Serratia* nuclease and I-*Ppo*I accepting deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) as substrate.

accepts deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) as a substrate and cleaves it at the same position as *Serratia* nuclease (Fig. 2b).

3.3. Proposal for a mechanism of phosphodiester bond hydrolysis of Serratia nuclease and I-PpoI

Based on the structural comparison and the biochemical data presented here for I-*Ppo*I and published recently for *Serratia* nuclease [9] we propose that both enzymes share a common catalytic mechanism (Fig. 3). In this mechanism a histidine residue acts as a general base to activate the attacking water and the Mg^{2+} ion bound to an asparagine residues serves as a Lewis acid to stabilize the transition state and the leaving group.

In the structure of I-*Ppo*I bound to its recognition site (PDB code: 1a74) His-98 is located optimally to position a water molecule for an in-line attack on the phosphodiester bond to be cleaved. Mutational analysis of the corresponding histidine residue in *Serratia* nuclease, His-89, supports the importance of this residue, as any amino acid exchange results in a mutant protein with almost no residual nuclease activity [6,7]. Further evidence for the role of His-89 as the general base is derived from the fact that the minimal substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) is cleaved by all mutants so far generated but not by those bearing amino acid exchanges at His-89 or Asn-119 [9], which leaves His-89 or the Mg^{2+} cofactor bound to Asn-119 as principal candidates for the general base or the Lewis acid, respectively, in the mechanism of cleavage. Based on the crystallographic data of the various I-*Ppo*I complexes, the most likely role of His-98 (His-89 in *Serratia* nuclease) is that of a general base which activates the water molecule for the nucleophilic attack rather than stabilization of the transition state.

The position of the 3′-oxygen group of the scissile phosphodiester bond is identical in both the substrate complex without Mg^{2+} (PDB code: 1a74) and the product complex with Mg^{2+}, and the position of the Mg^{2+} is similar in the product complex of I-*Ppo*I (PDB code: 1ipp) and the *Serratia* nuclease without substrate (M.D. Miller and K.L. Krause, unpublished). Therefore, we propose that the Mg^{2+} ion serves as the Lewis acid to stabilize the negative charge on the pentacoordinate phosphate transition state and the leaving group. The fact that the inert $(Co(NH_3)_6)^{3+}$ inhibits rather than substitutes for Mg^{2+} in the case of the *Serratia* nuclease (P. Friedhoff, unpublished) argues for the observed direct coordination of the Mg^{2+} to the oxygen(s) of the phosphate attacked in the case of *Serratia* nuclease, as also observed in the I-*Ppo*I•DNA•Mg^{2+} complex. The role of Asn-119 for the mechanism of cleavage both for *Serratia* nuclease and for I-*Ppo*I, therefore, is probably the binding and correct positioning of the Mg^{2+} cofactor. This is supported by the finding that all *Serratia* nuclease variants with substitution of Asn-119 display markedly reduced activity which can partially be rescued by the use of different divalent metal ion cofactors, i.e. Mn^{2+}, Co^{2+} or Ni^{2+}, for Mg^{2+} (P. Friedhoff, unpublished).

Interestingly, the sole direct amino acid ligand of Mg^{2+} is Asn-119 in both enzymes. The only other ligands, with the exception of water in I-*Ppo*I and presumably also in *Serratia* nuclease are one non-bridging oxygen and the 3′-oxygen of the phosphate attacked, similar to what was found for the metal binding site B of 3′→5′-exonuclease of Klenow polymerase [16]. The role of Arg-61 in I-*Ppo*I and Arg-57 in the *Serratia* nuclease, respectively, might be to stabilize the transition state and/or to bind to the 5′-phosphate group of the product DNA. In agreement with this important role is our mutational analysis according to which the *Serratia* nuclease R57A mutant shows a 1000-fold reduced k_{cat} [7] and the observation that Arg-61 in the I-*Ppo*I product complex is in contact with the 5′-phosphate of the former scissile phosphodiester bond [4].

It should be noted that although our mechanism is speculative it represents a reasonable alternative to the mechanism discussed for I-*Ppo*I by Stoddard and co-workers [4], in which a Mg^{2+} bound water is deprotonated by a general base and

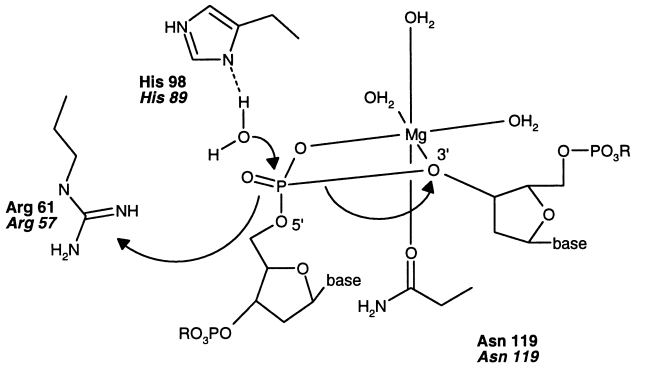

Fig. 3. Proposed mechanism of cleavage for *Serratia* nuclease and I-*Ppo*I based on structural and biochemical data. Essential amino acids are indicated for *Serratia* nuclease (in italics) and for I-*Ppo*I. A water molecule was placed in this scheme to allow for an in-line attack on the scissile phosphodiester bond with activation of this water molecule by the general base histidine.

attacks the phosphate while His98 serves as the Lewis acid to stabilize the transition state.

3.4. *Protein sequence alignment of the family of DNA/RNA non-specific endonucleases and the Cys-His box containing intron encoded endonucleases*

An extensive sequence database and sequence comparison revealed no significant overall sequence homology between the family of DNA/RNA non-specific nucleases, represented by the *Serratia* nuclease, and the family of Cys-His box containing intron encoded endonucleases, represented by I-*Ppo*I. However, during this search we found another family of nucleases, related to the DNA-entry nuclease of *Streptococcus pneumoniae* [17], which shares homology with the *Serratia* nuclease family in parts of the sequence which make up the active site (Fig. 4, Table 1). Therefore, we propose that this family of nucleases has the same active site and follows a similar mechanism of phosphodiester bond hydrolysis as the *Serratia* nuclease and I-*Ppo*I. This proposal is supported by the finding that for one nuclease of this family, i.e. mitotic factor nuclease of *Streptococcus pyogenes*, it has been shown that the conserved histidine residue (His-122) is important for catalytic activity [18].

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NUCG_HUMAN :SGFDRGHLAAAANHRWS------QKAMDDTFYL-SKVAPQVTH-LNQ--NAWNNLEKYSRSLT : 187
NUCG_BOVIN :SGFDRGHLAAAANHRWS------QKAMDDTFYL-SNVAPQVPH-LNQ--NAWNNLEKYSRSLT : 189
NUCG_MOUSE :SGFDRGHLAAAANHRWS------QRAMDDTFYL-SNVAPQVPH-LNQ--NAWNNLERYSRSLT : 184
Nuc Caeel  :SGFDRGHLAAAGNHRKS------QLAVDQTFYL-SNMSPQVGRGFNR--DKWNDLEMHCRRVA : 640
Nuc Drome  :SGFDRGHLAAAGNHHLQ------QNHCEDTFFL-TNIAPQVGQGFNR--SAWNNLEQYVRNLV : 129
NucC1 Cunec:SGFDRGHMAPAGDAVAT------QPAMDQTFYL-SNMSPQVGIGFNR--HYWAYLEGFCRSLT : 134
NUC1_YEAST :SGYDRGHQAPAADAKFS------QQAMDDTFYL-SNMCPQVGEGFNR--DYWAHLEYFCRGLT : 185
NUC1_SCHPO :SGYDRGHQVPAADCKFS------QEAMNETFYL-SNMCPQVGDGFNR--NYWAYFEDWCRRLT : 189
Nuc Trybr  :RGLSRGHLAAAQFHKSS------TVELAQTFNMNANTVPQDMT-MNA--VDWLRLENLTRKLR : 268
Y4FB_RHISN :NYFDRGHLVRRLDPVWG-EIRVAKQANDDTFQW-TNCSPQYWGF-NQGADLWQGLENFLLYNT : 541
Nuc Pseae  :ASFDKGHMVRREDPGWGDSDAVARQAEDDTFVY-TNAVPQVAQ-LNQ--RSWLSLEDYVLQNA : 513
K123 Chick :TGLNRGHLNPSGHHSDS-------SSRAATFSL-TNIVPQNEK-LNG--GAWNNYEQQTMMRR : 187
Nuc Borbu  :SGYDRGHIVSSADMSFS------ENAMKDTYFL-SNMSPQKSE-FNS--GIWLKLEKLVREWA :  91
Nuc Camje  :SGYTRGHTAPNASFSXX------KAAQNSVFLM-SNITPQNAQ-INX--KIWNXIEQRERNLA :  84
NUCA_ANASP :SGYDRGHIAPSADRTKT------TEDNAATFLM-TNMMPQTPD-NNR--NTWGNLEDYCRELV : 170
NUC_SERMA  :LKVDRGHQAPLASLAG------VSDWESLNYL-SNITPQKSD-LNQ--GAWARLEDQERKLI : 134
Consensus  :sg drGH                    tf   n  PQ    N     W  lE   r

Nuc3 Strpy :-VMNRGHLVGY------------QFCGLNDEP-RNLVAMT-AWLNTGAYSGANDSNPEGMLY : 162
Nuc2 Strpy :-LMDRGHLVGY------------QFSGLNDEP-KNLVTMT-KYLNTGFS----DKNPLGMLY : 161
Nuc Entfa  :-NHSRGHLIGR------------QMGGSGDDP-RNLTTLYQNPVNTPYM-----------TK : 149
DRN1_STREQ :HLFVASHLFA-------------DSLGGKSIR-KNAITGT-QMQNVGTR-------KGGMQY : 217
Nuc1 Strpy :YLFDRSHLIA-------------DSLGGRPFR-NNLITGT-RTQNVGNN-----DRKGGMQY : 223
MF25 Strpy :DFWNRSHLIA-------------DSLGGDALR-VNAVTGT-RTQNVGGR-----DQKGGMRY : 200
Nuc4 Strpy :-AVDRGHLLGYA-LVGG-----LKGFDASTGNP-DNIATQL-SWANQANK-----PYLTGQNY : 109
NUCE_STRPN :-AVDRGHLLGYA-LIGG-----LDGFDASTSNP-KNIAVQT-AWANQAQA-----EYSTGQNY : 203
Consensus  :    r HL                   g       N  t t    N           g  y

Nuc Porsp  : GEEASHTCH-NA---------------KCVNK-AHLTLES-GDLNK-SRIYCRLM------- : 122
Nuc Banat  : GEEASHRCH-NA---------------KCVNP-LHMAFES-GDVNK-SRLYCRLF------- :  94
Nuc Naesp  : EYVIRHTCG-CK---------------DCCNP-EHLKLGT-KSDNE-YDKGIHEYL------ : 151
Nuc1 Porte : DLTISHVCG-TH---------------HCLAA-HHLMLEP-KHVND-DRVHCHVFLGRVSDA : 142
Nuc2 Porte : RYGASHLCG-DK---------------RCVRP-SHMTLES-GALNK-TRSYCAYFR------ :  95
Nuc Necga  : GWHASHLCG-NP---------------LCLEP-AHIHVEP-KTANE-ARKGCREFV-----S :  90
I-DirI     : TFHSSHLCKGDG---------------SCMEL-KHTLRVP-AQTNLADHELCPAF------- : 205
I-NanI     : ARTISHLCG-NG---------------GCARP-GHLRIEK-KTVND-ERTHCHFLL-RRSQS : 216
I-PpoI     : TCTASHLCH-NT---------------RCHNP-LHLCWES-LDDNK-GRNWCPGP------- : 128
Consensus  :    sH C------------------C   - H   e   N   r  c
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Fig. 4. Alignment of the presumptive active site regions of three different families of nucleases. Protein sequence alignment of the family of (top) DNA/RNA non-specific endonucleases, (middle) nucleases homologous to the DNA-entry nuclease of *Streptococcus pneumoniae*, and (bottom) the Cys-His box containing nuclear homing endonucleases (for references Table 1). The catalytically relevant histidine and asparagine residues are shown in white with black background. Conserved residues within each family are shaded gray. The consensus sequence for each family is shown below the individual alignments. Note, the alignment in B has been edited to optimize the sequence homology with A, while the alignment in C has been optimized based on the structural similarity of *Serratia* nuclease and I-*Ppo*I in the active site region. The superimposed residues in *Serratia* nuclease and I-*Ppo*I in Fig. 1 are indicated by a horizontal line within the consensus sequences.

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