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The Extracellular Endonuclease of *Serratia marcescens*. II. Active Site Amino Acid Residues and a Hypothetical Mechanism of Enzyme Functioning

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Abstract—A catalytic mechanism of *Serratia marcescens* endonuclease [EC 3.1.4.9] is proposed, basing on X-ray crystallographic data and functional characteristics of the enzyme and on the amino acid sequences of related proteins. The mechanism involves histidine and glutamic acid residues as general acid–base catalysts, as well as aspartic acid and arginine residues. Similarities and differences between the active sites of the nuclease and pancreatic DNase I are analyzed.

Key words: *Serratia marcescens*, extracellular endonuclease, structure, mechanism

INTRODUCTION

The enzymes of nucleic acid metabolism, nucleodepolymerases in particular, play a key role in the processes utilizing genetic information. Nucleodepolymerases participate in regulation, are widely used as instruments in genetic engineering and are also known in biotechnological industry as promising antiviral and carcinostatic agents and chemical reagents. The possibilities of using nucleodepolymerases for basic research and practical purposes are limited by the current state of knowledge of the general principles of enzyme catalysis.

While extensive data on the spatial structures and mechanisms of RNases and DNases has been accumulated, only few structural data are available for nucleases, showing no specificity with respect to the sugar moiety of nucleic acid. That is why much attention is attracted to the extracellular endonuclease of a pathogenic Gram-negative bacterium *Serratia marcescens* (*Sm* nuclease) [EC 3.1.4.9].

Sm nuclease catalyzes the splitting of the 3'-phosphodiester bonds in single- and double-stranded DNA and RNA to yield 5'-phosphorylated nucleotides [1] and is one member of the superfamily of homologous nucleodepolymerases isolated from different taxonomically distant pro- and eukaryotic species. The amino acid sequences have been determined for three representatives of this superfamily—for the mitochondrial nucleases of *Saccharomyces cerevisiae* [2, 3] and *Bos taurus* [4] and for nuclease of *Anabaena* sp. PCC 7120 [5]. The percentage of identity between *Sm* nuclease and the nucleases of *S. cerevisiae* and *Anabaena* is 17%, and with the *B. taurus* nuclease, 19%. All these nucleases show no specificity with respect to the sugar moiety of nucleic acids, but some of them, for instance *B. taurus* nuclease, are specific for the nucleotide sequences of substrate and preferentially hydrolyze G-C base pairs in DNA [6].

Sm nuclease is a metal-dependent enzyme, requiring pH 6.5–8.0 and 5–10 mM Mg^{2+} for maximal activity; somewhat lower activity is supported by Mn^{2+}.

Initial characterization of *Sm* nuclease was carried out with homogeneous preparations isolated from wild-type bacterial strains [7–9]. Recent successes in gene expression, secretion and obtaining recombinant nuclease [10–12] have made it possible to obtain variant nucleases with expected properties.

Not long ago, two independent groups of investigators launched X-ray diffraction studies of *Sm* nuclease [13, 14]. As a result of this work, Miller *et al.* [15] have determined the structure at 2.1 Å resolution, and we have refined it to 1.7 Å.

In the present work, we analyze the structure of *Sm* nuclease active site and propose a mechanism of its

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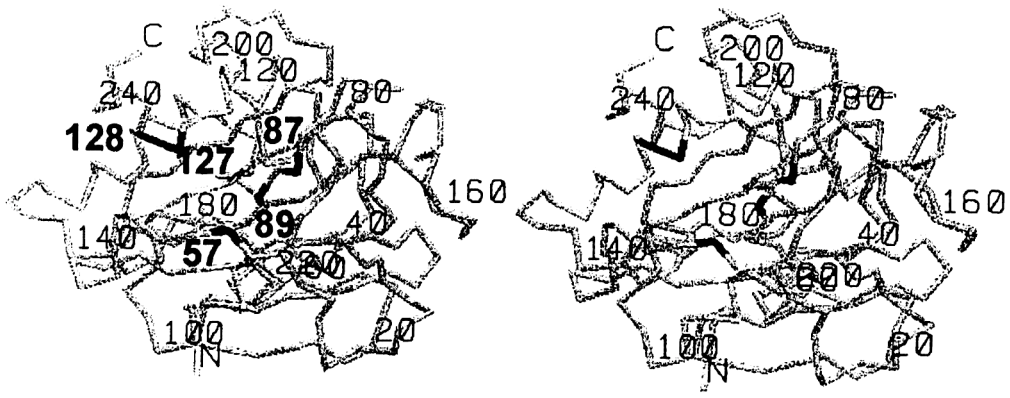

Fig. 1. A stereo representation of *Sm* nuclease C_α chain, as determined at 1.7 Å resolution [1]. Active site region is indicated by solid lines.

action, basing on the X-ray crystallographic data, comparison with functionally similar proteins and analysis of the functional properties of several known variants of the enzyme obtained by mutagenesis. The mechanism involves general acid–base catalysis by the side chains of glutamic acid and histidine residues as well as those of aspartic acid and arginine.

EXPERIMENTAL

Atomic coordinates for DNase I and *Sm* nuclease not containing nucleotide substrates were taken from Protein Data Bank and correspond to resolution of 2.0 Å [16, 17] and 1.7 Å [1], respectively.

Computer analysis of protein three-dimensional structures was carried out with an ESV 10/32 interactive graphical system using the programs FRODO and O [18, 19].

RESULTS AND DISCUSSION

Sm nuclease remains the only protein with known spatial structure in the family of homologous proteins. On the other hand, *Sm* nuclease is functionally similar to pancreatic DNase I, despite the absence of clear similarity of their primary structures [10, 11, 16]. Besides, the conformational topology of DNase I, α/β/β/α [16, 17, 20, 21], is in sharp contrast with that of *Sm* nuclease [1, 15] (Fig. 1).

The active site of DNase I is formed by two histidine residues (His-134 and His-252), which are hydrogen-bonded through ND-1 atoms to Glu-78 and Asp-212 carboxylates, and, possibly, by Arg-9, Glu-39 and/or Asp-251 [20, 21]. The guanidine group of Arg neutralizes the extra negative charge at the phosphorus atom of the scissile phosphodiester bond, while the side chains of Asp and Glu are coordinated to the essential Ca^{2+} or Mn^{2+} ions. In general, the donor–acceptor interactions in DNase I (Glu-His-H_2O) are reminiscent of the classical Asp-His-Ser catalytic triad of serine proteases.

Preliminary X-ray diffraction studies of *Sm* nuclease considered free enzyme, not containing any bound nucleotide. Therefore, the information on the active site and catalytic groups is scarce and limited to the indirect data obtained by computer modeling of a complex between *Sm* nuclease and DNA.

The DNA-binding site of *Sm* nuclease is localized between two basic N- (residues 1–114) and C- (residues 115–245) terminal subdomains (Fig. 1) and is flanked by two rows of clusters of positively charged residues (Lys-48, Lys-55, Arg-57, Lys-60, Lys-84, and Arg-87 of the N-terminal domain and Arg-15, Arg-131, Lys-132, Arg-136, and Lys-196 of the C-terminal domain) [1, 15], which, through electrostatic interactions with the phosphate groups of double-stranded DNA, allow the enzyme to recognize one complete turn of the B-form double helix. Also located here is a pair of residues, His-89 and Glu-127, which also belong to different subdomains, and is closest to the scissile bond (6.64 Å between His-89 ND1 and Glu-127 OE1) (Table 1).

The roles of different amino acid residues in catalysis by *Sm* nuclease have not been studied in detail and, with the exception of the Cys residues forming two intramolecular disulfide bonds (Cys-9–Cys-13 and Cys-281–Cys-243) [11], is primarily based on the results of site-directed mutagenesis studies [12]. Ten variants of *Sm* nuclease with Ala substituted for His-26, His-45, Asp-86, Arg-87, His-89, Asp-117, Glu-127, Arg-131, Asp-208, and Glu-211 have been described. The substitutions of His-26, His-45, Asp-117, and Asp-208 had no effect on *Sm* nuclease activity, whereas the substitutions at positions 86, 87, 89, 127, 131, and 211 all had a significant effect. The His89Ala substitution completely inactivated *Sm* nuclease, and the Gln127Ala, Arg87Ala, Glu211Ala,

Table 1. Interatomic distances in the active sites of *Sm* nuclease and DNase I

Amino acid residue atoms	Distance, Å	Amino acid residue atoms	Distance, Å
Sm nuclease		DNase I	
His-89ND1–Glu-127OE1	6.64	His-134NE2–His-252NE2	6.12
His-89ND1–Glu-127OE2	5.88	His-134NE2–His-252ND1	7.05
His-89ND1–Asp-128OD2	13.18	His-134ND1–Glu-78OE1	4.23
His-89ND1–Asp-86OD2	9.89	His-134ND1–Glu-39OE1	4.23
His-89ND1–Arg-87NH1	10.53	His-134NE2–Glu-39OE2	6.49
His-89NE2–Arg-87NH1	11.05	His-134ND1–Asp-251OD1	9.71
His-89ND1–Arg-131NH1	9.82	His-134NE2–Asp-251OD1	8.49
His-89NE2–Arg-131NH2	11.75	His-134NE2–Arg-9NH1	11.17
Glu-127OE1–Arg-87NZ1	15.31	His-134ND1–Arg-9NH2	13.03
Glu-127OE2–Arg-87NZ1	15.15	His-252ND1–Asp-212OD2	2.67
Glu-127OE1–Asp-86OD2	10.61	His-252NE2–Glu-39OE1	6.92
Glu-127OE1–Asp-128OD2	7.19	His-252ND1–Glu-39OE1	8.27
Glu-127OE1–Arg-131NH1	5.71	His-252NE2–Asp-251OD1	5.49
Glu-127OE2–Arg-131NH2	7.42	His-252NE2–Asp-251OD1	4.24
Arg-87NH1–Asp-86OD1	6.84	His-252NE2–Arg-9NH1	9.2
Arg-87NH1–Asp-86OD2	8.29	Arg-9NH1–Glu-39OE2	6.79
Arg-87NH1–Asp-128OD2	20.32	Arg-9NH2–Glu-39OE2	6.99
Arg-131NH1–Asp-86OD2	15.65	Arg-9NH1–Asp-251OD1	5.42
Arg-131NH2–Asp-86OD2	15.18	Arg-9NH2–Asp-251OD1	7.44
Arg-131NH1–Asp-128OD2	6.99	Glu-39OE1-Asp-251OD1	6.80
Asp-86OD1–Asp-128OD1	16.10		

Asp86Ala, and Arg131Ala substitution decreased k_{cat}/K_M for DNA hydrolysis 100–1000-fold [12].

Only the Glu211Ala variant of *Sm* nuclease (and Arg131Ala to a lesser extent) retain the substrate-binding properties of the wild-type enzyme. For all other variants, the value of K_M for DNA is increased more than 100-fold, supporting the hypothesis of the multipoint substrate recognition by enzyme through the combination of the electrostatic interactions of enzyme with the sugar-phosphate backbone of the nucleic acid and of specific hydrophobic interactions with the nucleotide bases.

Although the results of the site-specific mutagenesis analysis does not allow unambiguous identification of the functionally important residues as the catalytic residues of the active site, it is partly supported by comparison of the amino acid sequences of *Sm* nuclease and related proteins (Fig. 2). The data in Fig. 2 indicate that the identified residues are conserved among the homologous proteins.

By comparing the primary structures of *Sm* nuclease and nonspecific nucleases of *Anabaena* sp. PCC 7120, *S. cerevisiae*, and *B. taurus* one can identify five regions (21–57, 86–94, 107–150, 165–181, 199–235) with residues conserved in one or several proteins (Fig. 2). Besides, the Leu-3, Pro-67, Ala-68, and Asn-245 residues of *Sm* nuclease are identical with the corresponding residues in *Anabaena* and *S. cerevisiae* nucleases and Ala-74 is conserved in *B. taurus* nuclease. In total, only 23 residues are conserved in all four proteins, six of them being located in the most conserved region 86–94 of *Sm* nuclease. This region also includes three residues (Asp-86, Arg-87, and His-89) which were identified as catalytically important by site-directed mutagenesis. Other ionizable residues common for all the nucleases include the "catalytically important" Glu-127, Arg-131, and Glu-211, as well as Lys-172, whose importance for catalysis remains to be elucidated.

Basing on the structural and functional similarity of the nucleases under consideration, it is reasonable to expect that the spatial organization of their active sites formed by same polar groups is also similar. Although the spatial topologies and specific features of catalytic action of *Sm* nuclease and DNase I are clearly different, their active sites share several common features.

The two histidine residues of DNase I, His-134 and His-252, are assumed to act as conjugated acid–base catalysts, and any of them may function as both an

active nucleophile and electrophile [16,17, 20, 21]. Two catalytically important residues of *Sm* nuclease, His-89 and Glu-127, are on approximately the same distance from each other as the His-134 and His-252 in DNase I (Table 1) an may therefore be their functional equivalents in the capacities of a Lewis acid and a Lewis base, respectively. Two variants of active site architecture in DNase I and *Sm* nuclease, taking into account that His-89/Glu-127 of *Sm* nuclease and His-134/His-252 of DNase I are approximately equidistant from the corresponding catalytic residues Asp-86/Arg-87/Arg-131 and Arg-9/Glu-39/Asp-251, were analyzed by computer graphics-generated superposition of the active sites (Table 2 and Fig. 3).

The first variant (Table 2) assumes that Glu-127 and His-89 of *Sm* nuclease are equivalent to DNase I His-134 and His-252, respectively. When these pairs are superposed in the two enzymes, the "catalytic" residues Arg-87 and Asp-86 of *Sm* nuclease are placed in the closest position with respect to Arg-9 and Glu-39/Asp-251 of DNase I. The guanidino group of Arg-131 is outside the active site of *Sm* nuclease and is separated by 19–20 Å from Arg-87.

A characteristic feature of active site in *Sm* nuclease by comparison with DNase I is the absence of a hydrogen bond between the imidazole ring of His-89 and any carboxylate, including Glu-127. Glu-211, conserved in the family of the homologous nucleases (like Lys-172) is well off the active site (the His-89–Glu-211 and His-89–Lys-172 distances are about 25 and 10–11 Å, respectively), and its functional significance is not known. The Glu211Ala mutation does not result in any global conformational change, detrimental to nucleic acid binding in *Sm* nuclease [12]. On the other hand, the carboxylate group of Asp-86 is separated by 9.9 Å from the "active" His-89 and cannot affect to any extent the ionization of the imidazole group, which accepts proton in the course of catalysis. Thus, Asp-86 carboxylate appears to be the only suitable candidate for coordination of the essential divalent cations, like Gln-39/Asp-251 in DNase I.

In the alternative superposition of the active sites of the two enzymes (Table 2), His-89 of *Sm* nuclease is assumed to be equivalent to His-134 of DNase I and, likewise, Glu-127 equivalent to His-252. In this case, Arg-9 of DNase I is quite close to Arg-131 of *Sm* nuclease. Arg-87 of *Sm* nuclease is far from the scissile bond (n) and may interact with the (n + 2)th phosphoric acid residue of the substrate, taking into account that the distance between the closest phosphate residues in the nucleotide chain is ~6.2 Å. The Asp-86 carboxylate is outside active site and far from Glu-39 and Asp-251 of DNase I. The only structural and functional equivalent of DNase I Asp-251 is Asp-128 (Table 2 and Fig. 3).

Table 2. Distances between equivalent atoms in superposed structures of *Sm* nuclease and DNase I active sites

Sm nuclease	DNase I	Distance, Å
	Variant 1	
His-89ND1	His-252ND1	1.0–1.5
Glu-127OE1	His-134ND1	1.5–2.0
Glu-127OE1	His-134NE2	1.5–2.0
Asp-86OD1	Glu-39OE2	6.2
Asp-86OD2	Glu-39OE2	4.1
Asp-86OD1	Asp-251OD1	7.5
Asp-86OD2	Asp-251OD1	7.2
Arg-87NH1	Arg-9NH2	6.1
Arg-87NH1	Arg-9NZ1	6.2
	Variant 2	
His-89ND1	His-134ND1	1.0–1.5
Asp-128OD1	Asp-251OD1	4.2
Asp-128OD1	Asp-251OD2	2.4
Arg-131NH1	Arg-9NH1	4.5
Arg-131Nh2	Arg-9NH1	3.6
Glu-127OE1	His-252ND1	4.5–2.0
Glu-127OE2	His-252NE2	2.0

Although the functional significance of Asp-128 in *Sm* nuclease is not supported by any experimental data, it may participate in catalysis like Asp-86 carboxylate. It should be noted, however, that in the homologous nucleases this residue is conserved in only *Anabaena* sp. PCC 7120 enzyme and the substitutions for Ala of other partly conserved residues, such as His-45, Asp-117, and Asp-208, (Fig. 2) had no effect on enzyme activity.

Noteworthy, the second variant of superposition is supported by conservation of residues around His-89 of *Sm* nuclease (His-Gln-Ala-Pro) and His-134 of DNase I (His-Ser-Ala-Pro). In both variants, Glu-127 and His-89 are expected to take part in the catalytic action of *Sm* nuclease.

Basing on the above and also taking into account the spatial structure of *Sm* nuclease, the similarity of the sequences in the related proteins and the functional consequences of mutations of particular residues in *Sm* nuclease, we propose a mechanism of its action. The mechanism is based on general acid–base catalysis and involves glutamic acid, histidine and arginine residues as well as protein-bound Mg^{2+} ions.

The residues important for catalysis by *Sm* nuclease include Asp-86/Asp-128, His-89, Glu-127, and Arg-87/Arg-131, which are functional equivalents of Glu-39/Asp-251, His-134, His-152 (or His-152, His-134), and Arg-9 of DNase I, respectively. Stereochemically [22], the phosphodiester bond is hydro-

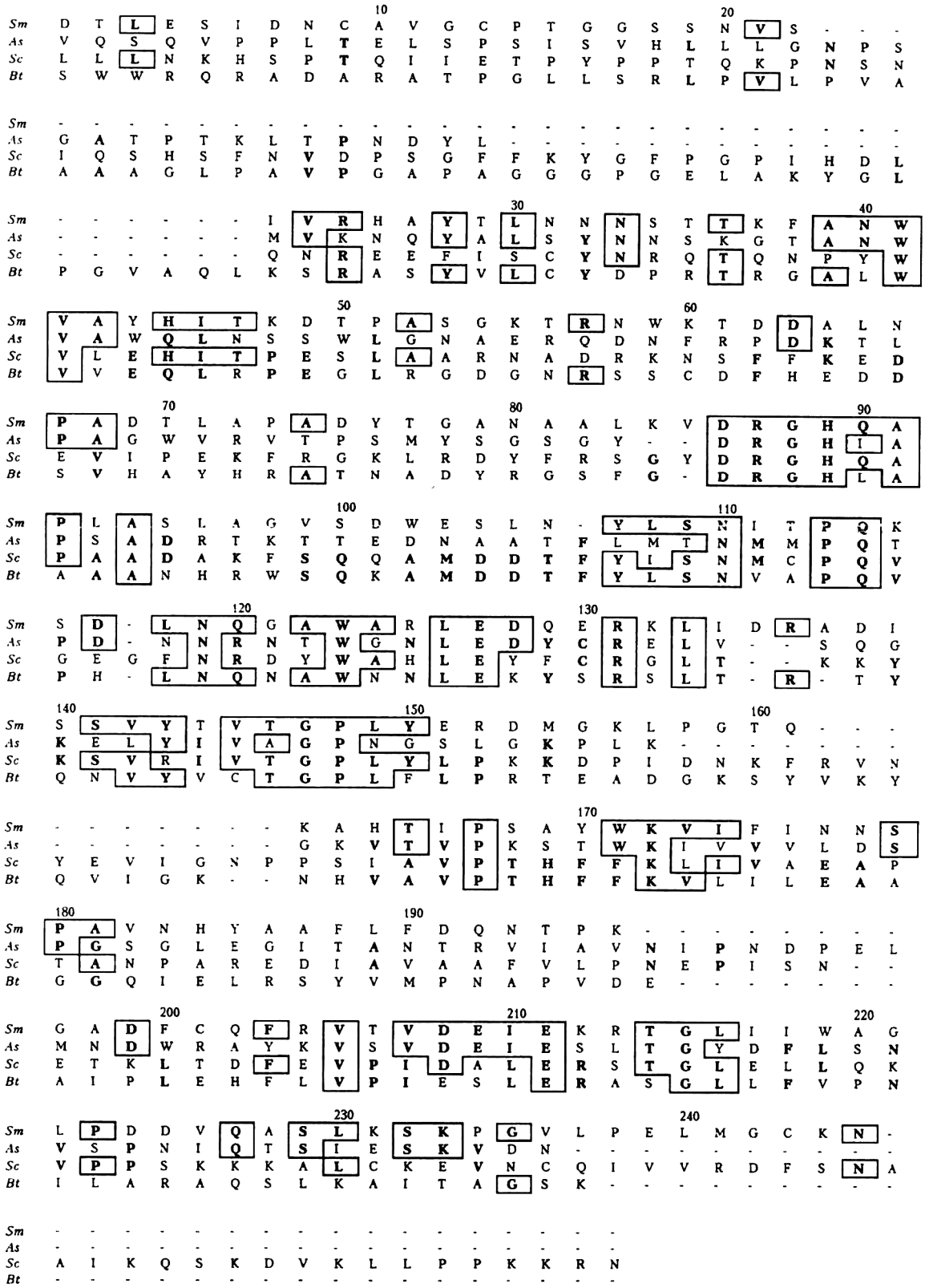

Fig. 2. Amino acid sequence alignment for nucleases of *S. marcescens* (*Sm*), *Anabaena* sp. PCC 7120 (*As*), *S. cerevisiae* (*Sc*), and *B. taurus* (*Bt*). Conserved residues are shown in boldface, residues identical to those of *Sm* nuclease are enclosed in boxes.

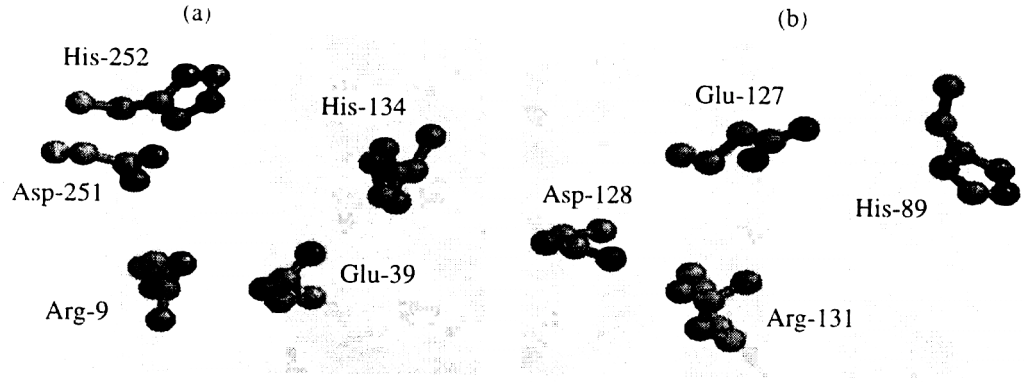

Fig. 3. Comparison of active site geometries in DNase I (a) and *Sm* nuclease (b) for variant 2 in Table 2.

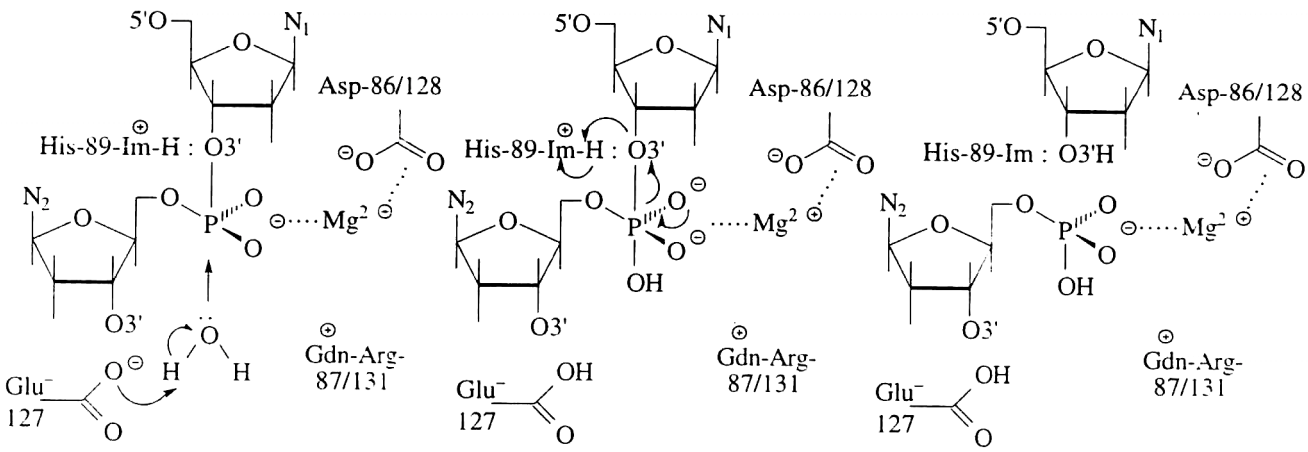

Fig. 4. Chemical mechanism of catalysis by *Sm* nuclease.

lyzed by nucleophilic substitution at the phosphorus atom as an addition-elimination reaction of water molecule to form a pentacoordinated intermediate having a geometry of a trigonal bipyramid with the phosphorus atom in the center, the attacking and leaving groups in the vertices and three oxygen substituents in the base plane. The mechanism of the nucleophilic substitution implies maximal concentration of positive charge on the central phosphorus atom in the transition state, when the anionic leaving groups is departing. The reaction is accompanied by inversion of the configuration at the phosphorus atom and occurs "in-line," i.e., the attacking nucleophile approaches from the side opposite to the leaving group.

In our scheme (Fig. 4), Glu-127 plays a role of a general Lewis base and activates water molecule by increasing the partial negative charge on its oxygen atom and, hence, its nucleophilicity prior to the attack on the phosphate group located in front of the O3' atom. The excessive negative charge near the pentacoordinated phosphorus atom is neutralized in the transition state by the guanidino group of Arg-87 or Arg-131 and by an Mg^{2+} ion coordinated with Asp-86 or Asp-128; the metal ion also properly positions the scissile bond with respect to the protein interior. The positively charged His-89 residue acts as a general acid to protonate the leaving O3' group.

In conclusion, while fairly possible in view of the available data, the proposed mechanism does not exclude other alternatives described for other nucleodepolymerases with enzyme-bound Mg^{2+} [23, 24]. The proposed role of Mg^{2+} in *Sm* nuclease is similar to that in DNase I, whereas *Escherichia coli* DNA polymerase utilizes two Mg^{2+} ions, one of which activates water molecule and the other is coordinated with the 3' oxygen of the leaving group [25]. In the restriction endonucleases *Eco*RI and *Eco*RV, the leaving groups

are protonated by the water shell of Mg^{2+} and the attacking water molecule is activated by the phosphate group of the scissile bond [26]. Clearly, the proposed candidate for the water-activating group should possess maximal basicity.

Refinement of the molecular mechanism of *Sm* nuclease requires additional information and analysis. At present, we are carrying out a systematic study of the hydrolysis kinetics for a series of natural and synthetic oligonucleotides and an X-ray crystallographic study of *Sm* nuclease complexed with minimal substrates and their analogs.

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