Structural Insights into the Mechanism of Nuclease A, a $\beta\beta\alpha$ Metal Nuclease from *Anabaena* *

Received for publication, February 17, 2005, and in revised form, May 12, 2005 Published, JBC Papers in Press, May 15, 2005, DOI 10.1074/jbc.M501798200

Mahuja Ghosh‡, Gregor Meiss§, Alfred Pingoud§, Robert E. London‡¶, and Lars C. Pedersen‡

From the ‡Laboratory of Structural Biology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 and the §Institut für Biochemie, FB08, Justus-Liebig-Universität, Heinrich-Buff-Ring 58, D-35392 Giessen, Germany

Nuclease A (NucA) is a nonspecific endonuclease from *Anabaena* sp. capable of degrading single- and double-stranded DNA and RNA in the presence of divalent metal ions. We have determined the structure of the Δ(2–24),D121A mutant of NucA in the presence of Zn$^{2+}$ and Mn$^{2+}$ (PDB code 1ZM8). The mutations were introduced to remove the N-terminal signal peptide and to reduce the activity of the nonspecific nuclease, thereby reducing its toxicity to the *Escherichia coli* expression system. NucA contains a $\beta\alpha$ metal finger motif and a hydrated Mn$^{2+}$ ion at the active site. Unexpectedly, NucA was found to contain additional metal binding sites ~26 Å apart from the catalytic metal binding site. A structural comparison between NucA and the closest analog for which structural data exist, the *Serratia* nuclease, indicates several interesting differences. First, NucA is a monomer rather than a dimer. Second, there is an unexpected structural homology between the N-terminal segments despite a poorly conserved sequence, which in *Serratia* includes a cysteine bridge thought to play a regulatory role. In addition, although a sequence alignment had suggested that NucA lacks a proposed catalytic residue corresponding to Arg$^{577}$ in *Serratia*, the structure determined here indicates that Arg$^{583}$ in NucA is positioned to fulfill this role. Based on comparison with DNA-bound nuclease structures of the $\beta\beta\alpha$ metal finger nuclease family and available mutational data on NucA, we propose that His$^{124}$ acts as a catalytic base, and Arg$^{583}$ participates in the catalysis possibly through stabilization of the transition state.

Nuclease A (NucA)$^1$ from *Anabaena* sp. PCC 7120 (1) is a member of a family of highly active, divalent metal ion-dependent, nonspecific nucleases, which are characterized by the DRGH prosite motif. NucA is able to degrade both single- and double-stranded DNA and RNA and functions optimally in the presence of Mn$^{2+}$ or Mg$^{2+}$ ions (2). The extracellular nuclease from *Serratia marcescens* (3), yeast Nuc1 (4), mitochondrial EndoG (5), and *Streptococcus pneumoniae* EndA (6) are some of the other family members. The best characterized of these is the *Serratia* nuclease (7), a homodimer (8–11) that contains two disulfide bonds per subunit (12) and is characterized by an unusual Mg$^{2+}$ binding site optimized for binding a hydrated metal ion (13, 14).

The prokaryotic nucleases of this family generally contain an N-terminal signal peptide and are secreted into the extracellular environment (15). It is believed that most of them serve nutritional purposes and possibly also function as bacteriocides, similar to the colicins of *Escherichia coli* (16). Because nonspecific nucleases will generally be extremely toxic to the cells that produce them, various mechanisms have evolved to deal with their toxicity. Many of the nucleases in this class contain disulfide bonds that activate the nucleases upon secretion into an oxidizing environment but when reduced in the intracellular environment render the enzyme inactive (15). If the cysteine residues in the N-terminal signal peptide are not included, there is only one additional cysteine in NucA, and hence it is not anticipated to contain disulfide bonds, which could prevent the nuclease from being active in the cell. Instead, NucA is paired with a specific inhibitor, NuiA, analogous to the colicin immunity proteins in *E. coli* (17). NuiA protects the cell from nuclease action by forming a very stable NucA-NuiA complex (2, 18, 19). The three-dimensional structure of NuiA has recently been determined (20).

NucA is a monomeric enzyme with secondary structure composition, as judged by circular dichroism spectroscopy, and a metal ion cofactor requirement similar to the *Serratia* nuclease (2). It prefers Mn$^{2+}$ and Co$^{2+}$ over Mg$^{2+}$ and shows little activity with Ni$^{2+}$ and no activity with Zn$^{2+}$. It has a pH optimum of 5.5–7.5 and a temperature optimum around 35 °C. With 8.4 × 10$^{8}$ Kunitz units/mg of protein ($k_{\text{cat}}$ = 2,055 s$^{-1}$), NucA is one of the most active nucleases known. For comparison, DNase I has 7.2 × 10$^{9}$ Kunitz units/mg of protein ($k_{\text{cat}}$ = 200 s$^{-1}$) (21). The general sequence preferences of NucA, e.g. avoiding d(A)-d(T) tracts (2), are similar to other nonspecific nucleases, such as the *Serratia* nuclease (22, 23).

NucA and *Serratia* nuclease share ~22% sequence identity (Fig. 1), which is most pronounced in the active site region and the central multistranded $\beta$-sheet core of the nuclease. According to a detailed mutational analysis, most amino acid residues important for phosphodiester bond hydrolysis by *Serratia* nuclease (9, 19, 24) have counterparts in NucA, suggesting that *Serratia* nuclease and NucA share a common mechanism of action, with differences in detail (19). It has recently become clear that the $\beta\beta\alpha$ Me finger motif (in this terminology “Me" refers to the bound metal ion) is shared by a broad class of nucleases (25) which includes the highly specific homing endonuclease I-PpoI (26–28), a member of the His-Cys box family (29), and the *Vibrio* nuclease Vvn from *Vibrio vulnificus* (30, 31).
Despite the overall similarities, the sequences suggest a number of significant differences, the reality of which can only be resolved by structural comparisons. For example, sequence alignment of NucA with the *Serratia* nuclease indicates that Arg^{57} of *Serratia*, a residue thought to be important for stabilizing the transition state, is not present in NucA (19). Thus, NucA either uses a different mechanism for stabilizing the transition state, or there is a structural alignment that is not immediately apparent from the sequence alignment. The resolution of this and related questions can only be resolved by a complete structural determination.

Here we report the crystal structure of NucA in complex with Mn^{2+} at a resolution of 1.9 Å. NucA bears a mixed αβ fold topology. Comparison with other nucleases reveals a similar structural arrangement in the endonuclease active site containing two β-strands and one α-helix with a centrally located divalent metal ion, as expected for a member of the ββα Me finger family of nucleases. Superpositions of the NucA structure with Vvn (31) with bound DNA suggest the position of the DNA binding site as well as specific catalytic roles for various residues. These results provide insight into the catalytic mechanism for NucA as well as structural variations relative to the *Serratia* nuclease.

**MATERIALS AND METHODS**

**Protein Construct and Site-directed Mutagenesis**—Expression of nucleases in *E. coli* cells generally requires some type of nuclease inactivation to limit cellular toxicity. In the present NucA studies, this was achieved by the introduction of a D121A mutation, which results in a reported 0.04% activity relative to the wild type enzyme (19). The full-length NucA sequence contains a signal peptide at the N terminus, which is responsible for the protein excretion to the periplasm. For this study we have used a construct in which N-terminal residues 2–24 were replaced by an αH_{121}M His tag, and a point mutation was introduced at position 121 to produce a D121A mutant. To obtain the D121A mutant, site-directed mutagenesis was performed using QuickChange kit (Stratagene) and verified by DNA sequencing. Residue numbering is based on the complete sequence of the enzyme (Fig. 1).

**Protein Expression and Purification**—Recombinant NucA was produced by transforming the plasmid into *E. coli* BL21 star (DE3). Cells were grown to mid-log phase (A_{600} ≈ 0.6) at 37 °C in LB medium containing 50 µg/ml carbenicillin. NucA protein expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside at 37 °C for 6 h. Cells were harvested by centrifugation at (7,000 × g), resuspended in 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl, and lysed by sonication with a Branson Sonifier 200 using a microtip probe. The lysate was centrifuged at 30,000 × g for 40 min. The nuclease was extracted from the inclusion bodies (for details, see Ref. 2) in the pellets using 50 mM Tris-HCl, pH 7.5, 6 M urea, and 200 mM imidazole. The eluted fraction containing protein was then concentrated using a Millipore concentrator to a desired volume of 10 ml (~10 mg/ml concentration) and renatured using flash dilution of 20-fold with moderate stirring at 4 °C. The renaturing buffer containing 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM MgCl₂, and 5 mM dithiothreitol was precooled to 4 °C. The renatured enzyme was then concentrated using an Amicon concentrator and applied to the Superdex-75 gel filtration 26 × 60-cm column and equilibrated with 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM dithiothreitol. The major peak of absorbance at 280 nm was found to be pure NucA protein as judged by SDS-PAGE. The peak fractions were concentrated using a Millipore concentrator, and the buffer was exchanged to 20 mM Tris-HCl, pH 7.5, 200 mM NaCl.

**Crystallography and Data Collection**—The NucA Δ(2–24) D121A mutant was concentrated to 10 mg/ml and dialyzed in 20 mM Tris, pH 7.5, and 200 mM NaCl. Crystals of this protein were obtained using the hanging drop vapor diffusion technique at 4 °C by mixing 2 µl of the protein solution with 2 µl of the reservoir solution consisting of 100 mM Mes, pH 6.5, 10 mM ZnSO₄, 300 mM NaCl, and 35% polyethylene glycol 550. For the data set used with manganese ion present, crystals were transferred and soaked in 100 mM Mes, pH 6.5, 300 mM NaCl, and 35% polyethylene glycol 550. For the data set with manganese, crystals were transferred and soaked in 100 mM Mes, pH 6.5, 10 mM MnCl₂, 300 mM NaCl, 35% polyethylene glycol 550, and 10 mM of the dinucleotide 5’-CG-3’.

For data collection, crystals were flash cooled by submersion in liquid nitrogen and placed on the goniometer in a stream of nitrogen gas cooled to −180 °C. Data were collected using a RU-H3R generator equipped with Osmic mirrors and a Raxis IV area detector. NucA crystallized in the orthorhombic space group P2₁2₁2₁, with one molecule/ asymmetric unit.

**Structure Determination and Refinement**—A model for molecular replacement was created from the crystal structure of *Serratia* endonuclease (PDB code 1G8T (14)) by replacing nonidentical side chains with alanines. The model was then refined by iterative cycles of model building using the program O (34) and refinement using the program CNS (35). The quality of the final structures were assessed using the programs Procheck (36) and MolProbity (37). The statistics for the data collection and results from refinement are reported in Table I. The structure of the Mn^{2+} enzyme has been submitted to the Protein Data Bank (PDB code 1ZM5).

**RESULTS**

**Overall Structural Characteristics of NucA**—The crystal structure of NucA reveals an αβ mixed globular fold (Fig. 2a). There are 13 helices and 8 strands giving rise to a large, six-stranded β-sheet and a small, two-stranded β-sheet. The active site of the enzyme contains one hydrated divalent metal ion, and one sulfate ion coordinated to the active site residues. An interesting outcome of this structural determination was the observation of a second and third metal ion binding site located ~26 Å from the metal ion in the active site (Fig. 2a).

**Structure of the Active Site in the Manganese-substituted Enzyme**—To obtain a structure of NucA with Mn^{2+} in the active site, crystals grown in the presence of Zn^{2+} were transferred into solution containing no Zn^{2+} and only Mn^{2+} as a divalent cation. The bond lengths and B-factors obtained from

---

**Fig. 1. NucA-Serratia nuclease sequence alignment.** The crystal structure of the Δ(2–24), D121A construct of NucA begins at residue Ile^{36}. The N-terminal His-tag as well as residues Gin^{37} through Ser^{39} are disordered. The deleted residues are indicated in magenta, the active site residues are highlighted in red, and the residues involved in coordinating the metal ions at the second binding site are colored green. The residues involved in the formation of the dimer interface in *Serratia* nuclease are highlighted in yellow.
Manganese ions, shown in the work of the molecule. The two metal binding sites bind a total of three metal ions (in stereo view showing the active site residues (in average length of 2.19 Å (Table II). The mode of interaction revealed an octahedral coordination of the divalent ion, with an Mn-O bond distances range from 2.04 Å for the bond to Asn155, to 2.36 for one of the bonds to water, with an average length of 2.19 Å (Table II). The mode of interaction with the divalent cation observed in NucA is typical of a series of other nucleases and ATPases with active sites that are optimized for binding a hydrated ion, e.g. Serratia nuclease, I-PpoI, and MutL (13, 38). In all of these examples, the active sites are optimized to interact with a hydrated metal ion rather than making direct contact with the metal ion, so that most of the protein interactions with the active site divalent ion are mediated by water molecules. For NucA, water molecule 1 (w1) is positioned by its interaction with His124, water molecules w2 and w3 are positioned by Glu163 (Fig. 2), and water molecule w4 by an interaction with Gln150. These positioning residues are themselves held in place by an additional network of hy-
dron bonds, e.g. the second carboxyl oxygen of Glu\(^{163}\) is hydrogen-bonded to the Ne of Trp\(^{139}\). The only direct bond formed between the divalent ion and the protein involves the Asn\(^{155}\) side chain carbonyl oxygen atom. In the native enzyme, the position of the Asn\(^{155}\) side chain would probably be stabilized by the interaction with Asp\(^{121}\), analogous to the interaction between Asn\(^{119}\) and Asp\(^{71}\) in the Serratia nuclease. However, the Asp\(^{71}\) side chain is not present in the D121A mutant that was studied. Apparently, the interaction between the Asn\(^{155}\) side chain and the Mn\(^{2+}\) is sufficient to define the position of this side chain in the absence of the Asp\(^{71}\) interaction. The sulfate ion observed in the active site interacts with the Mn\(^{2+}\), with the side chain amide of Asn\(^{155}\), and with the guanidino group of Arg\(^{89}\). If the sulfate is considered as a representative for a nucleotide phosphate group, this structure implies that the Asn\(^{155}\) and Arg\(^{89}\) residues would also interact directly with a phosphate oxygen of the substrate. Additionally, Arg\(^{89}\) is hydrogen bonded to Asp\(^{95}\) as a part of the additional hydrogen bond network around the active site which may exist to help stabilize the active conformation of Arg\(^{89}\). Finally, we note that although the dinucleotide 5'CG-3' was present in the crystallization solution, no electron density could be clearly identified for the dinucleotide.

**Additional Metal Binding Site**—An unexpected result of the crystal structure determination reported here is the presence of a second and third metal binding site located near the C terminus of the protein and ~26 Å from the active site of the enzyme. This site also represents an important crystal contact because both metal ions also coordinate with a Glu\(^{176}\) residue from a symmetry-related molecule. The site includes four acidic residues: Asp\(^{246}\), Glu\(^{249}\), Asp\(^{255}\), and Glu\(^{269}\), as well as carbonyl oxygen ligands derived from Phe\(^{256}\) and Glu\(^{265}\). These residues together bind two divalent ions (Fig. 3). The coordination of metal ion 2 (Mn2) is approximately octahedral, involving interactions with a single carboxyl oxygen from Asp\(^{246}\), Glu\(^{249}\), and Glu\(^{269}\), a water molecule, and the two carboxyl oxygen atoms from Glu\(^{136}\). The average metal-ligand bond length (Table II) is 2.27 Å (2.15 Å for the zinc enzyme), significantly longer than for Mn2.

**Electrostatic Surface of NucA**—The electrostatic surface of the NucA is very strongly positive, with an active site cleft that is flanked by positively charged amino acids. These residues include Lys\(^{101}\), Arg\(^{109}\), Lys\(^{248}\), Arg\(^{172}\), Arg\(^{93}\), and Arg\(^{167}\) (Fig. 4). A 180° rotated view of the electrostatic surface of NucA shows the other metal ion binding site flanked by negatively charged Asp\(^{246}\), Glu\(^{249}\), and Asp\(^{255}\) on the surface.

**Discussion**

**Comparison with Other Nuclease Structures**—NucA is a member of the family of extracellular nucleases that participate in DNA/RNA digestion in a nonspecific manner. It belongs to the superfAMILY OF ββα metal finger nucleases based on the structure. This ββα metal family comprises nucleases with diverse functions but with similar active sites; a few examples are: sugar nonspecific nucleases such as (a) Serratia nuclease (13, 14), NucA, and the apoptotic nuclease EndoG (39); (b) nonspecific DNases such as ColE7 (40) and ColE9 (41), Vvn nuclease (31), and the apoptotic nuclease CAD/DFF40 (42); (c) structure-specific nucleases such as T4endoVII (43); (d) type II restriction endonucleases such as KpnI (44); and (e) homing endonucleases such as I-Ppol (38) and I-Hmnl (45).

The active site of the ββα Me finger nucleases supplies one or two Mg\(^{2+}\) ligands as identified by crystal structure analyses: Asn\(^{155}\) in Serratia nuclease (13, 14); Asn\(^{155}\) in NucA, His\(^{44}\), and His\(^{409}\) in ColE7 (40); His\(^{102}\) and His\(^{127}\) in ColE9 (41); Glu\(^{79}\) and Asn\(^{127}\) in Vvn nuclease (31); Asp\(^{262}\) and His\(^{309}\) in CAD/DFF40 (42); His\(^{79}\) and Asp\(^{309}\) in T4endoVII (43); Arg\(^{119}\) in I-Ppol (38); Asp\(^{74}\) and Asp\(^{262}\) in I-Hmnl (45). Based on structure model building and biochemical data, the Mg\(^{2+}\) ligands are Asn\(^{174}\) in EndoG (39) and Asp\(^{148}\) and Gln\(^{175}\) in KpnI (44). The different modes of metal ion cofactor binding allow dividing the ββα Me finger nucleases into subgroups: Serratia nuclease, NucA, I-Ppol, and, based on computations, EndoG with an Asn residue as the sole Mg\(^{2+}\) ligand; Vvn nuclease, T4endoVII and I-Hmnl with a carbamate and a carboxamide as Mg\(^{2+}\) ligands; the colicins with two His residues as Mg\(^{2+}\) ligands and; CAD/DFF40 with a carbamate and a histidine as Mg\(^{2+}\) ligands. The different modes of metal ion cofactor binding allow dividing the ββα Me finger nucleases into subgroups: Serratia nuclease, NucA, I-Ppol, and, based on computations, EndoG with an Asn residue as the sole Mg\(^{2+}\) ligand; Vvn nuclease, T4endoVII and I-Hmnl with a carbamate and a carboxamide as Mg\(^{2+}\) ligands; the colicins with two His residues as Mg\(^{2+}\) ligands and; CAD/DFF40 with a carbamate and a histidine as Mg\(^{2+}\) ligands.
along with most other nucleases in this family, has a dimeric structure, whereas NucA is monomeric (19). The dimer interface in the *Serratia* nuclease includes a short loop from Arg<sup>136</sup> to Ile<sup>138</sup>, a short loop and β-strand region from Asn<sup>178</sup> to Tyr<sup>185</sup>, and a longer stretch of residues at the C terminus of the protein (residues 225–245). As can be seen from the alignment in Fig. 1, this region of the protein is generally not well conserved and includes many gaps. His<sup>184</sup>, which forms a hydrogen bond with the Ser<sup>229</sup> and with the Pro<sup>180</sup> carbonyl in the interchain region, corresponds to Arg<sup>222</sup> in NucA. It has previously been noted that the H184R mutant of the *Serratia* nuclease is monomeric (11). The interstrand salt bridge (Asp<sup>225</sup>-Arg<sup>136</sup>) in the *Serratia* structure would not exist in NucA, which has Asn and Gln residues at these positions. Similarly, the (Lys<sup>233</sup>-Glu<sup>239</sup>) interstrand salt bridge in *Serratia* would not be present in NucA. Of course, alternate stabilizing interactions could in principle be present in NucA; however, both biochemical (47) and the present crystal structure data support a monomeric structure for NucA.

The active site of NucA is observed to be homologous with that of the *Serratia* nuclease. A Mn<sup>2+</sup> ion is coordinated directly to the side chain carbonyl group of Asn<sup>155</sup>, four additional water molecules, and a sulfate oxygen forming a hexadentate octahedron. This pattern of coordination is analogous to that of the active site Mg<sup>2+</sup> ion observed in the Serratia nuclease (Fig. 5b). Compared with the surface residues of the *Serratia* nuclease only four of the basic residues are conserved or conservatively replaced in NucA, among which Arg<sup>122</sup> and Arg<sup>167</sup>, corresponding to Arg<sup>67</sup> and Arg<sup>131</sup> in the *Serratia* nuclease, are also important for activity based on earlier mutational analysis as mentioned in Ref. 19.

One region of considerable sequence disparity between the two nucleases is found at the N terminus (residues 5–33 for NucA; residues 36–70 for the *Serratia* nuclease) (Fig. 5c). In *Serratia*, this region includes two cysteine residues that form a disulfide bond that has been proposed to regulate the activity of the nuclease (12). Despite the complete lack of sequence homology and the missing disulfide bond, both structures occupy a similar region of the protein, filling a generally hydrophobic pocket located on the face opposite the active site. NucA contains an α-helix in place of the disulfide in *Serratia* nuclease. The extra secondary structure in this region of NucA may help stabilize the active conformation, whereas the presence of the disulfide in this region leads to active *Serratia* nuclease. The structural importance of this region in NucA was supported by our observation that a Δ59 construct of NucA failed to yield protein that could be refolded.

Several of the metal ion ligands present in the second and third metal ion binding site of NucA are also present in the *Serratia* nuclease; however, as indicated in the alignment of Fig. 1, Asp<sup>255</sup> and Glu<sup>269</sup> are not conserved in the *Serratia* sequence. No other metal binding sites were observed in two reported crystal structures of *Serratia* nuclease (13, 14). A survey of other related (DRGH motif-containing) nucleases also revealed no significant conservation of the residues involved in the second and third metal binding site except for Glu<sup>249</sup>, indicating that it is unique to NucA. Although the location of the second and third metal ion binding sites at a crystallographic interface introduces some uncertainty regarding the physiological significance of these sites, the presence of four anionic residues contributed by a single NucA molecule makes for interesting speculation. Potentially, this site could play a structural or regulatory role, it could be involved in anchoring or binding NucA to some other cellular component, similar to its role in creating a crystal contact, or it could possess an additional catalytic activity. The availability of the two metal ions for an intermolecular interaction would be consistent with the ability to interact with other substrates, suggesting that it might play a functional role.
This structure substantiates previous suggestions that NucA is the present study we report the first crystal structure of NucA.

Mechanism of Phosphodiester Bond Hydrolysis by NucA—In the present study we report the first crystal structure of NucA. This structure substantiates previous suggestions that NucA is a member of the ββα Me family of nucleases (19), providing insight into its catalytic mechanism. Drawing analogies to the proposed mechanisms for other ββα Me nucleases I-PpoI (38), ColE9 (41), and I-HmuI (45), the hydrolytic reaction pathway would proceed through an in-line displacement reaction mechanism whereby His124, acting as a general base, would deprotonate a water molecule which would attack the scissile phosphate coordinated to the divalent metal ion through a nonbridging oxygen and the leaving group 3' oxygen (Fig. 6). In both the Serratia nuclease (13, 14) and NucA structures, the proposed general base histidine forms a hydrogen bond with an inner coordination sphere water of the metal ion. Superpositions with I-PpoI (38) and I-HmuI (45), which were both crystallized in the presence of DNA, suggest that this water occupies the position of the nonbridging phosphate oxygen chelated to the metal ion. Thus, it is possible that upon substrate binding this water is replaced by the nonbridging oxygen of the phosphate, and the His124 side chain undergoes a slight conformational change to its catalytically active conformation, similar to the position of the catalytic histidine residue in the I-PpoI structure (Fig. 6). The divalent metal ion, along with the Arg93 guanidino group, would help stabilize negative charge buildup in the transition state through interactions with the nonbridging oxygen as well as enhancing the leaving group characteristics of the 3'-bridging oxygen.

Based on comparisons with the Vvn structure (31), the singly coordinated divalent metal only interacts with the nonbridging oxygen, suggesting that its role is to stabilize charge buildup on the scissile phosphate. It is suggested that a water molecule coordinated to the metal ion may help stabilize charge developed on the 3'-leaving group. It is interesting to note that a superposition of the structure of the Vvn product complex (31) positions the previous scissile phosphate directly on top of the sulfate coordinated to the Mn2+ ion in the NucA structure (Fig. 7). Whether or not this suggests that the mechanism of NucA is more similar to that proposed for Vvn is unclear. It should be noted, however, that in the Vvn structure (31) the divalent metal ion in the complex is Ca2+, which has significantly larger bond distances than Mg2+ or Mn2+, leading to a distorted active site. In either mechanism, Arg93 is in position to stabilize the transition state (Fig. 6) by interacting with the second nonbridging oxygen on the scissile phosphate or perhaps through stabilization of charge developed on the bridging oxygen of the 3'-leaving group. Arg93 is located in a position spatially similar to Arg57 in the Serratia nuclease (13, 14) and Arg96 in Vvn (31). We note, in addition, that in the DNA-bound model of NucA, the position of DNA on the surface of NucA near the catalytic site is consistent with the positive charge of the electrostatic potential map of the surface of NucA (Figs. 4 and 7).

In several nucleases for which nuclease-DNA co-crystal structures are available (ColE7, His545 (40); ColE9, His103 (41); Vvn nuclease, His80 (24); I-PpoI, His98 (38); and I-HmuI, His75 (45)) a histidine residue is ideally positioned to act as a general base to activate a water molecule for the nucleophilic attack on the phosphorus atom. Most probably, this is the case in other nucleases of ββα Me finger nuclease family. This assumption is supported by results of mutational analyses (Serratia nuclease, His59 (24); NucA, His124 (19); EndoG, His143 (39); ColE7, His103 (48); T4endoVII, His41 (49); KpnI, His149 (44)) and the analysis of the pH dependence of phosphodiester bond cleavage (pH half-maximal activity ~ 6 for Serratia nuclease (24) and NucA (2)). In general, most of these nucleases do not require an additional activation or orientation of the histidine residue involved in the catalysis. It is of particular interest that, in contrast with the other active site residues, His24 is not more rigidly positioned, e.g. by a hydrogen bond to the Nε of the ring. In general, a more rigid position would enhance its ability to interact with water w1, which is in the inner Mn2+ coordination sphere. The absence of such an interaction supports the idea of some positional variability, as illustrated, for example, in Fig. 6 as part of its catalytic function.

The position of Arg93 in NucA and its interaction with the sulfate ligand, as well as the simulated DNA complex based on the Vvn structure (Fig. 7), suggest that it may play a role in positioning the substrate and in stabilizing the transition state structure. A comparison of the active sites of some ββα Me finger nucleases suggests that in many nucleases a basic amino acid residue also is involved in neutralizing the extra negative charge on the phosphate oxygen in the transition state. Replacing Arg57 in Serratia nuclease (24), Arg61 in ColE9 (50), and Arg61 in I-PpoI (51) by alanine demonstrates that these arginine residues are essential for nuclease activity (in the case of I-PpoI a large effect is only seen at pH 10). In T4endoVII three histidine residues are candidates for transition state stabilization: their substitution by other amino acid residues leads to an inactive or almost inactive enzyme (43, 52, 53). A central role for Arg93 in the catalytic mechanism of NucA is supported by
the observation that the NucA R93A variant has only 0.12% residual activity (19) (for comparison the Serratia nuclease R57A variant has 0.6% residual activity (24)). We therefore conclude that in the NucA-catalyzed reaction, Arg93 (Fig. 6) is involved in transition state stabilization.

CONCLUSION

This work presents the three-dimensional structure of the nonspecific nuclease, NucA, from Anabaena sp., determined using x-ray crystallographic methods. NucA is one of the most active nonspecific nucleases that has been identified and is able to cleave both single- and double stranded DNA and RNA. In contrast with several other family members for which structural information is available, NucA is monomeric. However, it exhibits an αβ mixed fold that is similar to several other known nucleases, most closely resembling the Serratia nuclease. Despite limited sequence identity, the structure allows classification of NucA as a member of the βββα Me family, which includes both specific and nonspecific nucleases. The crystal structure also shows the presence of a second metal ion binding site near the C terminus of the protein which is ~26 Å from the active site of the enzyme. This site, which includes four acidic residues, binds two Mn2+ ions, but its physiological significance is unknown at present. Based on the crystal structure of NucA in the presence of manganese, the available mutational data, and a model of a NucA-DNA complex derived by homology with the previously determined Vvn-DNA complex (31), a single-metal ion mechanism is proposed for the DNA hydrolysis catalyzed by NucA. In this mechanism His124 would act as a general base, and both Arg93 as well as the active site divalent ion could stabilize the transition state, analogous to proposals for other ββα Me finger family nucleases.

Acknowledgments—We gratefully acknowledge the assistance of Dr. Tom Kirby in the preparation of the NucA mutant, Dr. Robert Petrovich of the Protein Expression Core Facility for protein expression, and Dr. Joseph Krahn for contributions to the structural refinement.

REFERENCES

1. Muro-Pastor, A. M., Flores, E., Herrero, A., and Flores, E. (1997) J. Mol. Biol. 268, 589–598
2. Meiss, G., Gimadutdinov, O., Haberland, B., and Pingoud, A. (2000) J. Mol. Biol. 297, 521–534
3. Kirby, T. W., Mueller, G. A., DeRose, E. F., Lebetkin, M. S., Meiss, G., Pingoud, A., and London, R. E. (2002) J. Mol. Biol. 320, 771–782
4. Doherty, A. J., Worrall, A. F., and Connolly, B. A. (1995) J. Mol. Biol. 251, 366–377
5. Meiss, G., Friedhoff, P., Hahn, M., Gimadutdinov, O., and Pingoud, A. (1995) Biochemistry 34, 11979–11988
6. Meiss, G., Gast, F. U., and Pingoud, A. M. (1999) J. Mol. Biol. 288, 377–390
7. Friedhoff, P., Meiss, G., Kolmes, B., Pieper, U., Gimadutdinov, O., Urbanke, C., and Pingoud, A. (1996) Eur. J. Biochem. 241, 572–580
8. Kuhlman, U. C., Moore, G. R., Krahn, J., Kleinknecht, C., and Hennings, A. M. (1999) FEMS Lett. 463, 1–2
9. Flick, K. E., Jurica, M. S., Monnat, R. J., Jr., and Stoddard, B. L. (1996) Nature 384, 96–101
10. Friedhoff, P., Franke, I., Meiss, G., Wende, W., Krahn, K. L., and Pingoud, A. (1996) Nat. Struct. Biol. 3, 112–113
11. Friedhoff, P., Franke, I., Krause, K. L., and Pingoud, A. (1999) FEMS Lett. 443, 209–214
12. Belfort, M., and Roberts, R. J. (1997) Nucleic Acids Res. 25, 3379–3388
13. Wu, S. I., Lo, S. K., Shao, C. P., Tsai, H. W., and Hor, L. I. (2001) Appl. Environ. Microbiol. 67, 82–88
14. Li, C. L., Hor, L. I., Chang, Z. F., Tsai, L. C., Yang, W. Z., and Yuan, H. S. (1995) EMBO J. 22, 4014–4025
15. Vinag, A., and Teplakov, A. (1995) J. Appl. Crystallogr. 339, 1922–1925
16. Bailey, S. (1994) Acta Crystallogr. Sect. D 50, 769–770
17. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
18. Brugger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gross, P., Gros, P., Kunz, R., Weinreb, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D 54, 905–921
19. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
20. Lovell, S. C., Davis, I. W., Arendall, W. B., III, de Bakker, P. I., Word, J. M., Prisant, M. G., Richardson, J. S., and Richardson, D. C. (2003) Proteins 50, 437–450
21. Galburn, E. A., Chevalier, B., Tang, W., Jurica, M. S., Flick, K. E., Monnat, R. J., Jr., and Stoddard, B. L. (1999) Nat. Struct. Biol. 6, 1096–1099
22. Schäfer, P., Scholz, S. R., Gimadutdinov, O., Cymerman, I. A., Bujnicki, M. J., Kuipers, C., Pingoud, A., and Meiss, G. (2004) J. Mol. Biol. 338, 217–228
23. Hins, K. C., Cheung, K. P., Cheng, Y. S., Ku, W. Y., and Yuan, H. S. (2004) Structure (Camb.) 12, 205–214
24. Mate, M. J., and Kleanthous, C. (2004) J. Biol. Chem. 279, 34763–34769
25. Woa, E. J., Kim, Y. G., Kim, M. S., Han, W. D., Shin, S., Robinson, H., Park, S. Y., and Oh, B. H. (2004) Mol. Cell. 14, 531–540
26. Raaijmakers, H., Toro, I., Birkenbihl, R., Kemper, B., and Suck, D. (2001) J. Mol. Biol. 308, 311–325
27. Saravanov, M., Bujnicki, J. M., Cymerman, I. A., Rao, D. N., and Nagaraja, V. (2004) Nucleic Acids Res. 32, 6129–6135
28. Shon, B. W., Landthaler, M., Shub, D. A., and Stoddard, B. L. (2004) J. Mol. Biol. 342, 43–56
29. Holm, L., and Sander, C. (1994) Proteins 19, 165–173
30. Franke, I., Meiss, G., and Pingoud, A. (1999) J. Biol. Chem. 274, 825–832
49. Gelz, S., Christoph, A., Birkenkamp-Demtroder, K., and Kemper, B. (1997) *Eur. J. Biochem.* 245, 573–580.

50. Pummer, A. J., Cal, S., Keeble, A. H., Walker, D., Evans, S. J., Kuhlmann, U. C., Cooper, A., Connoly, B. A., Hemmings, A. M., Moore, G. R., James, R., and Kleinhous, C. (2001) *J. Mol. Biol.* 314, 735–749.

51. Mannino, S. J., Jenkins, C. L., and Raines, R. T. (1999) *Biochemistry* 38, 16178–16186.

52. Giraud-Panis, M. J., and Lilley, D. M. (1996) *J. Biol. Chem.* 271, 33148–33155.

53. Birkenbihl, R. P., and Kemper, B. (1998) *EMBO J.* 17, 4527–4534.

54. Nicholls, A., Sharp, K. A., and Honig, B. (1991) *Proteins* 11, 281–296.