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Mechanism of DNA Cleavage by the DNA/RNA-non-specific *Anabaena* sp. PCC 7120 Endonuclease NucA and its Inhibition by NuiA

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A structural model of the DNA/RNA non-specific endonuclease NucA from *Anabaena* sp. PCC7120 that has been obtained on the basis of the three-dimensional structure of the related *Serratia* nuclease, suggests that the overall architecture of the active site including amino acid residues H124, N155 and E163 (corresponding to H89, N119 and E127 in *Serratia* nuclease) is similar in both nucleases. Substitution of these residues by alanine leads to a large reduction in activity (<0.1 %), similarly as observed for *Serratia* nuclease demonstrating that both enzymes share a similar mechanism of catalysis with differences only in detail. NucA is inhibited by its specific polypeptide inhibitor with a K_i value in the sub-picomolar range, while the related *Serratia* nuclease at nanomolar concentrations is only inhibited at an approximately 1000-fold molar excess of NuiA. The artificial chromophoric substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) is cleaved by NucA as well as by *Serratia* nuclease. Cleavage of this analogue by NucA, however, is not inhibited by NuiA, suggesting that small molecules gain access to the active site of NucA in the enzyme-inhibitor complex under conditions where cleavage of DNA substrates is completely inhibited. The active site residue E163 seems to be the main target amino acid for inhibition of NucA by NuiA, but R93, R122 and R167 (corresponding to K55, R87, R131 in *Serratia* nuclease) are also involved in the NucA/NuiA interaction. NuiA deletion mutants show that the structural integrity of the N and C-terminal region of the inhibitor is important for complex formation with NucA and inhibition of nuclease activity. Based on these results a mechanism of DNA cleavage by NucA and its inhibition by NuiA is proposed.

Keywords: nuclease; inhibitor; enzyme mechanism; structural model

Introduction

The non-specific endonuclease NucA from *Anabaena* sp. PCC 7120 (Muro-Pastor *et al*., 1992) is a highly active enzyme, which in the presence of Mn^{2+} or Mg^{2+} is able to degrade DNA as well as RNA in their single and double-stranded forms with nearly equal catalytic efficiency. Like many other non-specific nucleases NucA exhibits certain sequence preferences, suggesting that local structural features of the substrate influence the rate of cleavage of phosphodiester bonds. On double-stranded DNA of defined sequence NucA exhibits preferences for cleavage of phosphodiester bonds in stretches of d(C)·d(G) residues and in those sequences that have the propensity to adopt an *A*-like conformation (Meiss *et al*., 1998, 1999).

Homologues of NucA are widely distributed among heterocyst-forming multicellular cyanobacteria (Muro-Pastor *et al*., 1997). They belong to a superfamily of ubiquitous nucleases with diverse biological functions including the DNA/RNA non-

Abbreviations used: CAD, caspase activated DNase; ICAD, inhibitor of CAD; DFF40, DNA fragmentation factor 40 kDa subunit; DFF45, DNA fragmentation factor 45 kDa subunit; n.d.c., no detectable cleavage; npdTpn, deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate); NucA, DNA/RNA non-specific nuclease from *Anabaena* sp.; NuiA, inhibitor of NucA; wt, wild-type.

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specific endonucleases, the DNA-entry nucleases as well as the homing endonucleases of the Cys-His-box family. The biological roles of these nucleases are different depending on the family and organism. The prokaryotic nucleases of the family of DNA/RNA-non-specific nucleases, like *Serratia* nuclease and NucA, are secreted into the extracellular environment and are likely to serve nutritional purposes by providing the bacterial cell with nucleotides for the salvage pathway and/or phosphate when this is limiting in the environment (Baliaeva *et al.*, 1976). In addition, these extracellular nucleases could also function as bacteriocides, reminiscent of the endonuclease colicins of *Escherichia coli* (Kleanthous *et al.*, 1998). The DNA-entry nuclease EndA was shown to be important for efficient transformation of *Streptococcus pneumoniae* (Puyet *et al.*, 1990). The eukaryotic nucleases of the family of DNA/RNA-non-specific nucleases have again different functions: Nuc1 constitutes the main mitochondrial nuclease activity of *Saccharomyces cerevisiae* and is involved in DNA repair and recombination (Dake *et al.*, 1988; Zassenhaus *et al.*, 1988), while the related bovine endoG is responsible for the generation of primers for mitochondrial DNA replication (Ruiz-Carrillo & Cote, 1993).

Among the members of the family of DNA/RNA-non-specific nucleases the extracellular *Serratia* nuclease is the biochemically best characterized enzyme (for a review, see Benedik & Strych, 1998) as crystal structures are available (Miller *et al.*, 1994, 1999; Miller & Krause, 1996; Lunin *et al.*, 1997, 1999) and the active site of this nuclease has been identified and characterized by site-directed mutagenesis (Friedhoff *et al.*, 1994a, 1996a,b; Kolmes *et al.*, 1996) as well as crystallographic studies (Miller *et al.*, 1999). NucA from *Anabaena* sp. and the *Serratia* nuclease share 29.5% sequence homology. This homology is mainly restricted to the active site region and the central six-stranded β-sheet of the enzyme as seen in the *Serratia* nuclease structure. We have shown that the quaternary structures of both nucleases differ: NucA is a monomeric enzyme (Meiss *et al.*, 1998) while *Serratia* nuclease is a homodimer (Friedhoff *et al.*, 1994b; Miller & Krause, 1996; Franke *et al.*, 1998). The activity of the *Serratia* enzyme is dependent on two essential disulfide bonds that are formed in the oxidizing environment of the periplasm of *Serratia marcescens*. This protects the bacterial cell from the potentially toxic intracellular nuclease activity prior to secretion (Ball *et al.*, 1987, 1992). The nucleolytic activity of NucA is blocked intracellularly by a polypeptide inhibitor, NuiA (Muro-Pastor *et al.*, 1997), with which the nuclease forms a tight 1:1 complex (Meiss *et al.*, 1998), similarly as described for many different nucleases and inhibitors present in prokaryotic as well as eukaryotic organisms, e.g. the *Bacillus amyloliquefaciens* RNase, barnase, and its inhibitor barstar (for a review, see Hartley, 1997), the group of colicins of *E. coli* with nuclease activity and their corresponding immunoproteins (Kleanthous *et al.*, 1998, 1999), the RNases from the RNase A-superfamily and RI (Raines, 1998) or the caspase-3 activated apoptotic nuclease DFF40/CAD and its inhibitor DFF45/ICAD (Liu *et al.*, 1997; Enari *et al.*, 1998).

Here we report the results of a mutational analysis of catalytically relevant amino acid residues of *Anabaena* nuclease, suggesting that NucA essentially follows the same mechanism of phosphodiester bond hydrolysis as the related *Serratia* nuclease with differences only in detail. Based on homology modelling and supported by the results of a CD-spectroscopic study we present a structural model of NucA that provides an explanation why this nuclease is a monomeric enzyme in contrast to the dimeric *Serratia* nuclease. Furthermore, on the basis of inhibition experiments with active site mutants of NucA we present a first model for the mechanism of inhibition of this nuclease by NuiA.

Results

Homology modelling of NucA

On the basis of the three-dimensional structure of *Serratia* nuclease (PDB entry 1SMN; Miller *et al.*, 1994) and the sequence homology of approximately 30%, a structural model of NucA was obtained using the Swiss Model automated modelling server. The structural model comprises amino acid residues M60 to K271 of the 274 amino acid residues of NucA. The N-terminal residues M1 to L59 with the presumptive signal peptide (residues 1-24; Muro-Pastor *et al.*, 1992) and the C-terminal residues V272 to N274 are not included in this model. According to the model, NucA contains less α-helical structural elements than *Serratia* nuclease. One of the most prominent differences between the NucA structural model and the *Serratia* nuclease crystal structure concerns the long helix 1 of the *Serratia* enzyme, which has its counterpart in a shorter helix 1 of NucA (Figure 1(a)). This helix contains catalytically relevant amino acid residues. Otherwise, and this is the basis of our analysis of the importance of presumptive catalytic residues, the catalytic core is very similar in the *Serratia* nuclease structure and the structural model for NucA. The comparison of the *Serratia* nuclease structure and the NucA structural model further shows that the *Serratia* nuclease residue H184, which is involved in an intricate hydrogen bond network and is critical for dimerization of the enzyme, has its structural counterpart in R222 of NucA. Intriguingly, substitution of H184 in *Serratia* nuclease by arginine, threonine or asparagine leads to a monomeric form (Franke *et al.*, 1998). Besides this, the *Serratia* nuclease C-terminal helix 4, which contributes to the dimer interface of the *Serratia* enzyme, has no equivalent in the NucA structural model. In good agreement with the crystal structure of the *Serratia* nuclease

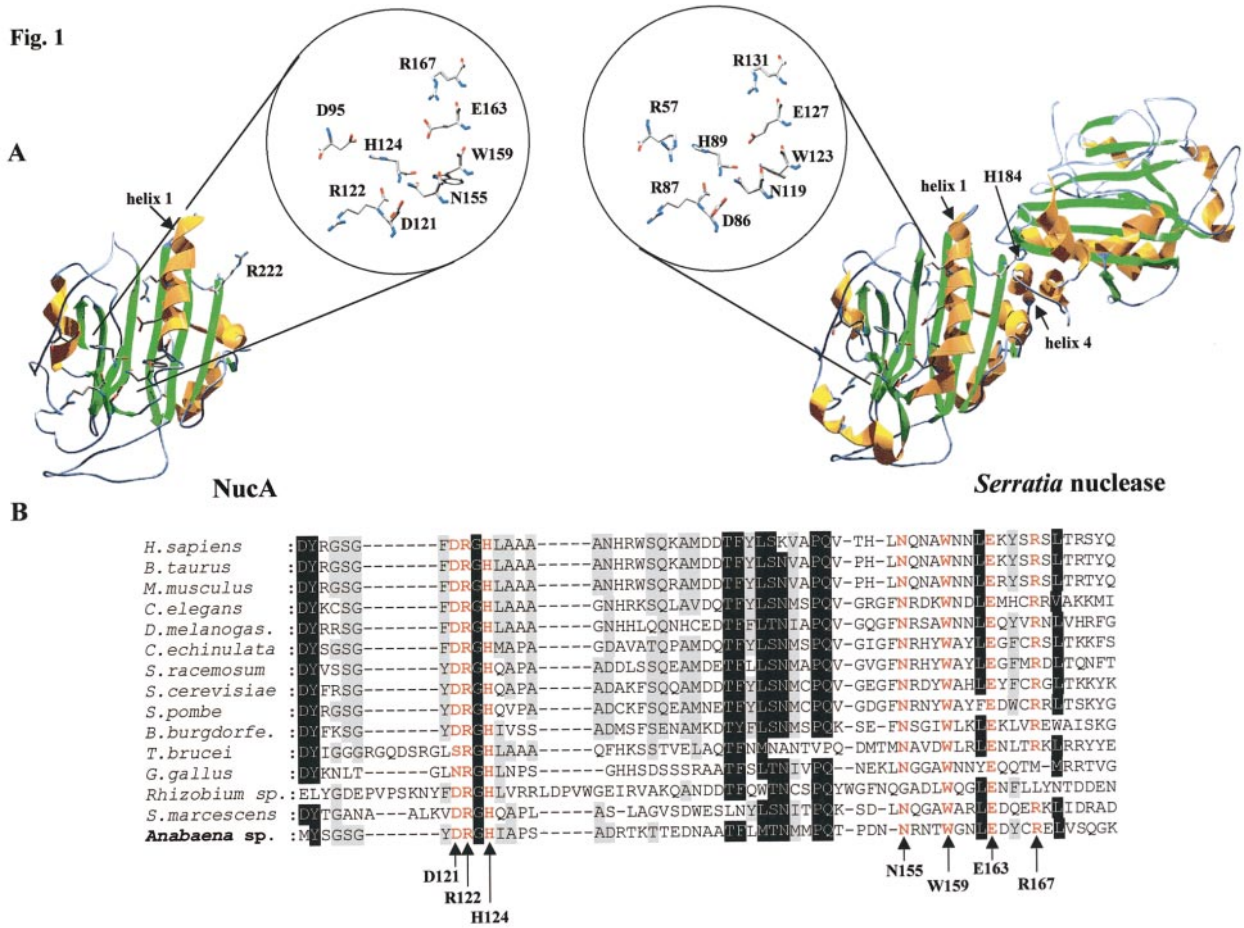

Figure 1. *Anabaena* and *Serratia* nuclease, two representatives of related DNA/RNA-non-specific endonucleases. (a) Structure of *Anabaena* nuclease as modelled by the Swiss Model Automated Modelling Server on the basis of the structure of *Serratia* nuclease (1SMN; PDB). While *Anabaena* nuclease is a monomer, *Serratia* nuclease is a dimer of identical subunits. The blow-up shows important amino acid residues located in the active sites of NucA and *Serratia* nuclease, respectively, that are also discussed in the text. (b) Sequence comparison among various members of the *Serratia* nuclease family. Only the amino acid residues comprising the regions of the active site including the DRGH-motif (Prosite motif PDOC00821) containing the active-site histidine (H124 in NucA, H89 in *Serratia* nuclease) are shown. *Homo sapiens* (Tiranti *et al.*, 1995); *Bos taurus* (Ruiz-Carillo & Cote, 1993); *Mus musculus* (Prats *et al.*, 1997); *Caenorhabditis elegans* (GeneBank AF003740); *Drosophila melanogaster* (GeneBank AC004340); *Cunninghamella echinulata var. echinulata* (Ho *et al.*, 1998); *Syncephalastrum racemosum* (Ho & Liao, 1999); *Saccharomyces cerevisiae* (Vincent *et al.*, 1988); *Schizosaccharomyces pombe* (GeneBank Z73099); *Trypanosoma brucei* (GeneBank U43702); *Borrelia burgdorferi* (Fraser *et al.*, 1997); *Camphylobacter jejunii* (WIT database (http://selkov-7.mcs.anl.gov/WIT/CGI/org.cgi) RCJ01399); *Anabaena sp.* (Muro-Pastor *et al.*, 1992); *Serratia marcescens* (Nestle & Roberts, 1969); *Pseudomonas aeroginosa* (WIT database RPA01594); *Rhizobium* sp. (GeneBank AE000072); *Gallus gallus* (NR database O73911). Highlighted in red are conserved amino acid residues that constitute the catalytic center of these nucleases.

(Miller *et al.*, 1994), the secondary structure composition determined by circular dichroism (CD) spectroscopy reveals 17 % α-helix (15 % in the crystal structure) and 27 % β-sheet (25 %). The structural model of NucA indicates that this protein has less α-helical elements than the *Serratia* nuclease. Indeed, we have shown that NucA shows a CD spectrum of a protein with only 13 % α-helix and 20 % β-sheet (Meiss *et al.*, 1998). The CD spectroscopical data thus support the structural model obtained for NucA.

Mutagenesis of catalytically relevant amino acid residues

Guided by the sequence alignment (Figure 1(b)) and the comparison of the *Serratia* nuclease structure and the NucA structural model we have produced and analyzed 11 variants of NucA having amino acid substitutions at eight different positions, *viz*. R93A, D121A, D121N, R122A, H124A, N155A, N155D, W159A, E163A, E163Q and R167A. At these positions, amino acid residues are

located that in the *Serratia* nuclease are known to be directly (H89, N119 and E127) or indirectly (D86) involved in catalysis and cofactor binding or in substrate binding (R87, R131 and W123). All NucA variants, obtained as N-terminally His_6-tagged proteins overproduced in *E. coli*, turned out to be soluble after renaturation from inclusion bodies. For the H124A, W159A and E163A variants it was shown by circular dichroism spectroscopy that these proteins have the same secondary structure composition and, therefore, most likely the same overall structure as the wild-type enzyme (data not shown). Activity measurement using the nuclease plate assay revealed that all NucA variants exhibited <0.5 % residual activity compared to the wild-type enzyme (Table 1), demonstrating that the amino acid residues substituted in these respective variants are important for the binding and/or cleavage of the nucleic acid substrates. Similar to results obtained for the analogous *Serratia* nuclease variants (Table 1), the variants with substitutions of the residues H124, N155 and E163 of NucA show the lowest residual activities (<0.1 %) among all variants analyzed, with the variants N155A and E163Q exhibiting only 0.02 % or 0.03 % residual activity, respectively. In contrast to the results obtained for *Serratia* nuclease, residue D121 of NucA (its counterpart D86 in *Serratia* nuclease is indirectly involved in catalysis by positioning the catalytic residue N119), when exchanged to alanine or asparagine leads to one of the most inactive nuclease variants. Variants in which the positively charged amino acid residues R93, R122 and R167 (like their putative counterparts K55, R87, R131 in *Serratia* nuclease thought to be involved in the positioning and binding of the substrate) were substituted for alanine exhibit residual activities below 0.2 %. Substitution by alanine of residue W159, strictly conserved in all members of the *Serratia* nuclease family, and possibly also involved in substrate binding (Meiss *et al.*, 1999), leads to a residual activity of NucA which is below 0.3 % (Table 1).

Cleavage of deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) by NucA variants

Deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) can be considered to be a minimal substrate for *Serratia* nuclease; its cleavage by *Serratia* nuclease variants indicates whether the catalytic center in these variants is affected by the amino acid substitution or not. As observed with *Serratia* nuclease, the hydrolysis of the synthetic chromophoric substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) by NucA is accompanied by a marked increase in absorbance at 400 nm, indicating *p*-nitrophenol to be the cleavage product. No measurable increase in absorbance at 330 nm, which would be indicative for the release of *p*-nitrophenyl phosphate could be detected. When this chromophoric substrate is incubated with wild-type NucA and the E163A variant cleavage occurs with equal catalytic efficiency (Table 2). In contrast, the NucA variants H124A and N155A exhibit no activity towards this substrate, indicating that similarly to *Serratia* nuclease, the function of the histidine and the asparagine residue is required for efficient cleavage of this artificial substrate, while that of the glutamic acid residue E163 is not (Table 2). Interestingly, both for the *Serratia* nuclease and NucA hardly any cleavage of DNA (see also] and no cleavage of deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) (Table 2) is observed for the W123A and W159A variant, respectively, suggesting that the aromatic side-chain is required for binding the substrate, in particular when it is a minimal substrate, like deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate).

Inhibition of NucA and the related *Serratia* nuclease by NuiA

We have determined the K_i value for the inhibition of NucA by NuiA by an agarose gel assay using plasmid DNA and titrating NucA with different amounts of NuiA (Figure 2). The value obtained for K_i' is 1.1×10^{-12} M (for a definition of

Table 1. DNA cleavage activity of several NucA and *Serratia* nuclease variants bearing homologous amino acid exchanges

NucA	Activity (%)	*Serratia* nuclease	Activity (%)
Wild-type	100	Wild-type	100
D121A	0.04	D86A	0.7
D121N	0.05	n.a.	n.a.
H124A	0.08	H89A	<0.001
N155A	0.02	N119A	<0.001
N155D	0.07	N119D	0.02
E163A	0.23	E127A	0.07
E163Q	0.03	E127Q	0.26
R93A	0.12	K55A	30
R122A	0.17	R87A	0.18
R167A	0.14	R131A	2.7
W159A	0.28	W123A	4.4

n.a, not available. Data for *Serratia* nuclease variants other than W123A and W123F are taken from Friedhoff *et al.* (1996a). Data for NucA were obtained using the nuclease plate assay described in Materials and Methods.

Table 2. Relative kinetic parameters

Enzyme	Rel. k_{cat}/K_M (DNA)	Rel. k_{cat}/K_M (npdTpn)
A. *wt NucA*	≡100	≡100
H124A	0.08	n.d.c.
E163A	0.23	100
W159A	0.28	n.d.c.
wt *Serratia* nuclease	≡100	≡100
H89A	<0.001	n.d.c.
E127A	0.1	100
W123A	4	n.d.c.
B. *E163A*	≡100	≡100
NucA + NuiA	n.d.c	100
E163A + NuiA	40	100

(A) Relative kinetic parameters for the cleavage of high molecular mass herring sperm DNA and cleavage of deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) by several variants of NucA and *Serratia* nuclease. (B) Relative kinetic parameters for the cleavage of DNA and deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) of wild-type NucA and the E163A variant in the presence of NuiA (1:1 complex) in comparison with the E163A variant in the absence of NuiA.

npdTpn, deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate); n.d.c., no detectable cleavage; wt, wild-type; data for *Serratia* nuclease and *Serratia* nuclease variants other than W123A are taken from Friedhoff *et al.* (1996a). DNA-cleavage data for NucA and its variants were obtained using the nuclease plate assay described in Materials and Methods. npdTpn-cleavage data were obtained using a spectrophotometric assay as described in Materials and Methods.

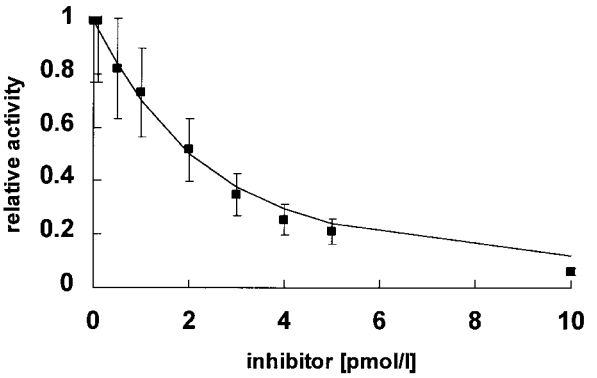

Figure 2. Effect of NuiA on the steady-state velocity of DNA cleavage by NucA. The plasmid cleavage assay was performed as described in Materials and Methods. The NucA concentration was 1.75 pM and NuiA concentrations were varied between 0.1 and 10 pM. Observed steady state velocities (■) were fitted (continuous line) to equation (1) (see the text) that describes tight-binding inhibitors (Leland *et al.*, 1998; Stone & Hofsteenge, 1986): A value of 1.1×10^{-12} M was obtained for K_i'.

K_i', see Stone & Hofsteenge, 1986), comparable to that obtained for the RNaseA/RI complex (Leland *et al.*, 1998; Suzuki *et al.*, 1999). Assuming competitive inhibition of NucA by NuiA for the cleavage of high molecular mass DNA this corresponds to a K_i value of 0.66 pM. In order to find out whether NuiA is able to inhibit the related *Serratia* nuclease we performed a steady-state kinetic analysis of the cleavage of high molecular mass herring sperm DNA in the presence of recombinant NuiA. Muro-Pastor *et al.* (1997) and our group (Meiss *et al.*, 1998) had shown that *Serratia* nuclease is not inhibited at equimolar amounts or a moderately high molar excess of NuiA. Here, however, we demonstrate also that *Serratia* nuclease can be inhibited by NuiA but that inhibition of *Serratia* nuclease by NuiA requires a more than a 1000-fold excess of NuiA over the nuclease at nM concentrations, while its nucleolytic activity remains unaffected in the presence of DFF45/ICAD, the inhibitor of the apoptotic nuclease DFF40/CAD which is a representative of a different family of nucleases (Halenbeck *et al.*, 1998; Enari *et al.*, 1998; Liu *et al.*, 1998; Mukae *et al.*, 1998) (Figure 3).

Inhibition of NucA active site variants by NuiA

We have tested the NucA variants R93A, D121A, R122A, H124A, N155A, W159A, E163A and R167A with respect to inhibition of their DNase activity by NuiA. In the presence of equimolar amounts of NuiA all nuclease variants at a concentration of 1 μM exhibit less than 10 % residual activity, with one exception: the NucA variant E163A retains approximately 40 % of its nucleolytic activity, indicating that NuiA binds to the E163A variant with reduced affinity (Table 3). We tested, therefore, the concentration dependence of the inhibition of this variant (Figure 4(a)). At a tenfold molar excess of NuiA over the E163A variant at a concentration of 1 μmol/l the residual activity of the enzyme is below 10 % and only at a 100-fold molar excess of NuiA is the nucleolytic activity virtually blocked, indicating that E163 directly or indirectly participates in the interaction between NucA and NuiA. Gelfiltration of equimolar amounts of the E163A variant, together with NuiA, shows that at μM concentrations complex formation takes place similarly as observed for wild-type NucA (Figure 4(b) and (c)).

Table 3. Inhibition of *Anabaena* nuclease variants by NuiA

Variant	Residual DNase activity[a] (%)
wt-NucA	n.d.c
E163A	40 (±8)
R93A	9 (±1.8)
R167A	7 (±1.4)
R122A	6 (±1.2)
W159A	3 (±0.6)
D121A	1 (±0.2)
H124A	1 (±0.2)
N155A	n.d.c

[a] Measured for a 1:1 ratio of nuclease and inhibitor at a concentration of 1 μM using the hyperchromicity assay. 100 % activity corresponds to the DNase activity of the respective variant in the absence of NuiA; n.d.c., no detectable cleavage; wt, wild-type.

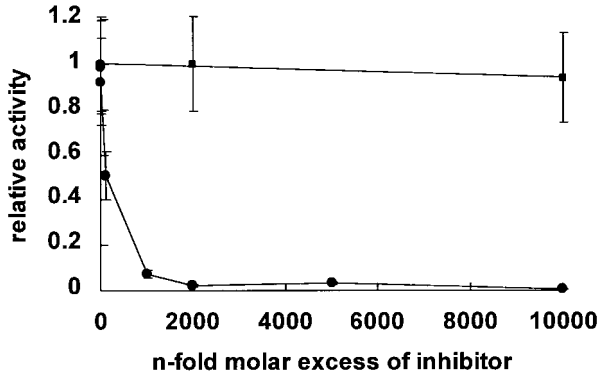

Figure 3. Inhibition of *Serratia* nuclease by NuiA. While NucA at nanomolar concentrations is completely inhibited at equimolar amounts of NuiA, the related non-cognate *Serratia* nuclease requires a high molar excess of NuiA (●) to be inhibited. In contrast, DFF45/ICAD (■), the inhibitor of the apoptotic nuclease, within the limits of error, shows no effect on the catalytic activity of *Serratia* nuclease. Data were obtained using the hyperchromicity assay as described (see Materials and Methods).

Inhibition of NucA by variants of NuiA

We have produced a series of seven variants of NuiA with truncations at the N terminus (Δ1-21), the C terminus (Δ134-135, Δ132-135, Δ131-135, Δ128-135) and carrying single amino acid substitutions (K131A, K131R) (Figure 5(a)). All NuiA variants could be expressed as soluble proteins and behaved like wild-type NuiA in the purification procedure. None of the deletion variants, when added in equimolar amounts, was able to inhibit NucA activity as determined in the hyperchromicity assay and none of them could form a complex with NucA at micromolar concentrations as shown in gelfiltration experiments under conditions, where wild-type NucA and NuiA as well as the NucA E163A variant and NuiA together form a 1:1 nuclease/inhibitor complex (Figure 4(b) and (c)). However, at a 100-fold molar excess over NucA, variants Δ134-135 and Δ132-135 are able to completey inhibit nuclease activity. In contrast, the variant Δ131-135 only partially (50 % at the highest concentration used) inhibits NucA and the variants Δ128-135 and Δ1-21 have almost no effect on the nucleolytic activity of NucA (Figure 5(b)). Since E163 of NucA is important for the inhibition of NucA by NuiA (Figure 4(a)), we assumed that a positively charged residue, for example K131, which is located in the C-terminal part of NuiA, whose integrity is critical for the inhibitory function of NuiA, might interact with this negatively charged active site residue (Figure 5(a)). The NuiA variants K131A and K131R, however, behaved like wild-type NuiA, inhibiting NucA activity at equimolar concentrations and forming a 1:1 complex in the gel filtration experiments (data not shown). As a further possible explanation for the reduced

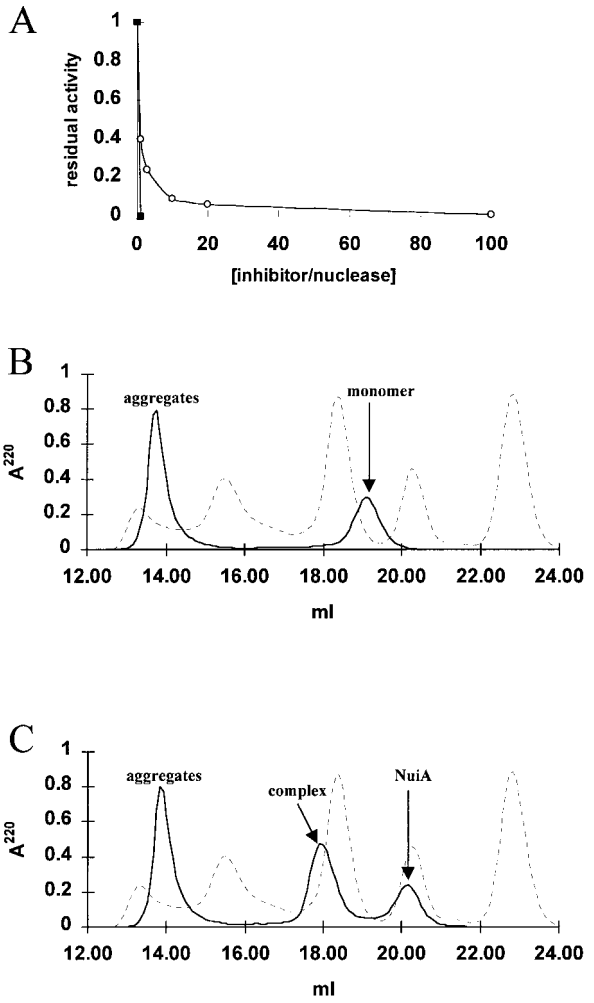

Figure 4. (a) Inhibition of wild-type NucA and the NucA variant E163A by varying amounts of NuiA. While at 1 μM NucA the nucleolytic activity of the wild-type enzyme (■) is completely blocked at equimolar amounts of nuclease and inhibitor, the activity of the *Anabaena* nuclease variant E163A (○) requires a much higher molar excess of NuiA over NucA. Data were obtained using the hyperchromicity assay as described (see Materials and Methods). (b) Elution profile of NucA E163A variant on a Superdex-75 HR10-30 column (Pharmacia). The peak representing active monomeric NucA is labelled ''monomer'' to distinguish it from the peak for inactive aggregates of NucA (Meiss *et al.*, 1998). (c) Complex formation of NucA variant E163A and wild-type NuiA on a Superdex-75 HR10-30 column (Pharmacia). The broken lines represent the elution profile of the standard proteins.

inhibitory capacity of the C-terminal deletion variants we then assumed missfolding to be responsible for the loss of inhibitory function. In order to determine the structural integrity of the NuiA-variants we performed CD-spectroscopic measurements that revealed that the degree of truncation is correlated with the loss of secondary structure (Figure 6).

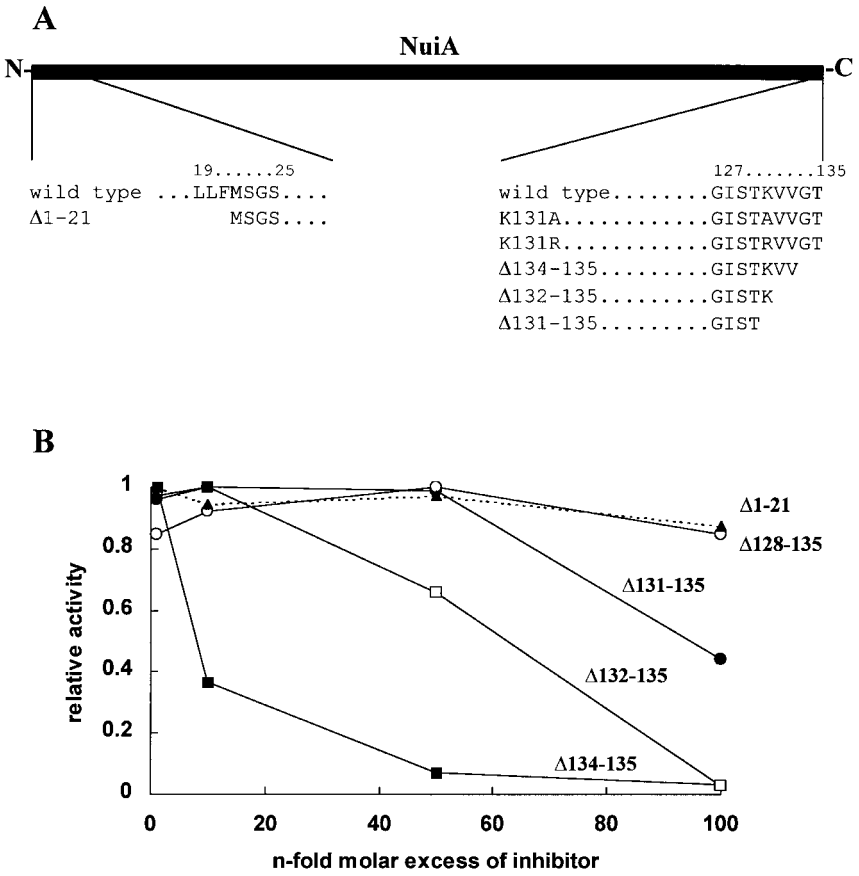

Figure 5. (a) Scheme of NuiA variants investigated for their inhibitory function. (b) Concentration dependence of the inhibition of wild-type NucA (10 nM) by deletion variants of NuiA as determined in the hyperchromicity assay with a 10, 50 and 100-fold molar excess of the respective variants (■, Δ134-135), (□, Δ132-135), (●, Δ131-135), (○, Δ128-135), (▲, Δ1-21). Under the conditions employed here NucA is completely inhibited by wild-type NuiA and the variants K131A and K131R at a 1:1 stoichiometry (data not shown).

Discussion

The *Anabaena* nuclease NucA belongs to a family of non-specific endonucleases whose best characterized member, both in structural and functional terms, is the *Serratia* nuclease (for a review, see Benedik & Strych, 1998). NucA shows approximately 30% homology with the *Serratia* nuclease. In contrast, however, to *Serratia* nuclease which is a homodimer, NucA is a monomeric enzyme. In addition, while intracellular activity of *Serratia* nuclease is suppressed by the absence of a critical disulfide bond (Ball *et al.*, 1992), the activity of *Anabaena* nuclease within the cell is blocked by a polypeptide inhibitor (Muro-Pastor *et al.*, 1997). These differences in quaternary structure as well as regulation of nucleolytic activity may explain why the sequence homology between the two enzymes is unevenly distributed along the amino acid sequence and most pronounced among the sequence stretches considered to represent the catalytic core. Using the structural model of NucA we have addressed three questions: (i) why is NucA in contrast to *Serratia* nuclease a monomer; (ii) what is the mechanism of action of NucA; and (iii) what is the mechanism of inhibition of NucA by NuiA?

Based on the sequence homology of NucA and the *Serratia* nuclease as well as the crystal structure of *Serratia* nuclease (Miller *et al.*, 1994) a model for the structure of NucA was generated (Figure 1) using the Swiss Model server (Peitsch & Jongeneel, 1993; Peitsch, 1995). Our structural model gives a straightforward explanation why NucA is a monomeric protein, while *Serratia* nuclease is a homodimer (Miller & Krause, 1996). The dimer interface of *Serratia* nuclease is formed in part by two symmetry-related α-helices (helix 4; Miller & Krause, 1996), which are absent in the NucA structural model. Furthermore, a critical subunit/subunit interaction in *Serratia* nuclease is formed by H184 which when replaced by arginine leads to monomerization (Franke *et al.*, 1998); in the structural model of the *Anabaena* nuclease the analogous residue is an arginine (R222). The absence of helix 4 and the presence of an arginine residue in a critical position, therefore, explains why NucA is a monomer.

The crystal structure analysis of the *Serratia* nuclease (Miller *et al.*, 1994) together with the results of site-directed mutagenesis experiments (Friedhoff *et al.*, 1994a, 1996a,b), which were based on a sequence alignment of six related nucleases, revealed five conserved amino acid residues to be of particular importance for nucleolytic activity: R57, R87, H89, N119 and E127. Among these, H89, N119 and E127 are the most important residues for catalysis, as their substitution by alanine leads to variants with very low DNA cleavage activity compared to the wild-type enzyme. A detailed biochemical investigation using the artificial substrate

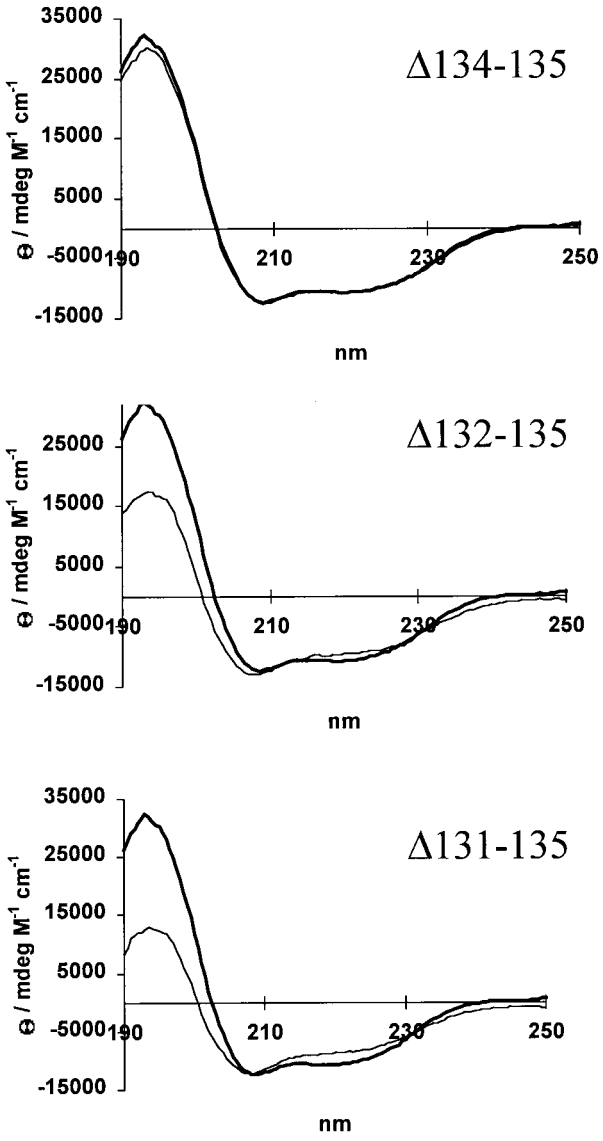

Figure 6. Circular dichroism spectroscopic analyses of NuiA variants carrying deletions in the C-terminal region of the inhibitor. The comparison of the three spectra demonstrates that the degree of truncation is correlated with a decrease in ellipticity at 195 and 220 nm.

deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) suggested that H89 acts as the general base (Kolmes *et al.*, 1996), which activates the attacking nucleophile, while E127 might be involved in protonation of the leaving group and together with N119 serves to bind the essential cofactor Mg^{2+}. Several positively charged amino acid residues, for example R87 and R131 which in the crystal structure flank a cleft region near the putative active site, are likely to constitute the central part of the substrate binding site of this enzyme. Interestingly, it has been demonstrated recently that the non-specific *Serratia* nuclease and the highly specific intron-encoded homing endonuclease I-*Ppo*I (Flick *et al.*, 1998) share a structurally conserved catalytic motif consisting of a histidine, an asparagine and an arginine residue and have a similar catalytic mechanism (Friedhoff *et al.*, 1999a,b; Miller *et al.*, 1999; Galburt *et al.*, 1999; Mannino *et al.*, 1999). In addition, it was recently shown that the colicin E9 DNase also has a similar active site as *Serratia* nuclease (Kühlmann *et al.*, 1999).

We have now carried out a mutational analysis of the corresponding homologous residues H124, N155 and E163 of NucA, which shows that the substitution of these residues by alanine, similarly as observed for the *Serratia* nuclease, leads to a decrease in catalytic activity by three to four orders of magnitude compared to the wild-type enzyme (Table 1). It must be emphasized, however, that the residues with the lowest residual activity among all variants tested, namely N155A and H124A, have a 10 to 100-fold higher activity than the homologous variants N119A and H89A of *Serratia* nuclease. On the other hand, the NucA variant E163Q is about one order of magnitude less active than the corresponding *Serratia* nuclease variant E127Q (Table 1). This could be due to the fact that these catalytic residues are embedded in slightly different surroundings, and probably does not mean that their particular roles differ. N119 is the principal metal ion ligand in *Serratia* nuclease and D86 is hydrogen-bonded to N119 (Miller *et al.*, 1999). Based on our mutational analysis we suggest that the interaction of N155 and D121 is also essential for the coordination of the divalent metal ion cofactor in NucA, although in comparison to residue D86 of *Serratia* nuclease, residue D121 of NucA, when substituted for alanine or asparagine, exhibits a more pronounced reduction in activity (Table 1). Since the two nucleases prefer different divalent cations for maximum activity, *Serratia* nuclease is more active with Mg^{2+} than with Mn^{2+} while the opposite is true for NucA (Nestle & Roberts, 1969; Meiss *et al.*, 1998), their metal ion binding sites are likely to differ in some detail. For this reason one is tempted to speculate that residue D121 in NucA is more critical for the positioning of N155 than its counterpart, D86, in *Serratia* nuclease for positioning N119. Cleavage of the artificial substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) has allowed us to determine the roles of the *Serratia* nuclease amino acid residues H89 and E127 in the mechanism of action of this enzyme (Kolmes *et al.*, 1996). As deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) in contrast to nucleic acid substrates, upon hydrolysis requires no protonation of the leaving group, the function of the presumptive general acid in the mechanism of catalysis is dispensible. The general base, however, is still needed to generate the attacking hydroxyl ion. As the H124A variant cleaves deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) with markedly reduced activity, while the E163A variant retains activity towards this artificial substrate (Table 2), it can be concluded that H124 like H89 in *Serratia* nuclease must be the gen-

eral base and in turn that E163 (like E127 in *Serratia* nuclease) might be or assist the general acid in the mechanism of catalysis of this enzyme. Recently, a crystallographic study of the *Serratia* nuclease complex with Mg^{2+} and a comparison with the I-*Ppo*I co-crystal structure has shown that the active site of *Serratia* nuclease contains a conserved magnesium-water cluster (Miller *et al.*, 1999). The divalent metal ion is octahedrically coordinated and the sole protein ligand is the amido function of N119 while five water molecules complete the coordination sphere. E127 is hydrogen-bonded to two of the water molecules in the coordination sphere of the Mg^{2+}. This suggests that a water molecule from the inner sphere of Mg^{2+} serves to protonate the leaving group rather than E127 itself. R57 of *Serratia* nuclease, which functions to stabilize the pentacovalent transition state, is also conserved in the intron encoded homing endonuclease I-*Ppo*I. Its structural counterpart in NucA is D95, as can be seen from the alignment as well as in the structural model of NucA. The negatively charged D95 could also serve to stabilize the transition state either by hydrogen bonding when uncharged or by binding to a second metal ion, similar to that suggested for the restriction endonuclease *Bam*HI which, different from other restriction endonucleases harboring the PD...D/EXK-motif, has a glutamic acid residue rather than a lysine in the last position of this sequence (Pingoud & Jeltsch, 1997; Kovall & Matthews, 1999). The mechanism of phosphodiester bond cleavage by the *Anabaena* nuclease, as derived from the mutational analysis and the comparison with *Serratia* nuclease and I-*Ppo*, is shown in Figure 7. Amino acid residues R87 and R131 of the *Serratia* nuclease are thought to be involved in the binding and positioning of the substrate. Our structural model and the results of the mutational analysis suggest that this is also true for the homologous amino acid residues R122 and R167 of NucA, because the variants in which arginine is substituted by alanine show a similar loss in activity as the respective *Serratia* nuclease variants (Table 1). In addition, W159 in NucA and W123 in *Serratia* nuclease are involved in the binding of the substrate, because these residues show an approximately 100-fold increase in K_M value when substituted by alanine (Meiss *et al.*, 1999). The analysis of the *Serratia* nuclease variant W123F also revealed that the aromatic nature of the side-chain is particularly important, since the exchange of one for another aromatic amino acid does not lead to a significant increase in K_M. W123 in *Serratia* nuclease and W159 in NucA are of particular importance for the cleavage of the minimal substrate deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) as the W123A and W159A variants of these enzymes show no activity with this artificial substrate, but a residual activity with DNA. It is tempting to speculate that this tryptophan residue, which is conserved in all members of the *Serratia* nuclease family, is involved in a π-π stacking interaction with a base of the nucleic acid substrate, either by intercalation or by binding to a flipped-out base, similarly as suggested for a tyrosine residue in the adenine DNA methyltransferase M·*Taq*I (Schluckebier *et al.*, 1998).

We have shown that NucA and NuiA, similar to other nucleases and their inhibitors, e.g. barnase and barstar (for a review, see Hartley, 1997), form a stable 1:1 complex (Meiss *et al.*, 1998). For NuiA, no homologues exist in the databanks, which, therefore, represents a new class of nuclease inhibitors (Muro-Pastor *et al.*, 1997). In order to obtain a first insight into the mechanism of inhibition of NucA by NuiA we tested several active site mutants of NucA that exhibit sufficient residual activity, such that their sensitivity towards inhibition by NuiA can be measured. This analysis shows that amino acid residue E163 of NucA, which may participate in cofactor binding or protonation of the leaving group is a target amino acid for the inhibition of the enzyme by NuiA. Residues R93, R122 and R167 are less involved than E163 in the interaction of the nuclease with NuiA, while several other amino acid residues, e.g. D121, H124, N155 and W159, that are also located at the active site of this enzyme, do not seem to participate significantly in this interaction. The nucleolytic activity of the E163A variant is only inhibited at an approximately 100-fold molar excess of NuiA over this nuclease variant. The complex between E163A and NuiA, although less stable than the wild-type NucA-NuiA complex can be detected by size exclusion chromatography. We assume that E163 of NucA is contacted by positively charged amino acid residues from NuiA, while the arginine residues could be contacted by negatively charged residues of NuiA. Replacing one of the charged residues of NucA by alanine weakens the interaction but does not completely abolish it. So far we have not identified any charged residue of NuiA involved in the interaction with NucA, but we suggest that the N and C-terminal parts of NuiA are involved in the interaction, as N and C-terminal deletion mutants of NuiA have lost their capability to inhibit the nucleolytic activity of NucA. The fact that these deletion mutants have an altered secondary structure, however, could also mean that the N and/or C-terminal parts are only indirectly involved in the NucA/NuiA interaction.

The fact that *Serratia* nuclease activity is inhibited at high molar excess of NuiA at nanomolar concentrations of the nuclease indicates that a complex between these two proteins can be formed. However, this complex is not sufficiently stable to be detected in gelfiltration experiments (data not shown). By X-ray crystallographic and mutational analyses it had been demonstrated that the *Bacillus amyloliquefaciens* extracellular RNase barnase is inhibited by its polypeptide inhibitor barstar by blocking the active site through interaction, among other residues, with H102 which acts as the general base in the barnase catalyzed cleavage of RNA (Buckle *et al.*, 1994). Thus, in the case of NucA and

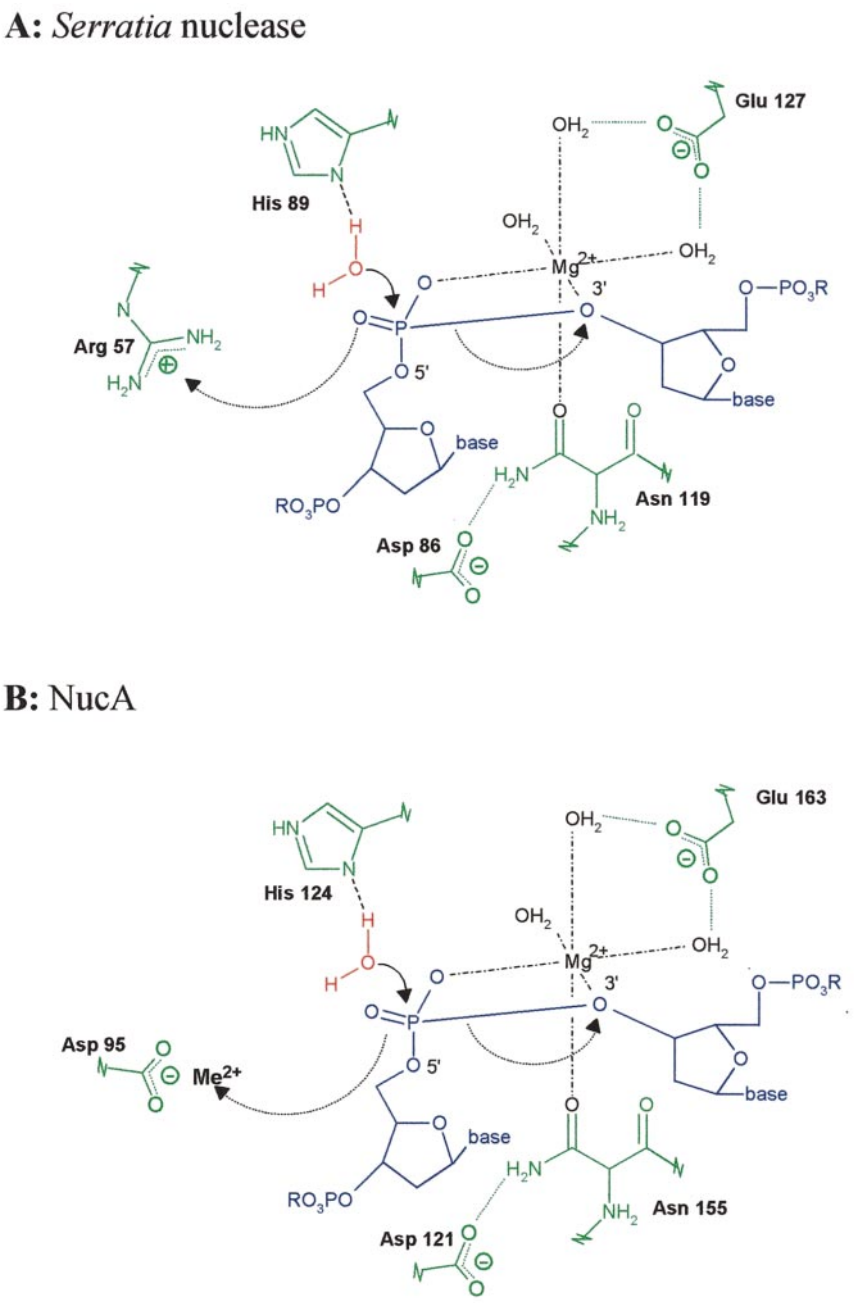

Figure 7. Proposed mechanism of catalysis of NucA. As with *Serratia* nuclease (a) the active site histidine residue of NucA (b) acts as a general base to activate a water molecule. The catalytically relevant Mg^{2+} is directly bound to the protein by only one asparagine residue, while several other active site residues, e.g. a glutamic acid residue, indirectly bind the metal ion *via* water molecules in the hydration sphere. In *Serratia* nuclease and in I-*Ppo*I (Friedhoff *et al.*, 1999b; Miller *et al.*, 1999) an arginine residue (Arg57 in *Serratia* nuclease) acts as an electrophile to stabilize the pentacovalent transition state. In NucA the corresponding residue is an aspartate residue (Asp95), which, however, could indirectly serve as an electrophile by binding a second divalent metal ion, as proposed for the restriction enzyme *Bam*HI, which in contrast to other restriction endonucleases harboring the PD…D/EXK-motif has a glutamic acid residue rather than a lysine in the last position of this sequence (Pingoud & Jeltsch, 1997).

barnase the respective inhibitor seems to directly interact with an active site residue. In contrast to this, it has been shown by a crystallographic analysis of the E9/Im9-complex (Kleanthous *et al.*, 1999) that the DNase activity of the endonuclease colicin E9 is not blocked by direct interaction of Im9 with an active site residue of the nuclease domain of E9 but rather by a partial occlusion and a change in the electrostatic potential of the enzyme's active site.

Materials and Methods

Materials

All chemicals, if not otherwise stated, were obtained from Merck. Restriction endonucleases were from Amersham, *Taq* DNA polymerase and the Expand™ Long Template PCR-System as well as T4 DNA ligase from Roche-Molecular-Biochemicals. All enzymes were used according to the respective manufacturer's recommendations. Herring sperm DNA was from Sigma. Deoxythymidine 3′,5′-bis-(*p*-nitrophenyl phosphate) was a kind gift from Dr I. Franke. The concentration of this analogue was determined by measuring the absorbance at 275 nm using an extinction coefficient of 22,500 M^{-1} cm^{-1} (Kolmes *et al.*, 1996).

Sequence alignments and homology modelling of NucA

Primary sequence analysis of the family of sugar non-specific nucleases was performed using MULTALIN (http://www.toulouse.inra.fr/multalin.html) (Corpet, 1988) and program GeneDoc (http://www.cris.com/~ketchup/genedoc.shtml) (Nicholas *et al.*, 1997). Homology modelling of NucA was performed using the automated knowledge-based protein modelling server Swiss Model (http://expasy.hcuge.ch/swissmod/SWISS-MODEL.html) (Peitsch & Jongeneel, 1993; Peitsch, 1995). Graphical analysis of the structural model was performed using the Swiss-PdbViewer v.3.1 (http://www.expasy.ch/spdbv/mainpage.htm) (Guex & Peitsch, 1997) and program POV-Ray (http://www.povray.org/nj-index.html).

Circular dichroism (CD) spectroscopy

CD spectra of the proteins were recorded on a Jasco J-710 dichrograph between 250 and 185 nm at ambient temperature in a cylindrical cuvette of 0.05 cm path length. Spectra of NucA and its variants and NuiA and its variants were recorded at a protein concentration of 5.0 μM in a buffer consisting of 20 mM Tris-HCl (pH 8.2), 0.01 % (w/v) lubrol. Spectra of *Serratia* nuclease were recorded similarly but without lubrol in the buffer. Concentrations of the proteins were determined by UV spectroscopy before recording the CD spectra.

Production of mutant enzymes

Wild-type NucA and the NucA variants R93A, D121A, D121N, R122A, H124A, N155A, N155D, E163A, E163Q, W159A and R167A were generated using an inverse PCR mutagenesis protocol and produced as His_6-tagged proteins in *E. coli* as described in detail by Meiss *et al.* (1998). Recombinant NuiA variants were produced by PCR mutagenesis using plasmid pHisnuiA as described (Meiss *et al.*, 1998). The *Serratia* nuclease variants W123A and W123F were produced and purified as described by Friedhoff *et al.* (1996a). Concentrations of the enzymes were determined by UV spectroscopy using extinction coefficients calculated according to Pace *et al.* (1995): 46,410 $M^{-1}cm^{-1}$ for NucA variant W159A, 51,910 $M^{-1}cm^{-1}$ for wt NucA and all other NucA variants; 39,670 $M^{-1}cm^{-1}$ for *Serratia* nuclease W123A variant; 45,170 $M^{-1}cm^{-1}$ for wt *Serratia* nuclease.

Steady-state kinetic assays

To measure the DNase activity of NucA variants two different activity assays were used. For the nuclease activity plate assay, that has been used previously to determine the catalytic efficiencies of active site variants of staphylococcal nuclease (Serpersu *et al.*, 1987; Weber *et al.*, 1991), nuclease variants were spotted at ambient temperature on 1 % (w/v) agar plates containing 25 μg/ml toluidine-blue, 1 mg/ml high molecular mass herring sperm DNA, 5 mM $MnSO_4$ and 50 mM Tris-HCl (pH 7.5). Upon hydrolysis of phosphodiester bonds the protons released cause a change in color of the indicator dye from deep blue to a light pink. Relative activities were obtained by comparing the areas of the halos produced by each nuclease variant with reference to the wild-type enzyme. In order to obtain absolute catalytic constants for the cleavage of nucleic acids by NucA and the *Serratia* nuclease we used a hyperchromicity assay with high molecular mass herring sperm DNA as substrate in a buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MnSO_4$ or $MgCl_2$, respectively, as described (Kunitz, 1950; Hale *et al.*, 1993; Friedhoff *et al.*, 1996b; Meiss *et al.*, 1998).

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

The rate of cleavage of the artificial substrate deoxythymidine-3′,5′-bis-(*p*-nitrophenyl phosphate) by NucA and the *Serratia* nuclease as well as variants of these enzymes was determined *via* the absorbance increase at 400 nm, typical for the cleavage product *p*-nitrophenol in its unprotonated form ($\varepsilon_{400} = 17{,}200$ M^{-1} cm^{-1}) (Kolmes *et al.*, 1996). The 35 μM substrate was incubated with 5-20 μM nuclease in a buffer consisting of 50 mM Tris-HCl (pH 8.2), 5 mM $MgCl_2$ in a 1 cm cuvette at 25 °C. Time scans were recorded at a wavelength of 400 nm on a Hitachi U-3000 spectrophotometer.

Nuclease inhibitor binding assays

Inhibition of NucA by NuiA was tested in the hyperchromicity assay as described above with wild-type NucA or NucA variants preincubated with different amounts of wild-type NuiA or NuiA variants, respectively. The residual nuclease activity was determined and normalized to the activity of the respective nuclease variant in the absence of inhibitor.

Inhibition of *Serratia* nuclease was tested in the hyperchromicity assay as described above using 1-5 nM *Serratia* nuclease in 50 mM Tris-HCl (pH 8.2), 5 mM $MgCl_2$ and 0.1 mg/ml high molecular mass herring sperm DNA. The concentration of NuiA was varied between 1 nM and 10 μM.

Values of K_i' for the inhibition of NucA catalyzed DNA cleavage by NuiA were determined by measuring the steady-state rate of sc plasmid DNA cleavage in the presence of NuiA using an agarose gel assay. Reactions were performed at 37 °C in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1.75 pM NucA and 50 ng/μl plasmid DNA. NuiA concentrations were varied between 100 fM and 10 pM. Values for K_i' were calculated by fitting steady-state rates to an equation that describes tight-binding inhibitors (Leland *et al.*, 1998; Stone & Hofsteenge, 1986):

$$v_s = \left(\frac{v_o}{2E_t}\right)\{[(K_i' + x - E_t)^2 + 4K_i'E_t]^{1/2} - (K_i' + x - E_t)\} \quad (1)$$

where v_0 is the steady-state rate of sc plasmid DNA cleavage in the absence of NuiA, v_s is the steady state rate, x the concentration of the inhibitor NuiA and E_t the total concentration of NucA.

Size-exclusion chromatography

Size-exclusion chromatography on a Superdex-75 HR10-30 column (Pharmacia) was used to examine complex formation between NucA, NucA variants, *Serratia* nuclease on one side and NuiA and its variants on the other. The flow rate was 0.5 ml/minute and the buffer contained 25 mM Tris-HCl (pH 8.2), 0.01 % (w/v) lubrol and 500 mM NaCl.

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