The Nuclease A-Inhibitor Complex Is Characterized by a Novel Metal Ion Bridge*

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Nonspecific, extracellular nucleases have received enhanced attention recently as a consequence of the critical role that these enzymes can play in infectivity by overcoming the host neutrophil defense system. The activity of the cyanobacterial nuclease NucA, a member of the ββα Me superfamily, is controlled by the specific nuclease inhibitor, NuiA. Here we report the 2.3-Å resolution crystal structure of the NucA-NuiA complex, showing that NucA inhibition by NuiA involves an unusual divalent metal ion bridge that connects the nuclease with its inhibitor. The C-terminal Thr-135NuiA hydroxy oxygen is directly coordinated with the catalytic Mg2+ of the nuclease active site, and Glu-24NuiA also extends into the active site, mimicking the charge of a scissile phosphate. NuiA residues Asp-75 and Trp-76 form a second interaction site, contributing to the strength and specificity of the interaction. The crystallographically defined interface is shown to be consistent with results of studies using site-directed NuiA mutants. This mode of inhibition differs dramatically from the exosite mechanism of inhibition seen with the DNase colicins E7/E9 and from other nuclease-inhibitor complexes that have been studied. The structure of this complex provides valuable insights for the development of inhibitors for related nonspecific nucleases that share the DRGH active site motif such as the Streptococcus pneumoniae nuclease EndA, which mediates infectivity of this pathogen, and mitochondrial EndoG, which is involved in recombination and apoptosis.

Nonspecific nucleases are involved in a broad range of functions that include extra- and intracellular digestion, programmed cell death, defense, replication, recombination, and repair (1–3). They also have proven useful for determining nucleic acid structures, mapping mutations, studying the interaction of DNA and RNA with various ligands (4), and sequencing of RNA (5). Most recently, an important role for these nucleases in microbial infectivity has been demonstrated, based on their ability to digest the DNA component of host neutrophil extracellular traps (6–8). Consequently, these nucleases are now recognized as significant drug targets, and information related to the inhibition of these enzymes is of potential use for inhibitor development. As a result of their ability to degrade nucleic acids nonspecifically, they also represent an endogenous toxic challenge. Therefore, regulation of their activity is critical for the cells that produce them.

The ββα Me superfamily of nucleases (9) comprises nonspecific, structure-specific, and sequence-specific enzymes that share a structurally conserved active site scaffold and utilize a divalent metal ion. They can be grouped according to sequence motifs into three families: His-Cys box nucleases (e.g. I-Ppol (10)), HNH nucleases (e.g. colicins E7 and E9 (11, 12) and I-HmuI (13)), and DRGH nucleases (e.g. the extracellular nuclease from Serratococcus pneumoniae (15), the Syncephalastrum racemosum nuclease (16), nuclease C1 from Cunninghamella echinulata (17), yeast Nuc1 (18), mitochondrial EndoG (19), and the Anabaena nuclease NucA (20)). Whereas the eukaryotic nucleases of the DRGH family represent the major mitochondrial nuclease activity, the prokaryotic members of this family are responsible for extracellular DNA degradation. Notably the DNA-entry nucleases EndA from Streptococcus pneumoniae and the related Streptodornase (Sda1) from Streptococcus sp. allow their host organisms to escape from neutrophil extracellular traps by digesting the DNA scaffold of these structures, thereby evading the first line of defense against microbial infection in mammals (6–8).

NucA, a member of the DRGH family, is one of the most potent nucleases known, and it degrades both single- and double-stranded DNA and RNA. Its activity is regulated by a potent and specific protein inhibitor, NuiA, which forms a tight 1:1 complex with picomolar affinity (21). The structure of the active site is closely analogous to that of the Serratia nuclease (22, 23), whereas the activity of the Serratia enzyme is dependent on the presence of cystine bonds and hence is determined by the redox level of the medium (24). A deletion analysis of NuiA had suggested that N- and C-terminal residues, directly or indirectly, are involved in the NucA-NuiA interaction (21). Nevertheless, the molecular basis for the strong inhibitory interaction has not yet been determined.

In comparison with the vast literature on proteinase inhibitors, nuclease inhibitors have received relatively little study. The most detailed investigations have focused on the Bacillus amyloliquefaciens RNase (barnase) inhibitor (barstar) (25), the RNase A inhibitor (26), and the immunity proteins that protect Escherichia coli from the colicin DNase activity (27, 28). Con-
sideration of the structures of these nuclease-inhibitor complexes, as well as the structure of the NucA-NuiA complex determined in the present study, suggests few common modes of inhibition.

Here we present the crystal structure of NucA (28 kDa) in complex with NuiA (15 kDa) at a resolution of 2.3 Å. Many of the features of the NucA-NuiA complex are unique. NuiA interacts directly with residues in the active enzyme site displaying target site mimicry and interacting directly with the active site Mg^{2+} ion through coordination with the C-terminal Thr-135 residue. Binding of NuiA results in no significant change of the backbone atoms of NucA (22) but does result in several minor side chain rearrangements. The structure of NucA-complexed NuiA shows some differences relative to the previously determined solution structure (29) of the uncomplexed inhibitor, primarily in the loop regions.

**MATERIALS AND METHODS**

**Protein Expression and Purification**—The recombinant NucA construct, containing a D121A mutation to reduce activity and related cellular toxicity, lacking the N-terminal export signal peptide, and containing an N-terminal His tag to facilitate purification, was produced as described previously (22). Recombinant NuiA, also containing an N-terminal His tag, was similarly produced as described previously (29). *E. coli* cells containing the required plasmid were grown to mid-log phase (A600 ~ 0.6) at 37 °C in LB medium containing 30 µg/ml kanamycin. NuiA protein expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside at 37 °C for 6 h. Cells were harvested by centrifugation (at 7000 × g), resuspended in 20 mM Tris-HCl, pH 8.0, 100 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 supernatant was applied to a Ni^{2+}-NTA resin (Qiagen) equilibrated with 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 2 mM dithiothreitol buffer. The protein corresponding to the major absorbance peak was then mixed together and applied to a Superdex-200 gel filtration 2.6 × 60-cm column previously equilibrated with 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 2 mM dithiothreitol. The peak corresponding to the NucA-NuiA complex was identified by SDS-polyacrylamide gel electrophoresis. Based on the same criteria of inhibition, Mg^{2+} ion through coordination with the C-terminal Thr-135 residue. Binding of NuiA results in no significant change of the backbone atoms of NucA (22) but does result in several minor side chain rearrangements. The structure of NucA-complexed NuiA shows some differences relative to the previously determined solution structure (29) of the uncomplexed inhibitor, primarily in the loop regions.

**Structure of NucA-NuiA Complex**

The recombinant NucA construct, containing a D121A mutation to reduce activity and related cellular toxicity, lacking the N-terminal export signal peptide, and containing an N-terminal His tag to facilitate purification, was produced as described previously (22). Recombinant NuiA, also containing an N-terminal His tag, was similarly produced as described previously (29). *E. coli* cells containing the required plasmid were grown to mid-log phase (A600 ~ 0.6) at 37 °C in LB medium containing 30 µg/ml kanamycin. NuiA protein expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside at 37 °C for 6 h. Cells were harvested by centrifugation (at 7000 × g), resuspended in 20 mM Tris-HCl, pH 8.0, 100 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 supernatant was applied to a Ni^{2+}-NTA resin (Qiagen) equilibrated with 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 2 mM dithiothreitol buffer. The protein corresponding to the major absorbance peak was then mixed together and applied to a Superdex-200 gel filtration 2.6 × 60-cm column previously equilibrated with 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 2 mM dithiothreitol. The peak corresponding to the NucA-NuiA complex was identified by SDS-polyacrylamide gel electrophoresis.

**Determination of Inhibition Constants**—Values of $K_{\text{app}}$ for the inhibition of NucA by NuiA were determined by measuring the steady-state rate of supercoiled plasmid DNA cleavage in the presence of varying NuiA concentrations using an agarose gel assay. Reactions were performed at 25 °C in a buffer consisting of 50 mM Tris-HCl, pH 7.0, 50 mM NaCl, 1 mM EDTA, 0.01% Triton X-100, 0.01% bovine serum albumin, and 5 mM MnCl₂, using a concentration of 5 pm NucA and 25 ng/µl plasmid DNA. Values for $K_{\text{app}}$ were calculated by fitting the steady-state rates to Equation 1, which describes tight-binding inhibitors,

$$ v = \nu_0 - \frac{[E] - [I]}{K + \sqrt{([E] - [I] - K)^2 + 4[E]K}} $$

(Eq. 1)

where $\nu_0$ is the steady-state rate of supercoiled plasmid DNA cleavage in the absence of inhibitor, [E] is the active enzyme concentration, [I] is the concentration of the inhibitor, and $\nu$ is the steady-state rate in the presence of inhibitor (30, 31).

For mutants other than those involved in phosphate charge mimicry and metal ion bridging, estimates of $K_{\text{app}}$ were calculated according to Equation 2 (30) by determining the rate of supercoiled plasmid DNA cleavage by NucA (5 nm) in the absence ($\nu_0$) and presence ($\nu$) of inhibitor (25 nm).

$$ K = \frac{[I] - [E](1 - \nu/\nu_0)}{(\nu/\nu - 1)} $$

(Eq. 2)

All DNA cleavage reactions were analyzed by electrophoresis on 0.8% agarose gels in Tris borate-EDTA buffer followed by ethidium bromide staining.

**Crystallization and Data Collection**—The NucA-NuiA complex purified by gel filtration chromatography was concentrated to 9 mg/ml and exchanged into 25 mM Tris, pH 7.5, and 17–21% PEG 6000. The crystals were transferred to 100 mM MES, pH 5.5, 100 mM NaCl, 2 mM dithiothreitol buffer. Crystals of the protein complex 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 5.5, and 17–21% PEG 6000. The crystals were transferred to 100 mM MES, pH 5.5, 100 mM NaCl, and 20% PEG 6000 buffer and soaked in 100 mM MES, pH 5.5, 100 mM NaCl, 20% PEG 6000, and 20% ethylene glycol as cryoprotectant.

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. A lower resolution data set was collected at 2.3 Å using a Rigaku 007HF x-ray generator equipped with a Saturn 92 detector. A higher resolution data set was then collected at 2.3 Å at the Southeast Regional Collaborative Access Team (SER-CAT) 22-ID beamline at the Advanced Photon Source, Argonne National Laboratory. Crystals of the NucA-NuiA complex belong to space group $P_{4}_{1}2_{1}2_{1}$.
Structure of NucA-NuiA Complex

and contain one molecule each of NucA and NuiA in the asymmetric unit.

Structure Determination and Refinement—The crystal structure of NucA (PDB ID code 1ZM8 (22)) was used as the model for molecular replacement using the 2.9-Å resolution data set.

### TABLE 1
Crystallographic data table for the NucA-NuiA complex

| Crystallographic data statistics | Data set       | NucA-NuiA                  | Unit cell       | a = b = 87.23 c = 138.98 |
|----------------------------------|---------------|---------------------------|-----------------|--------------------------|
| Space group                      | P4_1 n22      |                           | Resolution (Å)  | 50-2.3                   |
| No. of observations              | 283,171       |                           | Unique reflections | 24,604                  |
| Rsym (%) (last shell)            | 10.0 (57.3)   |                           | I/|/ (last shell)    | 9.4 (3.0)                |
| Mosaicity                        | 0.23–0.77     |                           | Completeness (%) (last shell) | 99.7 (97.5)            |

Refinement statistics

Rcryst (%)b | 20.1 |
| Rfree (%)c  | 24.1 |
| No. of waters | 229  |
| Mean B value (Å) | 46.9 |

r.m.s.d. from ideal values

Bond length (Å) | 0.008 |
| Bond angle (°) | 1.3   |
| Dihedral angle (°) | 24.0 |
| Improper angle (°) | 0.82 |

Ramachandran statistics

| Residues in favored (98%) regions (%) | 95.8 |
| Residues in allowed (>99.8%) regions (%) | 100 |

| a Rsym = Σ(I(I−<I>))/Σ<II> | 43212 |
| Rcryst = Σ|Fobs|−|Fcalc|||Σ|Fcalc| where Fobs is the intensity of the observation and |Fc|| is the mean intensity of the reflection. |
| Rfree = Σ|Fobs|−|Fc|||Σ|Fc| calculated from working data set. |
| r.m.s.d. was calculated from 5% of data randomly chosen not to be included in refinement. |
| r.m.s.d., root mean square deviation. |

RESULTS

Crystal Structure of the NucA-NuiA Complex—The crystal structure of the NucA-NuiA complex was determined at 2.3-Å resolution using recombinant NucA and NuiA expressed in *E. coli* (Table 1). A ribbon model representing the secondary structure of the complex is shown in Fig. 1A. As seen in the previously determined structure of NucA (22), the secondary structure is composed of 13 α-helices and two β-sheets. The root mean square deviation between the complexed and free NucA structure (Fig. 1B) is 0.34 Å for all Ca atoms. A divalent metal ion is located in the active site of NucA, and two additional divalent metal ions were observed in the secondary metal ion-binding site previously identified in the NucA structure (22). The coordination geometry of the active site metal ion is consistent with Mg^{2+}, the endogenous catalytic metal. Alternatively, the identities of the two metal ions at the secondary site were found to be most consistent with Ni^{2+} ions. These were presumably acquired or exchanged into the NucA molecule as a result of passage through the Ni^{2+}-NTA column (38). Consistent with the previously determined solution structure (29), NuiA (Fig. 1B) consists of four helices and a central six-stranded β-sheet arranged as an αβα sandwich. Helices A, C, and D are positioned on one side of the central β-sheet, and the distorted helix B is positioned on the other side. The three central strands of the β-sheet are located at the C terminus of the protein, and all six of the strands are antiparallel. Omitting the poorly aligned region at the end of helix 4 of NuiA (residues 71–90), the root mean square deviation for the backbone atoms of the NuiA structure determined in solution relative to that in the crystallized NucA-NuiA complex was 3.46 Å. We note that a more

![FIGURE 1. Overall fold of the NucA-NuiA complex.](image-url)
recent structural calculation using the same data set in combination with the program CYANA (39, 40) resulted in much closer agreement, with the root mean square deviation for the full structure falling to 3.1 Å.  

In the previously determined structure of NucA, the secondary metal ion-binding site was found to constitute a lattice contact, with two carboxyl oxygen atoms of a Glu-136 residue on a symmetry-related NucA molecule making contact with both metal ions in the secondary metal ion-binding site (22). In the structure of the NucA-NuiA complex, there is also a lattice contact involving the secondary metal ion-binding site on NucA and the Asp-87 residue of a second, symmetry-related NuiA molecule.

The NucA-NuiA Interface—The structure of NuiA can be described as an “open jaw” biting into one side of the NucA molecule in the complex (Fig. 1, C and D). The Thr-135 and Glu-24 residues of the upper jaw enter the NucA active site, whereas Asp-75 and Trp-76 residues of the lower jaw engulf a protruding section of the nuclease that includes a long loop of NucA running from Arg-93 to the beginning of β-strand 4 (Arg-122) (Fig. 2). Close contact is also made with several residues on coaxial helices H (Thr-151, Asn-155, and I (Thr-158–Gln-172) of NucA, including Asn-155 and Glu-163 of NucA (Fig. 2, B and C). The major hydrogen-bonding and salt bridge interactions between NucA and NuiA are summarized in Table 2 and illustrated in Fig. 2A. These include salt bridge interactions between Arg-93 and Glu-24 of NuiA and the C-terminal Thr-135 of NucA carboxyl oxygen, and Lys-101 of NucA and Asp-75 of NuiA in addition to a network of direct and water-mediated hydrogen-bonding interactions. The solvent-accessible surface areas calculated individually are 10,200 Å² for NucA and 7488 Å² for NuiA. After complex formation, the buried solvent-accessible surface area at the interface is calculated to be 1391 Å².

The substantial electrostatic contribution to the NucA-NuiA interaction is illustrated by the GRASP-generated surfaces in Fig. 3. To reveal the electrostatic potential of the interface, NucA is represented by its electrostatic surface, whereas NuiA is represented by a ribbon diagram (Fig. 3A). The representation is reversed in Fig. 3B. The electrostatic representations in Fig. 3 are consistent with the entries in Table 2, which include one acidic and five basic residues for NucA and one basic and seven acidic residues for NuiA. This electrostatic pattern is consistent with the proposal that, to a significant extent, NuiA binds to NucA as a substrate mimic. The principal hydrophobic contributions to the interface include Phe-97, Glu-24, Pro-99, Trp-76, and several other side chain interactions. However, there is no concentration of hydrophobic residues in the interface. The structure of the complex also contains two bound MES molecules, one of which contacts both NucA and NuiA. Its primary contacts include Glu-92 and Arg-106 of NuiA.

Effect of NuiA Interface Mutations—A previous deletion mutant of NuiA demonstrated the importance of the C terminus for tight binding to NucA (21). Our structural analysis confirms a critical role for Thr-135 of NuiA in NucA binding and identifies this residue as being involved in a novel metal ion bridge as part of the NucA-NuiA interface between the C-terminal end of NuiA and the NucA active site. Deletion of the C-terminal residue of NuiA (NuiA-Δ135) resulted in a 600-fold increase in the inhibition constant (Ki) relative to the value for the wild type inhibitor (Table 3). This result indicates the significance of metal ion bridging for tight binding of NuiA to NucA. In addition, our structural analysis indicates that Glu-24 of NuiA is inserted into the active site of NucA, apparently mimicking the negative charge of the scissile phosphate. To obtain quantitative data on the contribution of this residue to the inhibition of NucA by NuiA we generated three mutants of the inhibitor with a conservative (Asp), an isosteric (Gln), and a nonconservative (Ala) amino acid substitution at this position and determined the Ki values for these variants (Table 3). The Ki values obtained show the greatest increase (about 4 orders of magnitude) for the nonconservative and the isosteric substitutions (variants E24A and E24Q), whereas a wild type-like Ki was determined for variant E24D. Thus, a conservative amino acid exchange that preserves a negative charge at this position produces only a 3-fold increase in the inhibition constant (Table 3).

Based on initial NMR (28) and subsequent crystallographic data indicating the involvement of residues in the loop immediately preceding helix D of NuiA in the NucA-NuiA interface, we investigated the contributions of this second interaction site by mutating residues Gln-74 and Asp-75 of NuiA and Thr-135 of NucA. Replacement of Asp-75 with aspartic acid or glutamic acid resulted in dramatically increased Ki values (Table 3), consistent with an important contribution of the Asp-75–Lys-101 salt bridge to the stability of the complex. The W76A mutation, which would be expected to reduce the hydrophobic interaction surface with NucA residues Pro-99 and Thr-111, produced a weaker effect, whereas the Q74A mutation produced the smallest change in Ki (Table 3). Interestingly, the double mutant combining amino acid replacements Q74A and W76A was found to result in a significantly lower loss of inhibitory potency. The greater effect of the Q74A/W76A double mutant may result from an additional structural destabilization of this region of NuiA that also interferes with the Asp-75–Lys-101 salt bridge.

**Active Site of NucA in the Complex**—The structure of the NucA active site observed in the NucA-NuiA complex is essentially identical to that previously determined for isolated NucA (Fig. 2C, 22). It is characterized by similar divalent metal ion coordination geometry and hydrogen bonding network. The only significant conformational changes within the NucA molecule upon binding NuiA to the active site were the rearrangements of the side chains of residues Arg-93 and Asp-95 of NucA. The catalytic divalent metal ion coordinates with both Asn-155 of NucA and with the OG1 atom of the C-terminal Thr-135 of NuiA (Fig. 2B), as well as with four water molecules. The Thr-135 OG1 ligand thus substitutes for the coordinated sulfate oxygen ligand that was present in the structure of uncomplexed NucA determined previously and has been suggested to be the 5’-phosphate-binding site of the substrate (Fig. 2C) (22).
FIGURE 2. Interface and active site of the NucA-NuiA complex. A, stereo view of the interface residues involved in salt bridges, hydrogen bonds, or stacking interactions in the NucA (lavender)-NuiA (dark green) complex. The residues shown correspond explicitly to some of those identified as contributing to the interface as summarized in Table 2. NuiA residue Glu-26$_{\text{Nui}}$ which contacts NucA residue Gly-117$_{\text{Nuc}}$ is labeled in this view rather than Glu-24$_{\text{Nui}}$, which inserts into the active site. B, stereo view of the active site of NucA-NuiA complex showing the active site magnesium ion (green) and the six coordinating ligands (connected by orange lines): Asn-155$_{\text{Nuc}}$ (lavender), four water molecules (W1–W4), and the Thr-135$_{\text{Nui}}$ residue of NuiA (dark green). C, stereo view of the active site residues of NucA-NuiA complex (lavender and dark green) superimposed with NucA (cyan) and including their respective divalent metal ions, Mg$^{2+}$ in the NucA-NuiA complex and Mn$^{2+}$ in uncomplexed NucA. The OE1 and OE2 atoms of Glu-24$_{\text{Nui}}$ and OG1 atom of Thr-135$_{\text{Nui}}$ (dark green) occupy positions that approximate three of the oxygen atoms of the sulfate ion observed in uncomplexed NucA. The navy dotted lines represent the network of hydrogen bonds in all panels.
catalytically important Arg-93\textsubscript{Nuc} residue, which is positioned by an extensive secondary hydrogen bonding network, now forms salt bridges with the Thr-135\textsubscript{Nui} terminal carboxylate and with Glu-24\textsubscript{Nui} OE2. The other important active site residue of NucA, Asn-155\textsubscript{Nuc}, which is the only NucA residue directly coordinating the active site Mg\textsuperscript{2+}, also forms a hydrogen bond with the Glu-24\textsubscript{Nui} OE1 in the NucA-NuiA complex. The W1 water molecule, which is coordinated to the catalytic metal and previously has been suggested to function as the catalytic nucleophile required to break the phosphodiester bond, forms hydrogen bonds with Glu-24\textsubscript{Nui} and Thr-135\textsubscript{Nui}. In summary, Glu-24\textsubscript{Nui} and Thr-135\textsubscript{Nui} apparently play a significant role in forming the inhibitory complex by interacting with critical active site residues.

Other active site residues, particularly His-124\textsubscript{Nuc} and Glu-163\textsubscript{Nuc}, did not make direct contact with NuiA. Replacement of the mutated Ala-121\textsubscript{Nuc} residue with a modeled Asp-121 residue increased the computed NucA-NuiA interface area, indicating that this residue may also contribute to complex formation but is apparently not an absolute requirement. The side chain of Asp-121 was modeled by adding the preferred rotamer. In this position there are no steric conflicts, and the OD1 atom is 3.3 Å from OG1 of Ser-25\textsubscript{Nui}, 3.7 Å from NE of Arg-156\textsubscript{Nuc}, and 3.8 Å from ND2 of Asn-155\textsubscript{Nuc}. Thus, an Asp-121\textsubscript{Nuc}–Ser-25\textsubscript{Nui} interaction may also contribute to the stability of the complex.

DISCUSSION

The structure of the NucA-NuiA complex presented here is the first example of an inhibitor complex for a DNA/RNA-nonspecific nuclease. In the present structure, the C-terminal threonine residue of NuiA inserts directly into the active site, binds to the catalytic metal ion, and functionally replaces a coordinated sulfate molecule previously observed in the structure of uncomplexed NucA (22). As discussed previously, this sulfate ion appeared to mimic the 5′-phosphate group of the cleaved product. The structure of the NucA-NuiA complex is, to the best of our knowledge, the first example in which the catalytic divalent metal ion interacts directly with both a nucleophile and an inhibitor protein. In addition, one of the Glu-24\textsubscript{Nui} OG oxygen atoms of NuiA occupies a position close to that of a second sulfate oxygen in the uncomplexed NucA structure (22). Thus, the Glu-24\textsubscript{Nui} side chain presumably mimics the charge of a DNA phosphate oxygen. The quantitative importance of the Glu-24\textsubscript{NuiA} and Thr-135\textsubscript{NuiA} residues to the inhibitory potency of NuiA is indicated by mutational analyses demonstrating that the \(K_i\) value for the E24A mutant is increased by a factor of \(10^3\), and the value for the \(\Delta\)Thr-135\textsubscript{Nui} mutant is increased by \(\sim 600\) (Table 3).

Comparison of DNA and NuiA Binding by NucA—We previously obtained a useful model for the complex formed with cleaved or uncleaved DNA by superposition of our NucA structure (22) with the reported structures for DNA-complexed \(V\textit{vn}\) nuclease (1OUP) (41). This superposition places the DNA chain in a reasonable position relative to the catalytic groups of NucA. The 5′-terminal phosphate group of a hydrolyzed DNA substrate was found to superpose closely with the sulfate anion observed in the NucA structure (22). The superposition shown in Fig. 4 compares the NucA-DNA complex modeled as described previously with the NucA-NuiA complex obtained in the present study. Based on this comparison, oxygen atoms of Glu-24\textsubscript{Nui} and Thr-135\textsubscript{Nui} in NuiA occupy the position of the scissile phosphate of the \(V\textit{vn}\) DNA product complex. Note in particular the similar relative positions of the 5′-phosphate

![FIGURE 3. Electrostatic surfaces of NucA and NuiA in complex.](image-url) Electrostatic surface rendering of: NucA (A) and NuiA (B) generated using the GRASP software program. The protein-protein interface is revealed by using a coiled (yellow) representation of NuiA (A) and NucA (B), respectively.
from the DNA in Fig. 4 and the sulfate from the superposition shown in Fig. 2C. Additionally, the Thr-135Nui carboxyl group appears to be positioned near a bridging phosphate from the 3'-terminus of the cleaved DNA. The carbonyl oxygen of Glu-24Nui is also positioned fairly close to an oxygen atom in a bridging phosphate group at the 5'-side of the cleaved DNA. Thus, there is a more general mimicry of cleaved DNA by the NuiA inhibitor.

Comparison with Other Nuclease-Inhibitor Complexes—Although nucleases have often been suggested as drug targets (8, 42, 43), there is considerably less information on nuclease-inhibitor complexes than is available for proteinase-inhibitor complexes. Comparisons among these diverse structures reveal limited homologies. In general, the active sites of the nucleases are matched to the hydrophilic properties of the nucleic acid substrates so that inhibitors that mimic the substrates will exhibit analogous hydrophilic interactions.

The ribonuclease A-inhibitor complex studied by Kobe and Deisenhofer (26) is characterized by a largely hydrophilic inter-

**TABLE 3**

| Inhibitor  | $K_{\text{(app)}}$ [M] | Rel. $K_{\text{(app)}}$ |
|------------|------------------------|-------------------------|
| Wild type  | $3.2 \times 10^{-12}$ ($\pm 1.9$)* | 1                       |
| E24A       | $3.3 \times 10^{-8}$ ($\pm 2.1$)* | 10,300                  |
| E24D       | $8.8 \times 10^{-12}$ ($\pm 4.2$)* | 3                       |
| E24Q       | $2.9 \times 10^{-8}$ ($\pm 0.3$)* | 9,100                   |
| ?T135      | $1.9 \times 10^{-8}$ ($\pm 1.4$)* | 590                     |
| Q74A       | $7.5 \times 10^{-10}$ ($\pm 4.9$)* | 230                     |
| D75E       | $3.1 \times 10^{-8}$ ($\pm 2.0$)* | 9,700                   |
| D75N       | $6.8 \times 10^{-8}$ ($\pm 4.5$)* | 21,250                  |
| W76A       | $3.1 \times 10^{-8}$ ($\pm 0.2$)* | 970                     |
| Q74A/W76A  | $3.3 \times 10^{-8}$ ($\pm 4.2$)* | 16,600                  |

* $K_{\text{app}}$ values were determined using a plasmid cleavage assay by varying inhibitor concentrations in the presence of 5 pmol NucA and fitting the data to Equation 1 (see "Materials and Methods"). The $K_i$ values given are mean values of at least three independent experimental determinations.

**FIGURE 4. Modeled DNA interaction positioned in the NuiA-NucA complex.** The extent to which NuiA mimics the substrate is illustrated by this stereo view of a docked model of a cleaved DNA octamer in the active site of NucA. Selected residues from NucA and NuiA are indicated in *lavender* and *green*, respectively. The model is based on the alignment of active site residues in the NucA structure (Arg-122–Ile-125 and Met-147–Arg-150) with active site residues in the structure of *Vnu* in complex with DNA (Trp-78–Val-81 and Leu-119–Gly-128; PDB ID code 10UP). The coordination of the active site Mg$^{2+}$ (green) to four water molecules as well as to Asn-155Nui (lavender) and to a phosphate oxygen of the cleaved DNA strand (yellow) is indicated. The complementary strand of the DNA is shown in *blue*.

face, which relies primarily on electrostatic interactions. Although the inhibitor occupies most of the active site, it only partially mimics the RNase-RNA interaction and does not utilize the p1 phosphate-binding pocket of ribonuclease A, where a sulfate ion remains bound. In this example, the inhibitor forms a large, concave surface, which surrounds the ribonuclease so that a large contact area compensates for more modest shape complementarity (26). The barnase-barstar complex is the most extensively studied example of nuclease inhibition. Similar to the NucA-NuiA interface, the barstar-barnase interaction also relies primarily on electrostatic interactions (44). Barnase is base-selective, hydrolyzing at the 3'-end of guanosine in RNA, and also lacks a catalytic metal ion. Although NuiA mimics DNA using residues located at the edge of the central β-sheet, barstar inserts helix α2 into the active site of barnase (Fig. 5A) so that most of the interactions involve residues on and immediately preceding α2. Asp-39 on this helix mimics the charge of the scissile phosphate group, interacting with barnase residues Arg-83 and Arg-87. Barstar residues Thr-42 and Gly-43 at the end of helix α2 form hydrogen bonds with barnase residues Lys-27 and Arg-83, respectively (Fig. 5B). Barnase residue Arg-59, which forms a salt bridge with barnase Glu-76, is repositioned to stack against the G2 base in the nucleotide complex. Base A3, which stacks against barnase residue His-102, is replaced by barstar residue Tyr-29 in the inhibitor complex (Fig. 5B). Other significant actions that characterize the predominantly electrostatic barnase-barstar interface are summarized in Buckle et al. (Ref. 44, Table 2 therein).

Another excellent example of molecular mimicry by an inhibitor interacting with an enzyme acting on DNA is provided by the uracil DNA glycosylase-inhibitor complex, UDG-Ugi (45). The interacting surfaces display near perfect electrostatic and shape complementarity. A negatively charged ridge of the Ugi β-sheet binds to the positively charged DNA-binding site of UDG, thereby preventing access of the DNA substrate to the enzyme. Mimicry of the phosphate backbone of DNA has been discovered in a range of other proteins interacting with DNA (46).

The *E. coli* colicin DNase has been classified as a member of the ββα Me superfAMILY so that its inhibition by the *E. coli* immunity proteins might be considered to more closely approximate the mode of inhibition of NucA by NuiA observed here. However, in sharp contrast with the barnase and ribonuclease A-inhibitor complexes that involve direct insertion of the inhibitor protein into the active site, inactivation of the colicin DNase domains by the immunity proteins is more indirect, blocking critical binding interactions without directly filling the active site (47).

Unlike barstar, which uses interactions from an α-helix to mimic
residue Tyr-29 in the inhibitor complex. Base A3, which stacks against barnase residue His-102, is replaced by barstar between two proteins has recently been reported (48). In con-
inhibitor complexes, a copper-mediated binding interaction does not appear to have been reported for other nuclease-
in the active site, interacting with barnase residues Arg-87 and Arg-83. Bar-
the interacting residues of barnase (Ref. 44 (see Table 2 therein) are explicitly shown. This figure shows in partic-
the DNA binding, the unique feature of the structure of the NucA-NuiA complex appears to be the insertion of the C-terminal Thr-135Nui from the end of a β-sheet into the active site. This complexation effectively mimics the interaction of the DNA with the catalytic metal ion. Although this type of interaction does not appear to have been reported for other nuclease-inhibitor complexes, a copper-mediated binding interaction between two proteins has recently been reported (48). In con-
contrast to the NucA-NuiA complex, the Cu(I) ion mediates the reversible formation of a weak complex involving the copper chaperone Atx1 and the copper-binding domain of the Ccc2 ATPase.

The Second Metal-binding Site—As noted previously, NucA contains a second metal ion-binding site remote from the active site, which is capable of binding two divalent metal ions (22). Based on the structural parameters observed for this site, it appears to have picked up two Ni\textsuperscript{2+} ions from the nickel column used to purify the enzyme. In the previous study of NucA, this site contained either two Zn\textsuperscript{2+} ions, when the enzyme was crystallized in the presence of 10 mM ZnCl\textsubscript{2}, or two Mn\textsuperscript{2+} ions, after incubation of the enzyme with MnCl\textsubscript{2}. Interestingly, in both the previous structure of NucA as well as the present NucA-NuiA complex, the second metal-binding site is positioned at a lattice contact, although with a different molecule in each case. In both cases, a carboxylate ligand from a symmetry-related molecule interacts such that each carboxylate oxygen atom binds to a different metal ion. Although the role of the second divalent ion-binding site is unknown, its ability to support intermolecular complex formation suggests that metals may play a role in regulating NucA interactions with other proteins, possibly by anchoring NucA to the periplasm. Given that, as noted previously, this second metal site does not appear to be shared by other nucleases in the ββαα Me nuclease family, it may represent a unique mechanism for cellular localization of NucA.

Comparison with Chemical Shift Mapping Results—The identification of the NuiA interface on the basis of the crystal structure of the NucA-NuiA complex is in reasonable agree-
ment with the previous identification using chemical shift mapping (29). In that study, the NuiA residues Leu-20\textsubscript{Nui}, Met-22\textsubscript{Nui}, Ser-68\textsubscript{Nui}, Gln-74\textsubscript{Nui}, Trp-76\textsubscript{Nui}, Leu-107\textsubscript{Nui}, Gly-108\textsubscript{Nui}, Glu-109\textsubscript{Nui}, Val-133\textsubscript{Nui} and Glu-134\textsubscript{Nui} were identified as being near the interface on the basis of the amide shift differ-
ces observed between uncomplexed and NucA-complexed NuiA, although the NuiA resonances corresponding to the complex were not fully assigned (29). Examination of the structure of the NucA-NuiA complex indicates that most of these residues are positioned in or near the interface. In several instances, residues adjacent to interacting residues are identi-
fied, probably because of the use of the amide shift as the reporter group rather than the resonances of the interacting side chain. For example, the Gln-74\textsubscript{Nui} and Trp-76\textsubscript{Nui} amides were identified, whereas the Asp-75\textsubscript{Nui} side chain carboxyl forms a salt bridge with NucA. Similarly, the amides of Val-133\textsubscript{Nui} and Glu-134\textsubscript{Nui} were identified as part of the interface, although Thr-135\textsubscript{Nui} interacts most directly with the active site. Despite these limitations, the chemical shift mapping approach appears to have done a reasonable job of identifying the NuiA interface.

The structure of the NucA-NuiA complex presented here provides unique insights into the basis for inhibition of sugar nonspecific nucleases of the ββαα Me nuclease superfamily. Despite the significant cellular toxicity that expression of this class of enzymes can pose, little is known about how their activ-
ity is controlled; the nuclease-inhibitor complex reported here defines a structural basis for achieving such control. The struc-

FIGURE 5. Structural comparison of barnase-barstar complex. A, ribbon diagram showing the crystal structure of the barnase-barstar complex in which helix α2 of barstar (navy) is inserted into the active site of barnase (brick red). The active site nucleotide from the structure of a barnase-d(CGAC) complex (PDB ID code 1BRN, shown in light green) is superimposed to illustrate the basis for barstar inhibition. B, active site region of the barnase-barstar complex (PDB ID code 1BGX) overlaid with two nucleotides, G2 and A3, from the barnase-d(CGAC) complex (PDB ID code 1BRN, shown in light green). Several of the interacting residues of barnase (brick red) and barstar (navy) identified in Ref. 44 (see Table 2 therein) are explicitly shown. This figure shows in partic-
ular how barstar residue Asp-39 mimics the scissile phosphate. Several of the interacting residues are positioned in or near the interface. In several instances, residues adjacent to interacting residues are identi-
fied, probably because of the use of the amide shift as the reporter group rather than the resonances of the interacting side chain. For example, the Gln-74\textsubscript{Nui} and Trp-76\textsubscript{Nui} amides were identified, whereas the Asp-75\textsubscript{Nui} side chain carboxyl forms a salt bridge with NucA. Similarly, the amides of Val-133\textsubscript{Nui} and Glu-134\textsubscript{Nui} were identified as part of the interface, although Thr-135\textsubscript{Nui} interacts most directly with the active site. Despite these limitations, the chemical shift mapping approach appears to have done a reasonable job of identifying the NuiA interface.

The structure of the NucA-NuiA complex presented here provides unique insights into the basis for inhibition of sugar nonspecific nucleases of the ββαα Me nuclease superfamily. Despite the significant cellular toxicity that expression of this class of enzymes can pose, little is known about how their activ-
ity is controlled; the nuclease-inhibitor complex reported here defines a structural basis for achieving such control. The struc-
tecture reported here differs dramatically from previously determined other nuclease-inhibitor complexes in the Protein Data Bank. Based on the recently discovered role of similar nucleases as mediators of infectivity, resulting from their ability to destroy the DNA scaffold of neutrophil extracellular traps, these nucleases have emerged as potential drug targets (6–8). Hence, an understanding of the structural basis for the inhibition of these enzymes will be of increasing importance for the development of strategies to deal with infective microorganisms.

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