Mapping the Ribonucleolytic Active Site of Eosinophil-derived Neurotoxin (EDN)

HIGH RESOLUTION CRYSTAL STRUCTURES OF EDN COMPLEXES WITH ADENYLIC NUCLEOTIDE INHIBITORS

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Eosinophil-derived neurotoxin (EDN), a basic ribonuclease found in the large specific granules of eosinophils, belongs to the pancreatic RNase A family. Although its physiological function is still unclear, it has been shown that EDN is a neurotoxin capable of inducing the Gordon phenomenon in rabbits. EDN is also a potent helminthotoxin and can mediate antiviral activity of eosinophils against isolated virions of the respiratory syncytial virus. EDN is a catalytically efficient RNase sharing similar substrate specificity with pancreatic RNase A with its ribonucleolytic activity being absolutely essential for its neurotoxic, helminthotoxic, and antiviral activities. The crystal structure of recombinant human EDN in the unliganded form has been determined previously (Mosimann, S. C., Newton, D. L., Youle, R. J., and James, M. N. G. (1996) J. Mol. Biol. 260, 540–552). We have now determined high resolution (1.8 Å) crystal structures for EDN in complex with adenosine-3’,5’-diphosphate (3’,5’-ADP), adenosine-2’,5’-diphosphate (2’,5’-ADP), adenosine-5’-diphosphate (5’-ADP) as well as for a native structure in the presence of sulfate refined at 1.6 Å. The inhibition constant of these mononucleotides for EDN has been determined. The structures present the first detailed picture of differences between EDN and RNase A in substrate recognition at the ribonucleolytic active site. They also provide a starting point for the design of tight-binding inhibitors, which may be used to restrain the RNase activity of EDN.

The eosinophil-derived neurotoxin (EDN)† is an eosinophil protein stored in the matrix of the large secretory granules (1). It is a small, basic protein (2) that belongs to the pancreatic ribonuclease A (RNase A; EC 3.1.27.5) superfamily (3) and is also known as RNase-2 or RNase U. EDN was initially identified by its ability to induce the Gordon phenomenon (muscle stiffness, ataxia, incoordination, and spasmodic paralysis) when injected into rabbits (4, 5). Its neurotoxic effect is achieved through a selective killing of cerebellar Purkinje cells (6). The protein also displays cytotoxicity against helminths, single-stranded RNA viruses, and respiratory epithelial cells; and as a role as a host defense protein has been suggested (7). Damage of host tissues by EDN could contribute to the secondary effects associated with inflammatory disorders and hypereosinophilic syndromes (8).

EDN shares 36% amino acid identity with RNase A and 67% identity with a related eosinophil RNase, eosinophil cationic protein (ECP, also known as RNase-3) (7). EDN’s enzymatic activity is essential for its neurotoxic, helminthotoxic, and antiviral activities (9–11) and is 3- to 30-fold lower than that of RNase A, depending on the substrate used (9, 12).

The crystal structure of recombinant EDN in complex with sulfate has been determined previously at 1.83-Å resolution (13). The topology of the EDN molecule includes the RNase A fold and core ribonucleolytic active site architecture (14), which is conserved among all these molecules, although both ECP (15) and EDN exhibit significant differences at the peripheral substrate-binding sites (16).

The core of the catalytic site of RNase A consists of subsites B1, P1, and B2. These subsites accommodate the phosphate where phosphodiester bond cleavage occurs (P2) and the nucleotide bases on the 3’ and 5’ sides of the scissile bond (B1 and B2, respectively) (17). In addition, several studies (18–20) have identified additional sites, including P3 and P5. P3 interacts with the 5’-phosphate of a nucleotide base bound at B1 and P2 interacts with the 3’-phosphate of a nucleotide base bound at B2 (for recent reviews see Refs. 20–22). The three main catalytic residues of RNase A (His-12, Lys-41, and His-119 of the P1 subsite) are present in EDN as His-15, Lys-38, and His-129. The key B1 residues, Thr-45 and Phe-120 in RNase A, are conserved in EDN while the other components of this substrate differ. The B2 subsite is partially conserved between EDN and RNase A, but subsites P0 and P2

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† The atomic coordinates and structure factors (code 1HI2, 1HI3, 1HI4, and 1HI5) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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The abbreviations used are: EDN, eosinophil-derived neurotoxin; RNase A, bovine pancreatic ribonuclease A; ECP, eosinophil cationic protein; UpA, uridylyl-3’,5’-adenosine; 3’,5’-ADP, adenosine-3’,5’-diphosphate; 2’,5’-ADP, adenosine-2’,5’-diphosphate; 5’-ADP, adenosine-5’-diphosphate; d(CpA), deoxycytidyl-3’,5’-deoxyadenosine; 2’,5’-CpA, cytidyl-2’,5’-adenosine; d(Up), 2’,5’-deoxyuridyl-3’-phosphate; ppA-3’,5’-diphosphoadenosine 3’-phosphate; r.m.s., root mean square.

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are not. Although EDN and RNase A bind only pyrimidines at B1 and prefer purines at B2, differences in B1 and B2 site structures give rise to subtle changes in substrate specificity. With polynucleotide substrates, EDN has a 20-fold preference for cytidine over uridine; with dinucleotide substrates, EDN has a 2-fold preference for cytidine at B1 and a 100-fold preference for adenosine at B2 (9). Two sulfate anions were found in the EDN-sulfate complex structure (13) occupying two distinct subsites. One of these is subsite P1, whereas the other is a site not identified in the structure of RNase A, suggested to correspond to a new P-1 phosphate-binding subsite.

The biological properties attributed to EDN have been related to its ribonucleolytic activity (7). The analysis of its substrate specificity and the identification of the residues involved in substrate interaction would help in understanding its mechanism of action. In addition, the identification of nucleotide-based inhibitors may lead to therapeutic agents for use against the pathological conditions associated with eosinophil RNases. Here we present the first structures of recombinant EDN-nucleotide complexes (at 1.8 Å resolution) and make a detailed comparison with the EDN-sulfate structure at higher resolution (1.6 Å) than the previous reported structure (13). The structures of the complexes have revealed a detailed picture of critical residues involved in the P1 and B2 substrate-binding sites and their flexibility in interaction with different adenylic nucleotides. The analysis of the sulfate-containing structure and comparison with the nucleotide complexes also confirm the presence of a previously suggested P-1 subsite for EDN (13). In addition, kinetic results suggest that these nucleotides can serve as a starting point toward the design of potent inhibitors of EDN.

EXPERIMENTAL PROCEDURES

Materials—Uridylyl-3',5'-adenosine (UpA) was from ICN Biochemicals. Adenosine-5'-diphosphate (5'-ADP), adenosine-2',5'-diphosphate (2',5'-ADP) and adenosine-3',5'-diphosphate (3',5'-ADP) were from Sigma Chemical Co.

Protein Purification and Crystallization—Recombinant EDN was expressed in Escherichia coli and purified as described previously (13, 23). Briefly, a synthetic gene for human EDN was cloned into the pET1c expression vector, and the protein was purified by inclusion bodies. The recombinant EDN, which contains an additional methionine residue at the N terminus, has the same specific activity as the protein purified from the natural host (23) and does not have the post-translational modifications that are present in EDN isolated from human body fluids.

Crystals of recombinant EDN were grown using the hanging drop/vapor diffusion method from drops containing 9 mg/ml protein in 0.1 M sodium cacodylate buffer (pH 6.5), 0.75 M ammonium sulfate, and 2.5% ethanol. Drops were equilibrated against reservoirs containing 0.1 M MES NaOH, pH 6.5, at 25 °C using 0.5-cm path length cells. UpA was used as a substrate. Substrate and inhibitor concentrations were determined spectrophotometrically using the following extinction coefficients: ε295 = 23,500 M⁻¹ cm⁻¹ for UpA (31) and ε295 = 15,400 M⁻¹ cm⁻¹ for 5'-ADP, 2',5'-ADP, and 3',5'-ADP (32). The activity was measured by following the initial reaction velocities using the difference molar absorbance coefficient δε = 570 M⁻¹ cm⁻¹ for the transphosphorylation reaction of UpA (33). Kᵢ was determined by the Dixon method (34) using three different substrate concentrations from 0.15 to 0.4 μM of UpA, and eight inhibitor concentrations from 5 to 400 μM; UpA, where v₀ is the initial velocity, was plotted against the inhibitor concentration [I] (data not shown).

Accession Numbers—The final atomic coordinates for the four complexes of EDN (sulfate, 2',5'-ADP, 3',5'-ADP, and 5'-ADP) have been deposited with the RCSB Protein Data Bank (accession codes 1H12, 1H13, 1H4, and 1H15, respectively).

RESULTS

Overall Structures—The structures of EDN detailed here are very similar to the EDN-sulfate structure reported previously (13). The r.m.s. difference between Cα atoms in the two sulfate-bound structures is 0.15 Å. The corresponding values for the EDN-3',5'-ADP, EDN-2',5'-ADP, and EDN-5'-ADP complexes are 0.31, 0.29, and 0.28 Å, respectively. In the present EDN-sulfate structure, the additional methionine (Met-0) residue from the recombinant protein was observed.

In all free RNase A structures reported so far, the side-chain of His-119, which is part of the catalytic triad, was found in two conformations (A and B with χ₁ = 150° and χ₁ = 60°, respectively) (35, 36). Conformation A is compatible with nucleotide binding (37–39) whereas low pH or the presence of magnesium/stratophosphate in the EDN native form favors conformation B (40–42). The side chain of the corresponding EDN residue, His-129, is in conformation B in the EDN-sulfate (χ₁ = 65°), EDN-2',5'-ADP (χ₁ = 74°) complex structures, whereas in the EDN-3',5'-ADP (χ₁ = 152°) complex structure it is in conformation A.

The r.m.s. differences between the Cα atoms of the structures of the present 1.6 Å resolution EDN-sulfate complex and those of the 3',5'-ADP, 2',5'-ADP, and 5'-ADP complexes are 0.29, 0.27, and 0.27 Å, respectively. The corresponding values within the complexes, 3',5'-ADP against 2',5'-ADP and 5'-ADP are 0.27 and 0.31 Å, respectively, whereas the r.m.s. difference...
between the 2',5'-ADP and the 5'-ADP complexes is 0.18 Å. The differences between the four protein structures are very small, concentrated in the loop regions, and seemingly unrelated to the presence of the different inhibitors. The EDN-sulfate structure, determined at 1.6-Å resolution, contains 152 water molecules. The structures of the 3',5'-ADP, 2',5'-ADP, and 5'-ADP complexes are at a slightly lower resolution (1.8 Å) and contain 123, 110, and 132 water molecules, respectively. The residues at the active site are oriented similarly in all four complexes, with the exception of His 129 (noted above), and there are no significant conformational changes due to inhibitor binding. A number scheme and torsion angle definitions are shown in Fig. 1 using 5'-diphosphoadenosine 3'-phosphate (ppA-3'-p) as a reference molecule (38) for the three adenylic mononucleotides.

*The Binding of 3',5'-ADP to EDN*—The 3',5'-ADP molecule is reasonably well defined in the electron density map (Fig. 2A). The conformation of 3',5'-ADP when bound to EDN is very similar to that observed previously for B2-bound adenosine in the complexes of RNase A with d(pApA)2 (43), d(2'-A2TpApA2pG) (44), d(CpA) (37, 45), and 2',5'-CpA (45), as well as those frequently observed in free adenylic nucleotides (46). The glycosyl torsion angle χ adopts the anti-conformation, whereas the ribose is at the C2'-endo conformation. The γ torsion angle is in the unusual sp range (Table II), but its value (−29°) is very close to the highly favorable−sc region (−30° to −90°) (46) and the 5'-phosphate group is oriented similarly toward the adenine. The δ torsion angle, dictated by the orientation of the 3'-phosphate group, is in the +α region as commonly found in both free and protein-bound nucleotides (46).

The inhibitor binds to the P1–B2 region of the catalytic site of EDN in a manner similar to the binding of the analogous parts of d(ApTApApGpC) (44, 45), d(CpA) (37, 45), and 2',5'-CpA (45) to RNase A (Fig. 2B). The molecular surface of EDN calculated using the GRASP program (47) shows how the active site cleft accommodates the 3',5'-ADP nucleotide (Fig. 3A). EDN and 3',5'-ADP engage in seven hydrogen-bond interactions, and three water molecules form hydrogen bonds with the inhibitor (Table III). The 5'-phosphate binds to subsite P1 (the distance between the phosphorous to sulfur of the SO42− (A) of EDN-sulfate complex is 0.3 Å) and is involved in hydrogen bond interactions with the imidazole rings of His-15 and His-129. It also forms hydrogen bonds with the side chain of Gln-14, and the main-chain amide nitrogen of Leu-130 and makes a water-mediated interaction with the side chain of Lys-38 (Fig. 2B). The adenosine binds to the B2 subsite with its five-membered ring being almost parallel to the imidazole ring of His-129 (active conformation A) and thereby involved in ring-stacking interactions. In addition, atoms N1 and N6 of the adenine are located at hydrogen bonding distance from the side-chain atoms of Asn-70. The 3'-phosphate group forms a hydrogen bond with the α-amino group of Met-0 at the N terminus and participates in a water-mediated interaction with the side chain of Trp-10. There are also numerous van der Waals contacts between the inhibitor and Trp-7, Cys-62, Arg-68, Asn-70, Ala-110, Val-128, and His-129. With the exception of residues His-129, a shift in Ala-112 side-chain orientation and a slight movement of Arg-68 (−1.0 Å) from its position in the EDN-sulfate complex toward the inhibitor, there are no other significant conformational changes in the catalytic site of EDN upon 3',5'-ADP binding.

Comparison of the EDN-3',5'-ADP binding mode with the RNase AppA-3'-p complex (38) (PDB code 1AFK) shows equivalent interactions at the main phosphate site P1 and at the

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**TABLE I**

Crystallographic statistics

| Dataset | EDN · sulfate | EDN · 3',5'-ADP | EDN · 2',5'-ADP | EDN · 5'-ADP |
|---------|---------------|-----------------|-----------------|--------------|
| Space group | P212121 | P212121 | P212121 | P212121 |
| Cell dimensions | | | | |
| a (Å) | 53.4 | 52.4 | 52.5 | 52.7 |
| b (Å) | 57.2 | 56.1 | 56.2 | 56.3 |
| c (Å) | 42.2 | 41.4 | 41.5 | 41.8 |
| Resolution (Å) | 40.0–1.6 | 30.0–1.8 | 30.0–1.8 | 30.0–1.8 |
| Number of reflections measured | 131,724 | 11,016 | 11,016 | 11,016 |
| Number of unique reflections | 17,303 | 11,607 | 11,471 | 11,749 |
| Rsym (%)* | 6.5 | 6.0 | 5.9 | 6.4 |
| Completeness | 96.6 | 98.0 | 96.4 | 97.7 |
| (outermost shell) (%)* | (96.5) | (92.1) | (90.6) | (89.9) |
| I/σ (I) | 25.4 | 12.0 | 17.9 | 10.7 |
| Rcryst (%) | 17.7 | 19.8 | 19.3 | 19.3 |
| Rfree (%) | 21.9 | 27.0 | 25.9 | 25.3 |
| Number of protein atoms | 1,089 | 123 | 110 | 132 |
| Number of solvent molecules | 152 | 123 | 110 | 132 |
| r.m.s. deviation in bond lengths (Å) | 0.007 | 0.009 | 0.008 | 0.009 |
| Average B-factor for protein atoms | 13.7 | 17.8 | 14.9 | 15.2 |
| Average B-factor for ligand atoms (Å²) | 34.2/40.9 | 36.3 | 29.5 | 29.1 |

* Rsym = Σh |I(h)−1/2Σh I(h)|, where I(h) and I(h) are the hth and the mean measurements of the intensity of reflection h.
  * Outermost shell is 1.70–1.60 and 1.91–1.80 Å, respectively.
  * Rcryst = Σh |Fcalc|−|Fobs|/Σh Fcalc, where Fcalc and Fobs are the observed and calculated structure factor amplitudes of reflection h, respectively.
  * Rfree is equal to Rcryst for a randomly selected 5% subset of reflections not used in the refinement (27).
  * B-factors for the two sulfate ions.
secondary base site B₂ (Fig. 3B). Both of the 5′-phosphate of 3′,5′-ADP and the 5′-β-phosphate of ppA-3′-p are located at P₁. His-119 in RNase A and His-129 in EDN are both in the same plane and stack against the five-membered ring of adenine. Although the binding of adenine in both EDN-3′,5′-ADP and RNase A-ppA-3′-p is almost coplanar (as observed in RNase A complexes with substrate analogs d(CpA) (37) and d(ApTpApA) (44)), the six- and five-membered rings are reversed. Finally, the 3′-phosphates of ppA-3′-p and 3′,5′-ADP are located at the N-terminal ends of RNase A and EDN, respectively.

**The Binding of 2′,5′-ADP to EDN**—The structure of 2′,5′-ADP is very well defined within the electron density map (Fig. 2C). The conformation of 2′,5′-ADP is typical for protein-bound nucleotides and the deoxyribose takes up the energetically favored C2′-endo anti conformation (Table II) (46). The γ torsion angle around the C4′-C5′ bond of the adenosine is in the +sc range, and the δ torsion angle has a value of 158°. Both these angles are in the range frequently observed in protein-bound nucleotides (46).

Although 2′,5′-ADP binds to the same P₁-B₂ region of the EDN active site as 3′,5′-ADP (Fig. 2D), there are striking differences in their interaction with EDN. Interestingly, in the 2′,5′-ADP-EDN complex, a 2′- rather than the 5′-phosphate occupies the P₁ site. Indeed, the 2′-phosphate group forms a similar extensive set of hydrogen bonds with the side chains of Gln-14, His-15, and His-129 and with the amide nitrogen of Leu-130 (Table IV) as observed for the 5′-phosphate group in the 3′,5′-ADP-EDN complex. However, although the adenine is almost parallel to the imidazole of His-129, the five-membered ring does not stack against the imidazole ring as in the 3′,5′-ADP complex. Instead, the imidazole ring of His-129 adopts conformation B (inactive) and packs against the five-membered ring of adenine in a different orientation (Fig. 2D). There are three water molecules making hydrogen bond interactions with the inhibitor, and one of them mediates interactions between the adenine moiety and the side chain of Asn-70. A shift in orientation of both Arg-68 and Asp-112 is observed when compared with the sulfate bound EDN or the 3′,5′-ADP complex, although these residues are not directly interacting with the adenine. The 5′-phosphate is not involved in any direct interactions with EDN residues and is only involved in a water-mediated interaction with the α-amino group of Met-0 at the N-terminus (Fig. 2D). The binding of 2′,5′-ADP does not trigger any conformational changes at the active site of the EDN molecule and, apart from residues Gln-14, His-15, His-129, and Leu-130, 2′,5′-ADP does not interact directly with any other parts of EDN.

**The Binding of 5′-ADP to EDN**—The nucleotide is very well defined in the electron density map (Fig. 2E). The deoxyribose adopts the C2′-endo anti energetically favored conformation.
(46), and the γ torsion angle is in the \(+sc\) range. The \(\alpha\) and \(\zeta_{ap}\) torsion angles around the phosphoester bond are in the \(+sc\) and \(+ac\) range, respectively. These values are in the accepted range for both free and protein-bound nucleotides (Table II) (46).

5'-ADP is bound at the active site of EDN in an extended conformation with the β-rather than the α-phosphate group at subsite P1, and the adenosine located in a region away from the B3 subsite close to but not exactly at B1 (Fig. 2F). The β-phosphate group engages in a hydrogen-bonding network with the peptide nitrogen of Leu-130 and the side-chain atoms of Gln-14, His-15, and His-129 (found in conformation B). The α-phosphate forms a hydrogen bond with the ε-amino group of Lys-38, whereas the 2'-hydroxyl group of the ribose makes two hydrogen bonds with the main-chain atoms of Gln-40 (Table V). Furthermore, EDN and 5'-ADP participate in an extended water-mediated hydrogen bond network involving seven water molecules, and the residues Trp-7, Gln-14, Arg-36, Asn-39, Gln-40, Val-128, His-129, Leu-130, and Asp-131. In addition, 5'-ADP also has van der Waals interactions with His-82.

Inhibition of EDN by Mononucleotides 3',5'-ADP, 2',5'-ADP, and 5'-ADP—The inhibition constants for these adenylic mononucleotides have been determined spectrophotometrically. The \(K_i\) values for 3',5'-ADP, 2',5'-ADP, and 5'-ADP are \(32 \pm 2, 64 \pm 4\), and \(92 \pm 7 \mu M\), respectively. These results are consistent with the crystallographic analysis of EDN complexes described above, i.e. among the three complexes, 3',5'-ADP binds most avidly to EDN with a maximum number of contacts with the protein atoms. However, when comparing the determined \(K_i\) values with the reported ones in the literature, differences in pH and ionic strength of the assay mixture have to be taken into account. Kinetic assays were performed at pH 6.5, the pH used for the crystallization buffer. Previous kinetic characterization of eosinophil RNases indicated that they have a lower optimum pH for catalytic efficiency in comparison with RNase A. Sorrentino and Libonati (48) reported that of the optimum pH for catalytic efficiency is 7.5–8.0 for RNase A and 6.5–7.0 for EDN. Thus a lower pH optimum for the nucleotide interaction should be expected, because RNase A has an optimum pH of 7.5 for the catalytic constant and of 5.5 for the substrate affinity constant (49). Therefore, lower \(K_i\) values would be expected for the assayed mononucleotides if they are analyzed at a pH lower than 6.5. Moreover, when comparing the reported \(K_i\) with previous kinetic analyses of EDN, it should be considered that the present structural and kinetic characterization has been performed with the recombinant protein, whereas the reported kinetic assays used the natural enzyme (50). Native EDN has several N-glycosylation sites (8) and is also C-mannosylated at Trp-7 (51). The reported inhibition constants of the same adenylic mononucleotides for RNase A are in the range of 1 to 8 \(\mu M\) (52, 53), indicating that these nucleotides have a much higher affinity for RNase A. A 10-fold difference in the \(K_i\) values has also been observed while studying the affinity of dinucleotides for either RNase A or EDN (50), in agreement with kinetic data that show a lower catalytic efficiency for the RNA transphosphorylation reaction with EDN (9).

**Table II**

**Torsion angles for 3',5'-ADP, 2',5'-ADP, and 5'-ADP when bound to EDN**

| Definitions of the torsion angles are according to the current IUPAC-IUB nomenclature. For atom definitions see Fig. 1. |
|---|---|---|
| Backbone torsion angles (degrees) | 3',5'-ADP | 2',5'-ADP | 5'-ADP |
| O5'-C5'-C4'-C3' (γ) | −29 (sp) | 59 (+sc) | 59 (+ac) |
| C5'-C4'-C3'-O3' (δ) | 150 (+ac) | 158 (ap) | 144 (+ac) |
| C5'-C4'-C3'-C2' | −89 | −85 | −97 |
| C4'-C3'-C2'-O2' | −40 | −162 | −100 |

| Glycosyl torsion angle (degrees) | 3',5'-ADP | 2',5'-ADP | 5'-ADP |
| Glycose O4'-C1'-N9A-C4A (γ) | −40 (anti) | −91 (anti) | −100 (anti) |

| Pseudorotation angles (degrees) | 3',5'-ADP | 2',5'-ADP | 5'-ADP |
| C4'-O4'-C1'-C2' (ν5) | −27 | −12 | −19 |
| O4'-C1'-C2'-C3' (ν5) | 44 | 33 | 33 |
| C1'-C2'-C3'-C4' (ν5) | −43 | −40 | −34 |
| C2'-C3'-C4'-O4' (ν5) | 28 | 34 | 24 |
| C3'-C4'-O4'-C1' (ν5) | 0 | −14 | −4 |

| Phase (degrees) | 3',5'-ADP | 2',5'-ADP | 5'-ADP |
| Phase: C2'-endo | 162 | 180 | 167 |

**Phosphate torsion angles (degrees)**

| Phosphate O5A-PA-O5'-C5' (α) | 94 (pc) | 94 (sc) | 49 (ac) |
| PA-O5'-C5'-C4' (β) | 125 (+ac) | −168 (ap) | 132 (+ac) |
| PB-PA-O5A-O5' (z) | 99 (+ac) | |

*The α torsion angle for 3',5'-ADP and 2',5'-ADP cannot be defined, because the position of atom O3A is ambiguous (the phosphate group is free).*
DISCUSSION

All three ligands, 3',5'-ADP, 2',5'-ADP, and 5'-ADP bind to the catalytic site of EDN, each one in a significantly different manner. In the EDN-3',5'-ADP complex, the 5'-phosphate binds at the P1 site engaging in similar interactions with EDN to those observed for phosphate groups bound to RNase A subsite P1. This was anticipated, because subsite P1 is conserved between RNase A and EDN. The adenosine binds in an identical manner to that observed for adenosines bound to the subsite B2 of RNase A. Subsite B2 is only partially conserved between EDN and RNase A; residues Asn-67, Gln-69, Asn-71, and Glu-111 in RNase A are replaced by residues Asn-65, Arg-68, Asn-70, and Asp-112 in EDN. Atoms N6 and N1 of the adenosine of 3',5'-ADP form hydrogen bonds with Asn-70, and the adenine ring is engaged in stacking interactions with the imidazole of His-129. All these interactions are very similar to those observed for the adenosine bound to B2 in the RNase A complex with d(ApTpApApG) (44), d(CpA) (37), and 2',5'-CpA and 3',5'-d(CpA) (45). However, the replacement of RNase A residue Gln-69 by Arg-68 in EDN restricts EDN from forming an additional hydrogen bond with adenine as seen in the RNase A complex (44). In addition, the substitution of a Gln residue by Arg seems to force the adenine ring to occupy a slightly different position. The hydrogen bond between Arg-68 and Asn-65 observed in the sulfate-bound structure is not present in the 3',5'-ADP and 2',5'-ADP complexes, where the Arg-68 side chain can shift toward the base. The position of the 3'-phosphate of 3',5'-ADP indicates a possible location of subsite P2 in EDN. In RNase A, Lys-7 is the main

**FIG. 3.** A, the molecular surface of EDN calculated using the program GRASP (47). The 3',5'-ADP molecule is shown in red as a ball-and-stick model. The EDN residues interacting with 3',5'-ADP are shown in yellow. B, superimposed structures of EDN-3',5'-ADP and RNase A-ppA-3',5'-p (38). EDN and RNase A residues are shown in green and light gray, respectively. The nucleotides 3',5'-ADP and ppA-3',5'-p are drawn in dark green and black, respectively. Hydrogen bonds are indicated by dashed lines. Diagram was drawn with BOBSCRIPT (61).

**TABLE III**

Hydrogen bond interactions of 3',5'-ADP

| Donor      | Acceptor | Distance |
|------------|----------|----------|
| Met-0 N    | O3G      | 2.7      |
| Gln-14 Ne2 | O2A      | 3.0      |
| His-15 Ne2 | O1A      | 2.9      |
| Asn-70 N62 | N1A      | 2.6      |
| N6A        | Asn-70 O81| 2.7      |
| His-129 N51| O3A      | 2.3      |
| Leu-130 N  | O1A      | 3.0      |
| Water      | O1A      | 2.9      |
| Water      | O2A      | 2.8      |
| Water      | O1G      | 2.7      |

**TABLE IV**

Hydrogen bond interactions of 2',5'-ADP

| Donor      | Acceptor | Distance |
|------------|----------|----------|
| Gln-14 Ne2 | O2G      | 3.3      |
| His-15 Ne2 | O3G      | 2.6      |
| His-129 N51| O1G      | 2.7      |
| O3'        | Leu-130 O| 2.6      |
| Leu-130 N  | O3G      | 2.8      |
| Water      | O3G      | 2.8      |
| Water      | N3A      | 2.9      |
| Water      | O1A      | 2.9      |

**TABLE V**

Hydrogen bond interactions of 5'-ADP

| Donor      | Acceptor | Distance |
|------------|----------|----------|
| Gln-14 Ne2 | O2B      | 2.8      |
| His-15 Ne2 | O2B      | 2.8      |
| Lys-38 N7' | O1A      | 2.9      |
| O2'        | Glu-40 O | 3.1      |
| Gln-40 N   | O2       | 3.1      |
| His-129 N51| O1B      | 2.5      |
| Leu-130 N  | O2B      | 2.8      |
| N6A        | Water    | 3.0      |
| N7A        | Water    | 3.0      |
| Water      | N7A      | 3.0      |
| Water      | O2'      | 3.1      |
| Water      | O3'      | 3.1      |
| Water      | O1A      | 2.8      |
| Water      | O2A      | 2.9      |
| Water      | O2B      | 3.1      |

Hydrogen bond interactions were calculated with the program HBPLUS (59). Atom names are defined according to Fig. 1.
The 3'-phosphate group forms a hydrogen bond with the main-chain nitrogen of the N-terminal residue Met-0 (an interaction that should not exist in the natural EDN because Met-0 is an addition due to the expression system) and a water-mediated interaction with the side chain of Trp-10. In addition, this phosphate group forms several van der Waals contacts with Trp-10. Therefore, it appears that Trp-10 is the sole component of subsite P2 and contributes only through nonpolar interactions to substrate binding.

The observed binding mode of 2',5'-ADP to EDN was not anticipated on the basis of the 3',5'-ADP binding to EDN. In the EDN-2',5'-ADP complex, it is the 2'-phosphate that binds to the P1 subsite, whereas the 5'-phosphate points toward the N terminus and is involved only in water-mediated interactions with the N-terminal residue Met-0. In addition, there are no interactions between this phosphate group and Trp-10 (the sole component of subsite P2), which is 6.9 Å away. The adenosine binds to the purine binding subsite in a different mode to that observed in previous RNase A-nucleotide complexes (37–39, 44, 45). The adenine ring is in a different orientation and does not pack against the imidazole ring of His-129 due to steric hindrance. In doing so, His-129 adopts conformation B (as described for RNase A His-119, the inactive conformation). On
the other hand, the 2'-phosphate group of 2',5'-ADP forms hydrogen bond interactions with EDN at the main phosphate active site P₁ similar to those observed for the 5'-phosphate of 3',5'-ADP. From the present study, we are unable to explain why it is the 2'-phosphate instead of the 5' that binds to subsite P₁ of EDN. Modeling of the 2',5'-ADP molecule onto the 3',5'-ADP structure revealed that the EDN active site could easily accommodate 2',5'-ADP with the 5'-phosphate group in P₁ and the 2'-phosphate pointing toward the solvent. This mode of binding might also bring the adenine moiety to form stacking interactions with His-129. However, the binding mode of 2',5'-ADP to EDN shows the flexibility of the B₂ site in EDN, the ability of the P₁ site to accommodate either a 5'- or a 2'-phosphate and the low affinity for phosphate anions of subsite P₂ noted above.

In the EDN-5'-ADP complex it is the β-phosphate rather than the α-phosphate that occupies the P₁ subsite while the adenosine binds close to the EDN pyrimidine binding subsite B₁. The β-phosphate engages in interactions similar to those made by the phosphate groups and sulfate ion located at P₃ as seen in the other two EDN complexes described above. In addition, the α-phosphate group forms a hydrogen bond with Lys-38. This mode of binding for the pyrophosphate group has been observed previously in RNase A complexes. In previous structural studies of RNase A with three potent nucleotide inhibitors that had a 5'-pyrophosphate group (38, 39), the β- instead of the α-phosphate was found to bind to subsite P₁. That mode of binding drove the adenosine to adopt the syn instead of the anti conformation with the six- instead of the five-membered ring of the adenosine stacking against the imidazole ring of His-119. However, in EDN this does not occur. Instead, the binding of the β-phosphate group in the P₁ position forces the adenosine out of B₂ to a new location. Thus in this complex the binding of the adenosine to EDN is stabilized through two hydrogen bonds made by the ribose with Gln-40 and through van der Waals interactions of the adenosine ring with Lys-38, Gln-40, and His-82.

EDN is one of a relatively small array of proteins that are C-mannosylated which involves the attachment of an α-mannosyl residue via a C–C link to the indole moiety of the first tryptophan in the recognition sequence of Trp-X-X-Trp (55, 56). The site of mannosylation is Trp-7, and the modification is a biosynthetic modification but its role in structure and/or activity is not yet known. In the recombinant EDN-nucleotide complexes presented here, Trp-7 makes van der Waals interactions with the nucleotide in both 3',5'-ADP and 5'-ADP complexes at the active site of the protein. Based on these structural observations, we predict that these interactions will also be present in the natural protein. It is quite likely that the mannosyl residue(s) is(are) positioned on the opposite face of the active site and may not have a direct role on inhibitor binding to the protein. This hypothesis can only be tested through a structural study of one or more of these inhibitors with the C-mannosylated protein.

The EDN sulfate structure has confirmed the position of the two sulfate molecules at P₁ and P₃ sites. The presence of an additional anchoring site at P₃ was also proposed for ECP (α close homolog of EDN) both by structural analysis (15) and kinetic studies (16). Superpositions of the structures of the EDN-3',5'-ADP, EDN-2',5'-ADP, EDN-5'-ADP, and EDN-sulfate complexes (Fig. 5) reveal that the positions and the orientations of all three phosphate groups and sulfate A at the P₁ subsite are almost identical. All these groups participate in a similar hydrogen bond pattern with EDN and seem to be the anchoring point for each inhibitor. The binding of the adenosine is optimal only in the case of 3',5'-ADP, because this is the only inhibitor where the adenosine ring forms hydrogen bonds with EDN and binds in the purine binding subsite. The positions of the adenosine moiety in the other two complexes seem to be dictated by the binding of the phosphate in P₁. To optimize their interactions, each ligand adopts a different conformation, indicating that diversity in ligand binding can be achieved with subtle modifications to the parent ligand molecule mainly through the flexible side chain of His-129. Examples where analogous inhibitors adopt different binding mode/s are well documented (57). For example, 2-deoxy-n-glucose 6-phosphate and n-glucose 6-phosphate, which differ only in one hydroxyl group, bind in a totally different manner to glycogen phosphorylase (58).

The analysis of EDN-ligand complexes and the comparison with the new sulfate bound EDN at higher resolution (1.6 Å) allowed the identification of some of the key residues implicated in EDN substrate binding. The residues implicated in the main phosphate active site P₁ have been confirmed, and we have been able to analyze the B₂ site environment and its flexibility for different adenylic mononucleotides. Furthermore, we have confirmed that Trp-10 is the sole component of subsite P₂. Although the mononucleotides interact with the enzyme in quite different orientations, some common features are observed. In all three complexes, one of the phosphates is invariably located at P₃, even when the adenine does not directly interact with the protein (e.g. 5'-ADP complex) and adopts a completely different orientation. We can conclude that the interactions at P₃ are the main driving force for all the observed nucleotide binding analyzed. Interactions at P₁ are conserved for the three adenylic nucleotide complexes and the sulfate complex, as is the case in the well documented RNase A-nucleotide complexes. On the other hand, the position of the adenine base is considerably different for each complex. A position of the adenine analogous to the substrate interaction is only feasible in the 3',5'-ADP complex. The comparative analysis of EDN complexes and the sulfate-bound structure have allowed the identification of the residues directly involved in the ligand interaction. We can therefore conclude that adenine binding in EDN mainly involves ribonucleolytic active site residues Asn-70 and His-129.

The EDN-inhibitor complexes presented here suggest ways for further rational design of tight binding inhibitors of this enzyme against pathological conditions associated with eosinophil RNases. It also highlights that subtle alterations in the chemical structure of an inhibitor can generate significant changes upon binding to the protein. Thus the process of rational design may not follow a predictable course. However, the observations presented here emphasize the importance of crystal structure analysis intertwined with modeling studies toward achievement of significant enhancement in potency in the
inhibitor design process. Finally, the binding mode of nucleotides with EDN should also prove useful for the design of inhibitors of other biologically active RNase superfamily enzymes.

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Mapping the Ribonucleolytic Active Site of Eosinophil-derived Neurotoxin (EDN): HIGH RESOLUTION CRYSTAL STRUCTURES OF EDN COMPLEXES WITH ADENYLC NUCLEOTIDE INHIBITORS
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