Kinetic and Product Distribution Analysis of Human Eosinophil Cationic Protein Indicates a Subsite Arrangement That Favors Exonuclease-type Activity*

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Ester Boix‡, Zoran Nikolovski¶, Gennady P. Moiseyev‡‡, Helene F. Rosenberg¶, Claudi M. Cuchillo‡, and M. Victoria No"gues‡‡

From the ‡Departament de Bioquimica i Biologia Molecular, Facultat de Ciències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain and the ¶Laboratory of Host Defenses, NIAID, National Institutes of Health, Bethesda, Maryland 20892

With the use of a high yield prokaryotic expression system, large amounts of human eosinophil cationic protein (ECP) have been obtained. This has allowed a thorough kinetic study of the ribonuclease activity of this protein. The catalytic efficiencies for oligouridylic acids of the type (Up)nU>p, mononucleotides U>p and C>p, and dinucleoside monophosphates CpA, UpA, and UpG have been interpreted by the specific subsites distribution in ECP. The distribution of products derived from digestion of high molecular mass substrates, such as poly(U) and poly(C), by ECP was compared with that of RNase A. The characteristic cleavage pattern of polynucleotides by ECP suggests that an exonuclease-like mechanism is predominately favored in comparison to the endonuclease catalytic mechanism of RNase A. Comparative molecular modeling with bovine pancreatic RNase A-substrate analog crystal complexes revealed important differences in the subsite structure, whereas the secondary phosphate-binding site (p2) is lacking, the secondary base subsite (B2) is severely impaired, and there are new interactions at the p,B, and p-1 sites, located upstream of the P-O-5 residue, of the large specific granules of human eosinophils (1, 2). It is synthesized as a preprotein with a 27-residue signal peptide (3). Mature ECP is a single polypeptide with a molecular mass of 15.5 kDa, but several glycosylated forms ranging from 16 to 21 kDa have also been identified (4). The protein has a marked homology to the proteins from the pancreatic RNase A (EC 3.1.27.5) superfamily and shows a 67% amino acid sequence identity to another eosinophil-associated protein, the eosinophil-derived neurotoxin (EDN) (5). On the other hand EDN was found indistinguishable from the human nonsecretory ribonuclease (6). The EDN and ECP genes (7) arose from a duplication event that took place about 30 million years ago, and, while retaining the structural and catalytic requirements for RNase activity, both genes rapidly evolved under unusual evolutionary constraints to products with distinct biological properties (8, 9). ECP shows a reduced RNase activity and is extremely cationic (pI ~11) and toxic, whereas EDN shows a higher RNase activity but is much less cytotoxic (2).

Helminthotoxic and antibacterial activities of ECP have been described (10, 11), and a physiological function as a host defense protein has been suggested. Its cytotoxicity can damage the host epithelial tissues in vivo. Respiratory epithelial damage, similar to that observed in severe asthmatics, has been reproduced in vitro by purified ECP (2). Eosinophil granule proteins may also be responsible for the enhanced capacity of eosinophils to kill cancer cells (12). However, the mechanism of action of ECP remains unclear, and its toxicity against bacteria and helminths seems not to be related to its RNase activity. The ECP helminthotoxicity is not inhibited by the RNase inhibitor (10), and mutagenesis studies suggest that its toxicity for bacteria is also unrelated to the RNase activity (13). ECP can disrupt cell and artificial membranes by creating ion-selective channels (14) and in vitro lytic activity on the protozoa Trypanosoma cruzi was inhibited by heparin (1). However, both EDN and ECP appear to induce the neurotoxic Gordon phenomenon through their RNase activity (4, 15), and they participate in the host defense mechanism against single-stranded RNA viruses through their RNase activity (16, 17). The main catalytic residues required for RNase activity are conserved in ECP, as in all members of the mammalian RNase A superfamily, and site-directed mutagenesis at two of these residues (K38R and H128D) eliminates the RNase activity (13).

Early kinetic studies on the RNase activity of ECP indicated a pyrimidine specificity for the base position at the active site with a slight preference for cytidine in substrates of low mob-desorption ionization-time of flight; MES, 4-morpholinoethanesulfonic acid.

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¶ Recipient of a visitor fellowship from the Ministerio de Educación y Cultura, Spain. On leave of absence from the Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia.

† To whom correspondence should be addressed: Dept. of Bioquimica i Biologia Molecular, Facultat de Ciències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain. Tel.: 34-93-5811256; Fax: 34-93-5811264; E-mail: Victoria.Nogues@uab.es.

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Human eosinophil cationic protein (ECP) is a small cationic protein found in the matrix of the large specific granules of eosinophils (1, 2). It is synthesized as a preprotein with a 27-residue signal peptide (3). Mature ECP is a single polypeptide with a molecular mass of 15.5 kDa, but several glycosylated forms ranging from 16 to 21 kDa have also been identified (4). The protein has a marked homology to the proteins from the pancreatic RNase A (EC 3.1.27.5) superfamily and shows a 67% amino acid sequence identity to another eosinophil-associated protein, the eosinophil-derived neurotoxin (EDN) (5). On the other hand EDN was found indistinguishable from the human nonsecretory ribonuclease (6). The EDN and ECP genes (7) arose from a duplication event that took place about 30 million years ago, and, while retaining the structural and catalytic requirements for RNase activity, both genes rapidly evolved under unusual evolutionary constraints to products with distinct biological properties (8, 9). ECP shows a reduced RNase activity and is extremely cationic (pI ~11) and toxic, whereas EDN shows a higher RNase activity but is much less cytotoxic (2).

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luciferase mass but a clear preference for polyuridylic acid (poly(U)), in comparison with polycytidyllic acid (poly(C)) (18, 19). No activity toward either 2',3'-phosphate cyclic nucleotides, dinucleoside monophosphates, or double-stranded RNA was detected (20). Initial studies indicated that EDN and ECP have a similar substrate specificity, although the catalytic efficiency of ECP is substantially reduced in comparison to that of EDN (2, 18).

The structure-function studies with ECP have been delayed by the limited availability of human ECP purified from eosinophils and the difficulties in obtaining a good yield in a prokaryotic secretion expression system. We report here a different prokaryotic expression system, in which the vector pET11c and a synthetic gene for human ECP are used. This system allows recovery yields of about 5–10 mg of purified recombinant protein per 1 liter of culture. We have studied in detail the substrate specificity and polynucleotide substrate cleavage pattern of this recombinant protein, and the results have been analyzed by ECP molecular modeling based on the three-dimensional structure of EDN and RNase A-substrate analog complexes. The distinct putative subsites configuration of ECP favors an exonuclease mechanism in the same way as the endonuclease activity of RNase A is a result of its own multisubsite structure (22, 23).

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine pancreatic RNase A was purified according to Alonso et al. (24). Poly(C), poly(U), uridylyl(3',5')-adenosine (UpA), uridylyl(3',5')-guanosine (UpG), cytidylyl(3',5')-adenosine (CpA), uridine 2',3'-cyclic monophosphate (Up), cytidine 2',3'-cyclic monophosphate (Cp), carbenicillin, and dithiothreitol were purchased from Sigma; reagents for performing the polymerase chain reaction were obtained from Perkin-Elmer; vectors pET11c and pET22b, E. coli Novablue, and BL21(DE3) competent cells were from Novagen (Madison, WI). pFLAG-CTC expression vector was from International Biotechnologies, Inc. (New Haven, CT). Isopropyl-1-thio-D-galactopyranoside (IPTG), reduced glutathione (GSH), and oxidized glutathione (GSSG) were from Roche Molecular Biochemicals (Mannheim, Germany). Recombinant human placental ribonuclease inhibitor (PRI) was from Promega (Madison, WI). MicroBCA Protein assay was from Pierce. Mono-S (HR 5/5) and Resource S cation exchange columns were from Amersham Pharmacia Biotech. Dowex 50W-X8 cation exchanger was from Bio-Rad. Nova-Pak C18 (3.9 × 150 mm) was from Waters. All other reagents were of analytical grade.

**Construction of the Synthetic Gene and the Expression Plasmid**—The human ECP gene was synthesized using an *Escherichia coli* codon bias (25). Oligonucleotides were ligated and subjected to polymerase chain reaction to incorporate appropriate restriction sites for cloning in pFLAG-CTC expression vector. Plasmid construct was further subjected to polymerase chain reaction for subcloning of the ECP synthetic gene into pET11c plasmid using NdeI and BamHI restriction sites. The final sequence corresponded to ECP lacking the leader sequence and an additional N-terminal formylmethionine residue (pET11c/ECP). Alternatively, cDNA of ECP from the pFLAG-EC7 construct (13) was subcloned into the pET22b+ vector, behind the pelB leader coding sequence. *E. coli* Novablue-compliant cells were used for subcloning protocols, and the *E. coli* BL21(DE3) strain was chosen for expression of the recombinant protein.

**Expression and Purification of the Recombinant Protein**—*E. coli* BL21(DE3)-competent cells were transformed with the pET11c/ECP plasmid. The expression protocol was optimized in Terrific broth (TB) medium and in minimal medium M9 (26). For high yield expression, bacteria were grown in TB, containing 400 μg/ml carbenicillin. Recombinant ECP was expressed in *E. coli* BL21(DE3) cells after induction with 1 mM IPTG added when the culture showed an A600 of 0.6–1. The cell pellet was collected after 4 h of culture at 37 °C. Cells were resuspended in 10 mM Tris-HCl, 2 mM EDTA, pH 8, and sonicated at 50 watts for 10 min with 30-s cycles. After centrifugation at 15,000 × g for 30 min, the pellet fraction containing inclusion bodies was processed as follows: the pellet fraction was washed with 50 mM Tris-HCl, 2 mM EDTA, 0.3 M NaCl, pH 8, and after centrifugation at 20,000 × g for 30 min, the pellet was dissolved in 12 ml of 6 M guanidine HCl, 0.1 mM Tris acetate, 2 mM EDTA, pH 8.5, containing 80 mM GSH, and incubated under nitrogen for 2 h at room temperature. The protein was then refolded by a rapid 100-fold dilution into 0.1 M Tris acetate, pH 8.5, containing 0.5 M i-arginine, and GSSG was added to obtain a GSH/GSSG ratio of 4. Dilution in the refolding buffer should be adjusted to obtain a final protein concentration of 30–150 μg/ml. The protein was incubated in refolding buffer for 48–72 h at 4 °C. The folded protein was then concentrated, dialyzed against 0.15 M sodium acetate, pH 5, and purified by cation exchange chromatography on a Resource S column equilibrated with the same buffer. ECP was eluted with a linear NaCl gradient from 0 to 2 M in 0.15 M sodium acetate, pH 5 buffer. Further purification was achieved by a second cation exchange chromatography on a Mono S column using the same buffers as described for the Resource S chromatography. Protein concentration was determined either by microBCA assay using RNase A as a standard or spectrophotometrically by the calculated extinction coefficient using the formula obtained by Pace et al. (27) (ε280 = 17,460 M⁻¹ cm⁻¹), obtaining comparable values. The homogeneity of the purified proteins was checked by 15% SDS-PAGE and Coomassie Blue staining and by N-terminal sequencing.

**Kinetic Analysis by HPLC of Poly(C) and Poly(U) Digestion Products**—The high molecular mass substrates poly(C) and poly(U) were dissolved in 10 mM Tris-HCl, pH 7.5, at 25 °C. 25 μl of 5 mg/ml substrate solution were incubated with 5 μl of 0.35 μM RNase A, or 5 μl of 7 μM ECP for poly(U), and 5 μl of 28 μM ECP for poly(C) digestion. At different time intervals, between 0 and 90 min, the products of the reaction were analyzed as described previously (22). 15 μl of the reaction mixture were injected on a reversed-phase HPLC column, Nova-Pak C18 (3.9 × 150 mm), equilibrated with 10% ammonium acetate (w/v) and 1% acetonitrile (v/v) in water, and after a 10-min wash, a 50-min linear gradient was applied from 0 to 90% of elution buffer (10% ammonium acetate (w/v) and 11% acetonitrile (v/v) in water). The relative molecular mass of undigested substrates, poly(C) and poly(U), was estimated, as indicated by the manufacturer, to be about 260,000 to 1,200,000, and neither oligonucleotides nor other small molecular mass contaminants were observed in significant amounts. Product elution was detected from the absorbance at 260 nm. Peak identification was performed according to Moussaoui et al. (22). The mononucleotides 3’ UMP and 5’-p elute sequentially but very close in the first peak, and oligomers of increasing size elute with increasing retention times (Fig. 1). The identity of the oligonucleotides, from 2 to 6 units, was checked by mass spectrometry (see below). The relative amount of each oligonucleotide was determined by integration of the 260-nm peak area divided by the number of nucleotide residues. As the elution profile cannot distinguish between consecutive sizes after a length of 9–10 nucleotides, for the calculation of the relative distribution of each oligonucleotide. 

![FIG. 1. Reversed-phase HPLC elution profile of oligouridylic acids (Up)5,U>'](http://example.com/image1.png)

The following nomenclature was used: for 5'-p elute sequentially but very close in the first peak, and oligomers of increasing size elute with increasing retention times (Fig. 1). The identity of the oligonucleotides, from 2 to 6 units, was checked by mass spectrometry (see below). The relative amount of each oligonucleotide was determined by integration of the 260-nm peak area divided by the number of nucleotide residues. As the elution profile cannot distinguish between consecutive sizes after a length of 9–10 nucleotides, for the calculation of the relative distribution of each oligonucleotide. 

The RNase binding subsites are named as in de Llorens et al. (21). B, R, and p stand for the base, ribose-, and phosphate-binding subsites, respectively. B-R-p, is the main binding site where catalysis takes place. Subsites with subscript 2 are on its 3’ side in the nucleotide chain of the substrate, and subsites with subscript 0 or –1 are on its 5’ side.
gonucleotide, the area corresponding to all oligonucleotides with a size larger than 9 nucleotides was considered as high molecular mass product together with the undigested substrate fraction.

**Preparation of Oligouridylic Acids**—For the preparation of oligouridylic acids (Up), U-p, n = 1–4, 500 μl of 10 mg/ml poly(Up) solution in 10 mM Tris-HCl, pH 7.5, was incubated with 100 μl of 7 μM ECP at 25 °C for 15 min. Oligonucleotides were separated by reversed-phase HPLC as described previously (Fig. 1) and freeze-dried.

**Spectrophotometric Kinetic Analysis**—The relative activity of ECP with increasing amounts of Zn²⁺ was determined spectrophotometrically. Assays were carried out using 0.2 mM poly(U) as substrate in 50 mM MES-NaOH, pH 6.2, and 0.15 μM final enzyme concentration. The activity was measured by following the change in absorbance at 280 nm. 

The activity was measured by following the initial reaction velocities using the difference molar absorbance coefficients, in relation to cleaved phosphodiester bonds. The activity was measured by following the change in absorbance at 280 nm. ZnSO₄ was added from 0.2 to 1.2 μM final concentration in the assay reaction. 2 mM EDTA was added to all assay mixtures to abolish the inhibition by Zn²⁺ ions (see "Results") below.

Poly(U), poly(C), C-p, U-p, CpA, UpA, UpG, and oligouridylic acids (Up)–p, n = 1–4, were used as substrates, and the kinetic parameters were determined by the spectrophotometric method. Assays were carried out in 50 mM MES-NaOH, 2 mM EDTA, pH 6.2, at 25 °C using either 0.2- or 1-cm path length cells. Substrate concentration was determined spectrophotometrically using the following extinction coefficients: ε₂₈₀ = 6,200 M⁻¹ cm⁻¹ for poly(C) nucleotide residue, ε₂₈₀ = 9,430 M⁻¹ cm⁻¹ for poly(Up) nucleotide residue (28); ε₂₈₀ = 10,000 M⁻¹ cm⁻¹ for U-p, ε₂₈₀ = 8,400 M⁻¹ cm⁻¹ for C-p (29); ε₂₈₀ = 21,000 M⁻¹ cm⁻¹ for CpA, ε₂₈₀ = 23,500 M⁻¹ cm⁻¹ for UpA, and ε₂₈₀ = 20,600 M⁻¹ cm⁻¹ for UpG (31). For oligouridylic acids ((Up)n–p) the ε₂₈₀ values in each case were determined according to the following formula: ε₂₈₀ = 10,000 × (n + 1)ν, where n is the number of non-cyclic phosphodiester bonds.

The activity was measured by following the initial reaction velocities using the difference molar absorbance coefficients, in relation to cleaved phosphodiester bonds: Δε₂₈₀ = 2380 M⁻¹ cm⁻¹ for poly(C) and Δε₂₈₀ = 829 M⁻¹ cm⁻¹ for poly(U) (28); Δε₂₈₀ = 1450 M⁻¹ cm⁻¹ for CpA, Δε₂₈₀ = 570 M⁻¹ cm⁻¹ for UpA (30), and Δε₂₈₀ = 480 M⁻¹ cm⁻¹ for UpG (31) for transphosphorylation reactions; Δε₂₈₀ = 1450 M⁻¹ cm⁻¹ for C-p (30) and Δε₂₈₀ = 1000 M⁻¹ cm⁻¹ for U-p (32) hydrolysis reactions. Δε₂₈₀ = 700 M⁻¹ cm⁻¹ was used for the transphosphorylation reaction of oligouridylic acids (Up)–p, the Δε₂₈₀ value being practically the same for all the oligouridylic acids (n = 1–4) analyzed (32). Substrate concentration ranges were from 0.1 to 1 mM for cyclic mononucleotides and dinucleoside monophosphates, and from 0.01 to 0.1 mM for (Up)–p oligonucleotides. Final enzyme concentrations were in the range from 0.1 to 10 μM depending on the activity for each assayed substrate.

**Activity Staining Gels**—The RNase activity was used to follow the expression and purification of recombinant ECP and to detect the presence of RNase activities both in the soluble and insoluble intracellular fractions of the E. coli BL21(DE3) expression strain without the expression plasmid. The zymogram technique on 15% SDS-PAGE, containing either poly(U) or poly(C) as substrate, was used according to the method described by Bravo et al. (33).

**Mass Determination by MALDI-TOF Mass Spectrometry**—Mass determinations of purified recombinant ECP and of urydilyl oligonucleotides obtained from the HPLC separation of digestion products of poly(U) was carried out by MALDI-TOF mass spectrometry on a Bruker Biflex mass spectrometer (Bremen, Germany).

For protein analysis a sinapinic acid matrix was used. For urydilyl oligonucleotide substrates the protocol of Wang and Biemann (34) and Hahner et al. (35) was optimized in the following way: oligonucleotides were freeze-dried and resuspended in deionized water. 1 μl of NH₄OH exchange polymer beads (Dowex 50W-X8) was loaded to the target inert metal surface, and excess of solvent was removed. After solvent evaporation, 0.7 μl of 0.5–10 mM oligonucleotide was added. Ions were generated by irradiation with a 357-nm nitrogen laser with an acceleration ion voltage of 19 kV. All spectra were taken in the reflectron positive ion mode.

**Ribonuclease Inhibitor Interaction**—Analysis of ECP affinity for plasental ribonuclease inhibitor was calculated using a modification of the methodology of Vicentini et al. (36) for a competitive slow tight binding inhibition mechanism (37). 1 mM poly(U) was used as substrate, and the reaction was performed at 25 °C in 50 mM MES-NaOH, pH 6, 125 mM NaCl, 2 mM EDTA, 1.2 mM dithiothreitol, 0.1% (w/v) poly(ethylene glycol), and 0.2 mg/ml bovine serum albumin. ECP was added at a 50 mM final concentration, and the remaining RNase activity was followed for 90–120 min. Several inhibitor concentration ranges from 0.01 to 200 mM were used. Product formation was determined by following the decrease in absorbance at 280 nm.

**RESULTS**

**Expression and Purification of Recombinant ECP**

The expression protocol using a synthetic gene as described in this paper is the first reported method to obtain ECP in high yield. The previously reported expression system was rather inefficient one (13). The production of secreted soluble protein using the cDNA from ECP cloned behind the bacteria pelB leader peptide in pET22b+ vector was also very low (results not shown). Indeed, purification from human eosinophils is also a very limited source (1–20 μg/liter of serum and 5 μg/10⁶ eosinophils) (42).
FIG. 3. Analysis by reversed-phase HPLC of the products obtained from digestion of poly(U) (A) and poly(C) (B) by ECP at different time intervals. Panels 1–5 were taken under conditions in which the amount of remaining undigested oligonucleotides of a size larger than 9 nucleotide units was 100, 90, 60, 40, and 20%, respectively. Note that in each case the best ordinate was chosen.
Our prokaryotic expression system combines the use of a synthetic gene for ECP with bacterial preferential codons and the pET11c vector. Induction of the T7 lac promoter leads to the expression of intracellular protein in high yield that aggregates and accumulates in inclusion bodies. A yield of around 100 mg of protein in inclusion bodies per liter of medium is obtained in TB medium, and expression in minimal medium M9 yielded around 30 mg of protein per liter of medium. ECP represents more than 70% of the total protein in inclusion bodies after 2–5 h of culture following IPTG induction. Longer incubation leads to the accumulation of other cellular proteins and, consequently, a lower yield in the refolding step. Refolding of recombinant protein is achieved by rapid dilution of denatured reduced protein in renaturing buffer. The best yield was obtained when the GSH/GSSG ratio was 4, and the protein was added to the refolding buffer at a final concentration of 50 \( \text{mg/ml} \). Protein concentration could rise up to 200 \( \text{mg/ml} \) if added stepwise, allowing 1 h incubation between each loading. The renatured protein was easily purified to homogeneity by cation exchange chromatography with a final yield of 5–10 mg of purified protein per 1 liter of culture. The purified recombinant protein was analyzed by 15% SDS-PAGE stained with Coomassie Blue (Fig. 2A) or by SDS-PAGE containing either poly(C) or poly(U) as substrates for activity staining (Fig. 2B and C). Analysis of the E. coli BL21(DE3) expression strain without the expression plasmid by means of SDS-PAGE activity staining indicated that there was no other protein with RNase activity in the insoluble intracellular fraction (Fig. 2, B and C). However, a band at around 30 kDa corresponding to E. coli RNase I, as confirmed by N-terminal sequencing, was found in the soluble protein fraction (data not shown). Amino-terminal sequencing of the purified expressed recombinant protein confirmed the correct processing of the mature protein, including the (−1)-formylmethionine residue. The molecular mass of the purified recombinant protein was also checked by MALDI-TOF mass spectrometry.

**Kinetic Characterization**

The low catalytic activity of ECP and its limited availability have delayed a thorough kinetic study. We have analyzed the catalytic efficiency of ECP toward low and high molecular mass RNase substrates. We have also studied the cleavage pattern produced using high molecular mass substrates, such as poly(C) and poly(U), which has provided some insight on the catalytic mechanism.

**Effect of Zn\(^{2+}\) on RNase Activity**—ECP was found to have a high affinity for Zn\(^{2+}\) with 2–3 mol of bound Zn\(^{2+}\) per mol of protein (43). This property was used to purify the enzyme on a zinc-containing affinity resin (44). Differences in the effect of Zn\(^{2+}\) on the catalytic activity of pancreatic and nonpancreatic RNases, EDN, and ECP have been explained as due to structural differences at the active site (45). These authors observed that whereas human pancreatic RNase was markedly inhibited by the divalent cation, the non-pancreatic EDN was virtually
The oligouridylic acids were obtained as described under "Experimental Procedures". The spectrophotometric method was used. Reaction conditions were as follows: 50 mM MES-NaOH, 2 mM EDTA, pH 6.2 at 25 °C. Substrate concentration ranges were from 0.1 to 1 mM for the cyclic mononucleotides and dinucleoside monophosphates and from 0.01 to 0.1 mM for the oligonucleotides. Final enzyme concentrations were in the range 0.1 to 10 μM.

Table I

| Substrate | k_{cat} | K_{m} | k_{cat}/K_{m} |
|-----------|---------|-------|---------------|
| C\rightarrow U | (1.4 ± 0.2) \times 10^{-2} | 1.5 ± 0.3 | 11 |
| U\rightarrow p | (4.3 ± 0.4) \times 10^{-3} | 1.0 ± 0.2 | 4 |
| CpA \rightarrow p | 4.2 ± 0.3 | 2.4 ± 0.3 | 1750 |
| UpA \rightarrow p | 6.2 ± 0.4 | 5.4 ± 0.5 | 1150 |
| UpG \rightarrow p | ND |
| (Up)_{2}U \rightarrow p | 0.56 ± 0.03 | 1.4 ± 0.1 | 400 |
| (Up)_{3}U \rightarrow p | 1.2 ± 0.2 | 0.7 ± 0.18 | 1714 |
| (Up)_{4}U \rightarrow p | 1.4 ± 0.1 | 0.17 ± 0.02 | 8255 |

* ND, not detected (reaction too slow).
* Value obtained from the slope of the Lineweaver-Burk plot as the actual K_{m} value is too high to be measured.

Unaffected. We analyzed the effect of Zn^{2+} on the ECP catalytic activity, using poly(U) as substrate, and the results showed that increasing amounts of Zn^{2+}, with a Zn^{2+}/ECP molar ratio ranging from 1.3 to 80, reduce the enzymatic activity down to 50%. The activity could be restored by adding EDTA to 2 mM final concentration. Thus, all kinetic spectrophotometric assays were carried out in a buffer containing 2 mM EDTA.

**Pattern of Product Formation in the ECP Cleavage of Poly(C) and Poly(U)—**Fig. 3 shows the elution profile obtained by reversed-phase HPLC of oligonucleotides produced from poly(C) and poly(U) digestion by ECP at different time intervals. Different cleavage patterns between ECP and RNase A are clearly observed when comparing the relative percentages of oligonucleotide formation (Fig. 4). For comparison with the RNase A degradation of poly(C), the results of Moussaoui et al. (22) were considered. The elution profile of the digestion products shows that with ECP the cyclic mononucleotide appears early in the incubation, especially with poly(C) as substrate, whereas most of the high molecular mass substrate still remains uncleaved.

Comparison of the (Cp)_{n} \rightarrow p and (Up)_{n} \rightarrow p, n = 0–8, relative distribution at various digestion times (Fig. 4) provides further information. Although the digestion pattern of poly(U) by RNase A is essentially equivalent to that of poly(C) by RNase A (22), there is a clear difference in the case of ECP. An accumulation of mononucleotides from the beginning of the reaction and a slow appearance of intermediate oligonucleotides is observed. This trend is more pronounced for the poly(C) substrate than for poly(U). These results can be interpreted by a predominant exonuclease-like behavior and a low efficiency endonuclease activity of ECP. The product distribution pattern is quite different from that obtained with RNase A (Fig. 4 and Ref. 22). In the case of RNase A, with a described endonuclease mechanism, there is a clear accumulation of intermediate products.

Detailed analysis of the degradation of poly(U) and the relative accumulation of oligouridylic acids (n = 1–5) and U\rightarrow p as a function of time (Fig. 5) indicated that oligonucleotides, n = 1–5, initially accumulate at the same rate up to 20 min of digestion time and then are sequentially degraded as the time increases. The catalytic efficiency of their cleavage increases as a function of size, as observed when calculating the kinetic parameters of isolated (Up)_{n} \rightarrow p oligonucleotides (Table I). Longer incubation times, from 45 min up to 12 h (data not shown), lead to a sequential degradation of n = 5 to n = 2 and, finally, n = 1. The dinucleotide continues to accumulate when no other higher oligonucleotides are left and is only degraded at the end of the reaction. It is noteworthy that even after 12 h digestion, there is still some undigested dinucleotide. These results are in agreement with the low catalytic efficiency found with the dinucleotide UpU\rightarrow p and the increasing efficiency up to (Up)_{4}U\rightarrow p (Table I).

The relative percentage of U\rightarrow p to 3'UMP during the digestion of poly(U) (Table II) indicated the slow efficiency of the hydrolysis step in comparison to the transphosphorylation; after 45 min digestion only 3% of the mononucleotide is 3'UMP. The ratio of the rates of hydrolysis to transphosphorylation is even much lower than the one obtained for RNase A (22, 46).

**Kinetic Parameters—**The kinetic parameters for the cyclic mononucleotides U\rightarrow p and C\rightarrow p and the dinucleoside monophosphates CpA and UpA are indicated in Table I. No activity could be detected with UpG using 2 mM substrate concentration and up to 20 μM final enzyme concentration. Relative values of catalytic efficiency, k_{cat}/K_{m}, are compared with RNase A values (47) (Table III). The relative CpA/UpA value for ECP is comparable to that for RNase A and to the one reported for EDN (15). The rate of hydrolysis of cyclic mononucleotides with ECP is much slower than the rate of transphosphorylation, with ratios similar to those for RNase A (Table III). These results are in agreement with the low 3' UMP formation during poly(U) cleavage by ECP (Table II). A similar ratio was observed for the poly(C) cleavage by RNase A (22). No hydrolysis of the cyclic mononucleotides had previously been detected either with EDN (15) or with ECP (20), a fact very likely due to the limited amount of available protein.

In the case of both poly(U) and poly(C) no accurate values for K_{m} and k_{cat} can be given due to the sigmoidal-like behavior of the progress curve (Fig. 6). This abnormal behavior could be a result of the predominating exonuclease activity of ECP. A similar sigmoidal curve was observed for the exonuclease snake venom phosphodiesterase.3 At the start of the reaction the long polynucleotide chain is cleaved relatively slowly because of the low concentration of 3'OH ends and the low efficiency of the endonuclease activity. As the incubation proceeds, formation of intermediate oligonucleotides by the slow endonu-
ECP Kinetics and Subsite Structure

Molecular Modeling

To explain the kinetic behavior of ECP, we tried to identify the residues involved in the putative secondary binding sites. We superimposed the EDN crystal structure (38) and the ECP predicted three-dimensional structure to RNase A-d(CpA) (40) and RNase A-d(ApTpApApG) (41) enzyme-substrate analog complexes (Fig. 7). The results have been compared with the corresponding subsites structure analysis of EDN carried out by Mosimann et al. (38). We first analyzed in detail the B1- and B2-binding sites by superimposing the EDN and ECP modeling to the RNase A-d(CpA) complex.

At the B1 site, Thr-42 in ECP and EDN would be equivalent to Thr-45 in RNase A (Fig. 7A). Crystal structure analysis of RNase A-substrate analog complexes (40, 50) indicate that B1 can accommodate either cytidine or uracil depending on the conformation of the hydrogen bonds with Thr-45. Mutagenesis at Thr-45 (51) confirmed that this amino acid is mostly responsible for the B1 pyrimidine specificity of RNase A. Similar results were obtained in the case of angiogenin for the role of Thr-44 in B1 (52).

The amino acids corresponding to Asn-71 and Glu-111 which belong to B2 in RNase A could be identified as Asn-70 and Asp-112 in both EDN and ECP (Fig. 7A). However, the B2 site in both EDN and ECP has a considerable different conformation due to important changes in the loop 56–72 (RNase A numbering). In ECP, Asn-70 is further away from the purine base of the substrate in comparison to Asn-71 in RNase A (the O6-1 and N6-2 atoms of Asn-70 of ECP are about 4 Å from the N-6 and N-1 atoms of adenine, respectively, instead of the less than 3 Å in RNase A). Besides, the shorter Asp-112 side chain cannot mimic the interaction of Oe-2 atom of Glu-111 with the N-1 atom of adenine.

Additional binding sites were analyzed taking the RNase A-d(ApTpApApG) complex as a model. A comparative study of the primary sequences of ECP and EDN with that of pancreatic RNases showed that both eosinophil RNases presented non-conservative replacements in the region corresponding to the secondary phosphate-binding site p5 (Trp-10 in ECP instead of Lys-7 in RNase A). The hydrogen bond at the p5 site (Lys-7 Nε-O-1–P) is lost when lysine is substituted by a tryptophan (Fig. 7B). The kinetic characterization of the K7Q RNase A mutant (53) confirmed that the positive charge of a lysine was necessary for the operant p5 subsite.

The involvement of the p5 subsite of RNase A in the binding of RNA was deduced from kinetic and x-ray crystallography studies (54, 55). Lys-66 was ascribed to p5 in RNase A (21, 55). However, further crystallographic (41) and kinetic studies (56) concluded that the interaction of the substrate at p5 would be rather weak. In fact, the position of the Nε atom of Lys-66 in the crystal is at about 5 Å of the corresponding O-1–P atom (41). The nonconservative substitution at Lys-66 may be counteracted by the positive residue His-64 in ECP (Fig. 7C). The atom Nε of His-64 in the ECP modeling is located at about 2 Å from atom O-2–P corresponding to p5. However, the actual position of His-64 cannot be accurately predicted as in that position there is a non-conservative substitution in EDN.

Mosimann et al. (38) analyzed the subsite structure of EDN using the RNase A-d(Tp)3 complex model (57). This together with molecular modeling analysis revealed additional putative binding sites in ECP at the 5′ side of the main catalytic site, which are not observed in RNase A. The additional subsites found in ECP may correspond to the residues interacting in the EDN crystal structure with a second sulfate anion, SO42− (named B position). In the EDN x-ray crystal structure there are two SO42− anion-binding sites as follows: the position of the first SO42− anion mimics the p5 site, interacting with Gln-14,
His-15, His-129, and Leu-130 of EDN; the position of the second sulfate was suggested to represent a new binding site, $p-1$ (38). The region Arg-36—Asn-41, conserved in EDN and ECP, but with a different conformation in RNase A, could represent a new substrate binding region unique to the eosinophil RNases. The putative $B_0$ and $p-1$ subsites would be located in this region. In the model, the positions of Asn-39 and Gln-40 in both ECP and EDN are equivalent. In the ECP molecule, the Ne-2 of Gln-40 is about 3 Å from the 5’-O of the ribose, and this corresponds to the site that can be occupied by the $p-1$ phosphate in the RNase A. The Asn-39 Nε-2 atom is less than 4 Å from the N-1 of the adenine corresponding to $B_2$, and the Oδ-1 atom could interact with the N-3 of $B_0$ (Fig. 7D). In pancreatic RNases the $p-1$ $B_0$ region is structurally equivalent to the neighborhood of the residue Pro-42, which cannot interact with the substrate. Residues Asn-39 and Gln-40 are found to be conserved only in all primate eosinophil RNases EDN and ECP (8) and are flanked by two strictly conserved regions in all members of the RNase superfamily.

Molecular modeling analysis at the $B_0$ site, on the 3’ side of the catalytic center, did not reveal any putative substrate binding site in ECP.

**DISCUSSION**

ECP is an eosinophil secretion protein with RNase activity involved in the host defense mechanism. It is toxic against bacteria, helminths, and single-stranded viruses. The physiological function of the RNase activity of ECP is still unclear. Mutagenesis of the catalytic center residues leaves the antibacterial activity intact (13); its helminthotoxicity is not inhibited by the PRI (10), yet its RNase activity, necessary for neurotoxicity (15), is inhibited by human PRI. The structural
requirements for catalytic activity and ribonuclease inhibitor interaction have been conserved, whereas a strong evolutionary pressure has increased in a short period its basicity and toxicity (8, 9). Recently, the involvement of the eosinophil RNases, EDN (17) and ECP (48), in the host defense mechanism against the single-stranded RNA virus respiratory syncytial virus, through both their RNase and membrane disruptive properties, has been reported. Ackerman (1) suggested that ECP degrades or widens tight junctions between cells in helminths, and Young et al. (14) described the formation of transmembrane pores in cell and artificial membranes. However, it is still unclear the physiological meaning of the ribonuclease activity of ECP and its specific substrate in vivo. Thus, a detailed analysis of the ECP catalytic mechanism and its substrate preference will help us to understand its physiological function.

The preparation of recombinant ECP in relatively large amounts has enabled us to analyze in detail its kinetic properties toward a number of substrates. The N-terminal formylmethionine present in the recombinant protein did not alter the ECP specific activity. The side chain of (−1)-formylmethionine in the closely related EDN was found not to interact with any region of the molecule in its crystal structure (38), and recombinant Met-1 EDN displayed the same properties as eosinophil purified EDN (58). We have thoroughly analyzed the substrate specificity for cyclic mononucleotides, dinucleoside monophosphates, and (Up)U>p oligonucleotides (Table I). Because of the sigmoidal-like behavior of the progress curves obtained with the high molecular mass substrates poly(U) and poly(C) (Fig. 6), no kinetic parameters for these polynucleotides could be calculated. On the other hand, the cleavage pattern of polynucleotides by ECP was deduced from the distribution of digestion products by reversed-phase HPLC analysis (Figs. 3–5). The results can be interpreted on the basis of its unique arrangement of phosphate and base subsites as has been deduced from molecular modeling.

The results of kinetic studies with mononucleotides (C>p and U>p) and dinucleoside monophosphates as substrates (Table I), which indicate a preference for cytidine, much in the same way as EDN (15) and RNase A (Table III), can be explained by the predicted structure at the B2 site; Thr-42 of ECP and EDN would be equivalent to Thr-45 in RNase A that is responsible for the B2 pyrimidine specificity. On the other hand, the different conformation at the B2 site of ECP in relation to RNase A (Fig. 7A) may explain its lower catalytic activity for dinucleoside monophosphates (Table I) due to important changes in the loop 56–72 (RNase A numbering) that move Asn-70 away from the purine base and to the fact that the Asp-112 side chain is shorter than the corresponding Glu-111 in RNase A. These differences could explain the extremely low catalytic efficiency found for ECP with UpG as substrate as it has been shown that RNase A mutants at Glu-111 have a reduced catalytic efficiency only when a guanine is located at the B2 site (59).

The dramatic increase of $k_{cat}/K_m$ for (Up)U>p oligonucleotides from $n = 2$ to $n = 4$ (Table I), which is not observed for cytidine derivatives with RNase A (46), might be due to a new binding site (p2B2), which would enable longer oligonucleotides to interact more efficiently. On the basis of the catalytic efficiency for the cleavage of oligouridylidic acids by RNase A (32), an active site of about three nucleotides of size was postulated. The kinetic parameters $K_m$ and $V_{max}$ for RNase A did not show important changes going from $n = 2$ to $n = 5$. Only a slightly higher catalytic efficiency for substrates with a length of three nucleotides was observed (32). Similar results were obtained by Moussaoui et al. (46) in the case of RNase A with oligocytidylidic acids (C$p)$, from $n = 2$ to $n = 5$, with a maximum catalytic efficiency for the trinucleotide.

A reduced interaction at the B2 site and the lack of p2 can explain the lower catalytic efficiency of ECP for all assayed RNase substrates. Yakovlev et al. (60) showed that the $k_{cat}$ values depend significantly on the conformation of the cleavable P−O−5′ bond. The productive conformation of the P−O−5′ bond could be better achieved if the base of the O−5′ nucleotide is fixed at the B2 site of the enzyme. It has been shown in the case of RNase T2 that the $k_{cat}$ value decreases by about 2 orders of magnitude when the O−5′ nucleoside does not bind to the enzyme subsite (61). Similarly, the lack of p2 and an impaired B2 site should considerably reduce the effective cleavage of the P−O−5′ phosphodiester bond in ECP. In addition, mutants with a deleted p2 subsite such as K7Q RNase A and K7Q plus R10Q RNase A show much lower $k_{cat}$ values than the native enzyme (53).

The kinetic properties of ECP with polynucleotide substrates can also be interpreted as a result of the ECP putative subsites structure (Fig. 7) and compared with those found with RNase A. RNase A digestion of poly(C) was analyzed by Moussaoui et al. (22), and the product distribution profile was explained by a clear preference of RNase A for the polynucleotide or oligonucleotides of high molecular mass, according to its multisubsites structure (62). Formation of the enzyme-substrate complex would be mainly driven in RNase A by interactions between phosphate groups of the substrate and the active site (p1) and adjacent phosphate-binding subsites at both sides (p1 and p2). Additional electrostatic interactions between phosphate groups and basic amino acid residues would enhance the catalytic efficiency in RNase A. In comparison to the endonuclease activity of RNase A, the ECP exonuclease preference (Fig. 4) would be related to the different subsites distribution. ECP lacks the secondary phosphate subsite p2 and displays some important changes at the secondary base subsite B2. Deletion of p2 in the RNase A K7Q mutant was found to change the endonuclease activity to exonuclease activity (22). Moreover, the additional subsites B1 and p1 in ECP would preferentially align the substrate at the 5′ side of the catalytic center p1B1, and cleavage at p1 would result in the release of mononucleotides, as expected for an exonuclease-type enzyme.

Due to the sigmoidal-like behavior of the progress curve of ECP toward polynucleotides, no accurate values for $K_m$ and $k_{cat}$ can be given. However, even at the steepest part of the curve a lower apparent catalytic activity is observed, as compared with short oligonucleotides, and this could also be dependent on the location of additional positive charges on the enzyme surface. Positive charges in ECP and in EDN distributed over the enzyme surface, located far away from the active site, could produce enzyme-substrate complexes with a less effective alignment of the substrate (63, 64). From the properties of an RNase A-d(Ap)_4 crystal complex, McPherson et al. (65) designed a virtual RNA strand of 10–12 oligonucleotides that could fit the active cleft. The interaction between the phosphate groups of the polynucleotides and the clusters of positively charged groups of the enzyme would be essential in the formation of the enzyme-substrate complex in RNase A (62). On the other hand, the evolutive pressure that led to an increased number of arginines on the surface of ECP after its divergence from EDN resulted in an increased toxicity, probably due to its membrane disruption capacity. However, the position of these positive residues does not coincide with that of the RNase A homologous residues. This may determine that the distribution of the surface positive side chains in ECP does not favor an optimal alignment of the polynucleotide substrate for its cleavage. Moreover, the destabilizing capacity of the DNA secondary
structure and the catalytic activity against double-stranded RNA, present in pancreatic RNases but absent in eosinophil RNases, have also been related to the different distribution of positive charges (66).

An impaired B2 site together with the absence of p2 in ECP could also enhance the differences of the interaction at the B1 subsite for cytosine and uracil when cleaving the homopolymeroligonucleotides poly(U) and poly(C). Different contacts at B1 and B2 for cytosine or uracil may be a determinant for a more pronounced exonuclease pattern for poly(C) than for poly(U).

An additional factor that could influence the difference in the cleavage pattern between poly(C) and poly(U) is their different conformation in solution; poly(C) adopts a single helical stranded conformation (67), whereas poly(U) does not form any secondary structure at room temperature (68).

We conclude that the substrate specificity and the exonuclease cleavage pattern found with ECP is mainly determined by its subsites structure; interactions at BP2 are weaker or absent, and there is a new interacting region, at the 5' side of the cleaved P—O-5' phosphodiester bond, that would correspond to p1, Bp, and p0.

To confirm our hypothesis further studies will be carried out by site-directed mutagenesis and by comparative analysis with EDN. To understand better the ECP substrate specificity and its physiological function, we will also analyze its catalytic efficiency toward different cellular RNAs.

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