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The sulfate-binding site structure of the human eosinophil cationic protein as revealed by a new crystal form

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ABSTRACT

The human eosinophil cationic protein (ECP), also known as RNase 3, is an eosinophil secretion protein that is involved in innate immunity and displays antipathogen and proinflammatory activities. ECP has a high binding affinity for heterosaccharides, such as bacterial lipopolysaccharides and heparan sulfate found in the glycocalix of eukaryotic cells. We have crystallized ECP in complex with sulfate anions in a new monoclinic crystal form. In this form, the active site groove is exposed, providing an alternative model for ligand binding studies. By exploring the protein–sulfate complex, we have defined the sulfate binding site architecture. Three main sites (S1–S3) are located in the protein active site; S1 and S2 overlap with the phosphate binding sites involved in RNase nucleotide recognition. A new site (S3) that is unique to ECP is one of the key anchoring points for sulfated ligands. Arg 1 and Arg 7 in S3, together with Arg 34 and Arg 36 in S1, form the main basic clusters that assist in the recognition of ligand anionic groups. The location of additional sulfate bound molecules, some of which contribute to the crystal packing, may mimic the binding to extended anionic polymers.

In conclusion, the structural data define a binding pattern for the recognition of sulfated molecules that can modulate the role of ECP in innate immunity. The results reveal the structural basis for the high affinity of ECP for glycosaminoglycans and can assist in structure-based drug design of inhibitors of the protein cytotoxicity to host tissues during inflammation.

1. Introduction

The human eosinophil cationic protein (ECP) is an eosinophil secretion protein with antipathogen activities and is involved in the host immune defense response (Boix et al., 2008; Venge et al., 1999). Mature ECP has a MW ranging from 15 to 21 kDa due to several glycosylation grades (Eriksson et al., 2007; Rubin et al., 2009). The protein is highly basic (p$I \sim 11$), and it targets a wide range of pathogens, including helminths, protozoa and bacteria (Boix et al., 2012), suggesting a rather nonspecific mechanism of action. Indeed, structural and functional studies have identified protein regions that are involved in a membrane destabilizing mechanism (Carreras et al., 2003; Sanchez et al., 2011; Torrent et al., 2007, 2009a,b).

ECP (ID:P12724; EC:3.1.27.*), also known as RNase 3, was first identified as an eosinophil cytotoxic protein and ascribed to the RNase A superfamily by sequence homology (Gleich et al., 1986; Rosenberg et al., 1989). The first ECP crystal structure (Boix et al., 1999a) confirmed that the protein overall topology followed the RNase A superfamily fold, and the structure of ECP in complex with the 2′5′ ADP nucleotide (Mohan et al., 2002) revealed the main structural traits of the catalytic site. RNase A binds RNA via several subsites, which recognize the phosphate, base and ribose units and are designated as pn, Bn and Rn, respectively (Cuchillo et al., 2011; Nogues et al., 1998; Pares et al., 1991; Raines, 1998). p1 being the main site where the phosphodiester bond is cleaved. Following an equivalent nomenclature, the corresponding RNA binding sites for the distinct members of the RNase A family were assigned. The protein reduced RNase catalytic efficiency (Boix et al., 1999b; Sorrentino and Libonati, 1994) and the distinct polynucleotide cleavage pattern were related to impaired interactions at substrate binding secondary sites (Boix et al., 1999a,b).

On the other hand, ECP has a high affinity for sulfated heterosaccharides. In fact, ECP was first reported to bind heparin when it was originally purified from eosinophils (Olsson and Venge, 1974; Venge et al., 1999), and key interacting residues were recently located at the active site cleft by molecular docking prediction (Torrent et al., 2011a) and NMR spectroscopy (García-Mayoral et al., 2010). Binding

Abbreviations: ECP, eosinophil cationic protein; EDN, eosinophil derived neurotoxin; GAGs, glycosaminoglycans; LPS, lipopolysaccharides; pn, protein interaction site for phosphate anions; Sn, protein interaction site for sulfate anions.

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to heparan sulfate may facilitate adhesion to eukaryote extracellular matrix and promote the RNase cytotoxic activity (Chao and Raines, 2011; Chao et al., 2010; Fan et al., 2007). Because heparin and heparan sulfate derivatives modulate key biological cell paths, the pharmaceutical industry is applying a great effort to unravel the underlying structural determinants for protein binding (Boix et al., 2011; Capila and Linhardt, 2002; Imberty et al., 2007). Target recognition is primarily driven by electrostatic interactions that depend on the particular arrangement of protein basic clusters (Capila and Linhardt, 2002; Cardin and Weintraub, 1989; Fromm et al., 1995). These interactions can favor binding to sulfated saccharides and other anionic polymers, such as nucleic acids. Not surprisingly, the Cardin and Weintraub motif for heparin recognition shows a preference for sulfate anion binding and secondarily for phosphate groups (Boix et al., 2011). Indeed, ECP represents a nice illustrative example of protein affinity for both sulfated and phosphorylated polymers.

We report here the structure of ECP bound to sulfate anions, which provides the opportunity to analyze the putative binding sites for sulfated ligand molecules of biological significance. We also compare the new monoclinic crystal form to the previously reported hexagonal and tetragonal crystal forms (Boix et al., 1999a; Mallorqui-Fernandez et al., 2000) and to the NMR solution structure (Laurents et al., 2009).

2. Materials and methods

2.1. Protein expression and purification

Recombinant ECP was expressed and purified in *Escherichia* coli BL21(DE3) cells as described previously (Boix, 2001). Briefly, a synthetic gene for human ECP was cloned into the pET11c expression vector, and the protein was purified from inclusion bodies. The protein was purified by cationic exchange FPLC on a Resource-S column followed by reverse phase chromatography on a Vydac C4 column.

2.2. Protein crystallization

Crystallization conditions were screened at the High Throughput Crystallization facility of the EMBL Grenoble Outstation, starting from a protein sample of 14 mg/ml in 20 mM Na Cacodylate, pH 5. A single crystal appeared after two months of incubation at 20 °C in 0.2 M lithium sulfate monohydrate, 0.1 M Tris hydrochloride, pH 8.5, and 15% PEG 4000, which corresponds to condition number 17 from the Crystal Screen Lite, Hampton Research. A cryoprotectant was prepared by supplementing the reservoir crystallization solution with 20% PEG 400.

Crystallization conditions were also set to reproduce and optimize the previously reported ECP tetragonal crystal form (Mallorqui-Fernandez et al., 2000) using the hanging drop methodology in a 1 μL:1 μL protein/precipitant mixture. The precipitant concentration was slightly reduced to increase the crystal size. A sample of 15 mg/ml of protein in 20 mM Na citrate, pH 5.2 was incubated at 16 °C in 0.1 M Na citrate, pH 5.2, 6% Jeffamine M-600, 8 mM KH_2PO_4, 2 mM K_2HPO_4, and 10 mM $FeCl_3$. Crystals appeared after 5 days and were soaked in a cryoprotectant by supplementing the crystallization buffer with 30% 2-methyl-2, 4-pentanediol (MPD).

2.3. Data processing and structure refinement

Diffraction data for the new ECP crystal form were collected at 100 K at the BM14 beamline station at the European Synchrotron Radiation Facility (ESRF), Grenoble. One hundred fifty images were collected at a 1° oscillation range. Diffraction data for the ECP tetragonal crystal form were collected at 100 K at the X13 beamline station at the Deutsches Elektronen Synchrotron (DESY)-EMBL, Hamburg. Two hundred images were collected at a 0.5° oscillation range.

Data processing was performed with the XDS software, and scaling of integrated reflections was performed with SCALA. The ECP tetragonal crystal form (1DYT (Mallorqui-Fernandez et al., 2000)) was used as a starting model to solve both structures. Molecular replacement was performed with PHASER, and refinement was achieved with REFMAC5 (Murshudov et al., 1997), as implemented in the CCP4 suite package (CCP4i, 1994). The model was improved by alternate cycles of refinement and manual rebuilding with COOT (Emsley and Cowtan, 2004). Water molecules were incorporated with COOT at several steps of refinement when electron density peaks of either (Fo–Fc) or (2Fo–Fc) maps were higher than 3 or 1.5σ, respectively, and were located within a hydrogen bond distance from appropriate atoms. A final refinement cycle with TLS Motion Determination (Painter and Merritt, 2006) was performed, and the stereochemistry of the final structure was validated with PROCHECK (Laskowski et al., 1993).

3. Results and discussion

3.1. A new crystal form

A new crystal form for ECP was identified by screening multiple crystallization conditions using the High Throughput Crystallization facility of the EMBL Grenoble Outstation (https://htx-lab.embl.fr). The new crystal form belongs to the space group C2 and contains two molecules in the asymmetric unit. Data to 1.7 Å resolution were collected, and the final refined parameters are summarized in Table 1. The protein molecules are tightly packed in the crystal with a solvent content of 24%. A total of fourteen sulfate anions were fit unambiguously to the (Fo–Fc) electron density map. A sigma-A weighted map was also built to confirm the anion positions (Fig. S1). Five sulfate anions are associated with molecule A and nine with molecule B (Fig. 2 and Table S1). Three sulfate anions overlap in both molecules. Sulfate binding sites were classified and named S1 to S11, according to their relative position. The final average B factors are below 15 $Å^2$ for the two protein molecules in the asymmetric unit, where only segment 89–93 in loop L7 shows higher B factors (Fig. 1B). Few side chains show only partial density (Arg 28, Trp 35, Arg 36, Gln 58, Arg 73, Phe 76, Asn 92, Arg 101 and Arg 105), and residues Ile 13, Asn 53, Ser 59, Asn 69, His 82, Ile 86 and Arg 105 were built with alternate conformations.

Comparison of molecules A and B in the asymmetric unit reveals no major differences (RMSD between both main chains is 0.56 $Å^2$). The side chain of Tyr 122 has a different orientation in both molecules, which can be attributed to its position at the interface between the two asymmetric unit molecules. The interface between molecules A and B is mainly driven by interactions between loops L2 and L8 and the external side of α-helices 1–4 (Fig. 2 and Table S2). Two sulfate anions, A306 (S6) and B310 (S10), connect both molecules at each end of the interface. Segment 115–122, which showed high B factors in previous structures, is fairly rigid in our structure, as it is involved in the interface between molecules A and B.

The intermolecular packing interactions between symmetry related molecules are detailed in Table S3. In particular, exposed Arg residues in loop regions are the critical elements connecting symmetry molecules, often through sulfate molecules, providing tight packing and contributing to the reduced mobility of some of the exposed loops, such as L5 and L8 (Fig. 1B).

A close side to side comparison was performed with the other two available crystal forms, PDB ID 1QMT (Boix et al., 1999a) and