BBA - General Subjects 1862 (2018) 2090–2103

Contents lists available at ScienceDirect

BBA - General Subjects

journal homepage: www.elsevier.com/locate/bbagen

Crystal structure and substrate specificity of ExoY, a unique T3SS mediated secreted nucleotidyl cyclase toxin from *Pseudomonas aeruginosa*

Basavraj Khanppnavar[a,b], Saumen Datta[a,b,*]

[a] Department of Structural Biology and Bioinformatics, CSIR-Indian Institute of Chemical Biology (CSIR-IICB), Kolkata, India
[b] Academy of Scientific and Innovative Research (AcSIR), India

ARTICLE INFO

Keywords:
X-ray crystallography
Nucleotidyl cyclase toxins
T3SS
Anthrax toxA family
Non-canonical secondary messengers
One-metal-ion dependent catalysis

ABSTRACT

Background: The nucleotidyl cyclase toxin ExoY is an important virulence determinant of *Pseudomonas aeruginosa* that causes severe acute and chronic infections in immune-compromised individuals. Additionally, this unique T3SS effector shows a striking preference for cUMP, a newly identified non-canonical secondary messenger. Thereby, ExoY is also considered as a potential tool to study unexplored cUMP signaling pathways.
Methods: The crystal structure of ExoY was determined at 2.2 Å resolutions by in-situ proteolysis assisted crystallization and Rosetta-molecular replacement method. Additionally, isothermal calorimetric (ITC) and molecular dynamic (MD) simulation studies were also carried out to gain molecular insights into its substrate specificity and catalysis.
Results and conclusion: ExoY is a partially unfolded protein with higher propensity to form soluble higher-order oligomers. However, with meticulous attempts of removing of disordered regions by proteases, the recalcitrant ExoY could be successfully crystallized. The crystal structure of ExoY revealed similar overall structural fold present in other anthrax toxA family of nucleotidyl cyclases, with two-to-three distinctly conserved regions conferring specificity to eukaryotic binding partner. The in-vitro catalytic preference of ExoY is in the following order: cGMP > cUMP > cAMP > cCMP. The substrate specificity of ExoY mainly depends on its ability to bind NTP in proper geometrical orientations. ExoY also seems to prefer one-metal-ion dependent catalysis than two-metal-ion dependent catalysis.
General significance: Our results provide much needed structural insight on ExoY, an important virulence determinant of *Pseudomonas aeruginosa* and an exciting tool to study non-canonical cNMP signaling pathways.
Accession numbers: The structure factors and coordinate files have been deposited in the Protein Data Bank with accession number 5XNW.

1. Introduction

Pseudomonas aeruginosa is a ubiquitous environmental pathogen that causes acute and chronic infections in immune-compromised individuals and is also a major cause of chronic infections in cystic fibrosis patients [1]. The nucleotidyl cyclase toxin ExoY is one of the important virulence determinants of *P.aeruginosa* that is secreted directly into host cells via type III secretion systems (T3SS) [2, 3]. The ExoY exhibits numerous pathological functions inside the host cell that facilitate the virulence of *P. aeruginosa*. For example, ExoY inhibits host innate immune response by suppressing the activation of transforming growth factor-beta-activated kinase 1 (TAK1) [4]. ExoY derived cNMP pool activates protein kinases A and G, which result in phosphorylation of tau proteins, leading to disassembly of microtubule network [5, 6]. Another, the long-term pathological implication of hyperphosphorylated tau leads to insolubility of tau, that may result in tauopathies associated in many chronic neurodegenerative diseases such as Alzheimer diseases, argyrophilic grain disease, Pick disease, and hereditary fronto-temporal dementias [6, 7]. ExoY can also mediate reorganization of actin cytoskeleton causing bleb-niche formation in

Abbreviations: ExoY, exogenous toxin Y; T3SS, Type III secretory systems; AA-NC, actin-activated nucleotidyl cyclase; EF, edema factor; cNMP, cyclic mononucleotide phosphates; cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cCMP, cytidine 3′,5′-cyclic monophosphate; cUMP, uridine 3′,5′-cyclic monophosphate; cIMP, inosine 3′,5′-cyclic monophosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; DLS, dynamic light scattering; ITC, isothermal calorimetry; MD, molecular dynamics; TAK1, transforming growth factor-beta-activated kinase 1; Pa, Pseudomonas aeruginosa; Vv, Vibrio vulnificus; MR, molecular replacement; RMSD, root mean square deviation; RRCAT, Raja Raman Center for Advanced Technology; IPTG, isopropyl-b-D-thiogalactoside; NTA, nitrilotriacetic acid

* Corresponding author at: Department of Structural Biology and Bioinformatics, CSIR-Indian Institute of Chemical Biology (CSIR-IICB), Kolkata, India.
E-mail address: saumen_datta@iicb.res.in (S. Datta).

https://doi.org/10.1016/j.bbagen.2018.05.021
Received 30 January 2018; Received in revised form 15 May 2018; Accepted 25 May 2018
Available online 30 May 2018

epithelial cells [8, 9]. Hence, it can act as edema factor that can initiates bleb niche formation, inter-endothelial cell gap formation and increase macromolecular permeability [8, 10, 11]. Importantly, these observations has given us fundamental insight on cAMP compartmentalization in control of endothelial cell barrier integrity, where cAMP generated by membrane adenyl cyclases is barrier protective and cAMP generated by soluble adenyl cyclases is barrier disruptive [10]. ExoY intoxication not only induces inter-endothelial cell gap formation but can also manifest long-term impairment of cell migration and proliferation [12]. Since migration and proliferation are the fundamental cellular traits essential for tissue repair following injury [13]. Hence, it can also prevent the normal recovery from pneumonia-induced lung injury [12, 13].

ExoY shares sequence homology with class II adenylate cyclase family of toxins, which includes hemolysin-adenylate cyclase CyaA from *Bordetella pertussis* and anthrax toxin edema factor (EF) from *Bacillus anthracis* [2, 14–16]. However, unlike CyaA and EF, which require calmodulin as a cofactor for its complete nucleotidyl cyclase activity [15, 16], ExoY requires different eukaryotic cofactor i.e. actin for its stimulated nucleotidyl cyclase activity [14]. Based on this fact, ExoY has been recently classified in a new subgroup of actin-activated nucleotidyl cyclase (AA-NC) toxins within class II adenylyl cyclase family. Moreover, ExoY is secreted via T3SS, whereas EF is auto-transported [17] and CyaA is secreted via Type I secretory system (T1SS) [18]. Additionally, ExoY shows a striking preference for cGMP and cUMP production in various mammalian cell lines, whereas, its counterpart CyaA and Edema factor are ineffective in producing cGMP. CyaA shows a major preference for cAMP and, to a lesser extent, for cCMP and cUMP [19].

The striking preference of ExoY for cUMP production in various mammalian cell lines is found to be of immense fundamental significance. As until now, among all cyclic nucleotides, only cAMP and cGMP were considered to be as secondary messengers whereas the existence and relevance of other cyclic nucleotides, as secondary messengers remained controversial for many decades [20, 21]. Although, today it is a well-known fact that other cNMPs such as cCMP, cUMP and cIMP are also bonafide non-canonical secondary messengers that are postulated to play numerous cellular functions across the tree of life [20–23]. However, so far, the number of studies addressing the specific functions of each cNMP is very limited. The major reason for these limitations is the unavailability of effector proteins that can specifically generate or degrade particular cNMP [20]. The majority of nucleotidyl cyclases identified till now, including mammalian membrane-bound adenylate cyclase (mACs) and periplasmic guanylate cyclase (pGCs), prokaryotic nucleotidyl cyclase (non-toxins), selectively prefer in producing canonical cyclic nucleotides cAMP or cGMP. The non-canonical cyclic nucleotides cUMP, cCMP and cIMP, so far are being produced in a promiscuous manner by previously known adenylate or guanylate cyclases and certain bacterial nucleotidyl cyclase toxins [20, 23]. Since, specific nucleotidyl cyclases with the ability to synthesize particular cNMP are critical tools for carrying out systematic experiments, in order to assign specific functions of particular cNMP. Hence, further research in the field of non-canonical secondary messengers largely depends on identification of nucleotidyl cyclases that can exclusively generate specific cNMPs. Taking into account that nucleotidyl cyclase toxin ExoY, that show a striking preference for cUMP production in various mammalian cell lines, can also be specifically translocated into eukaryotic cells via T3SS, it is considered as an exciting tool to study cUMP signaling pathways [20]. Moreover, by redesigning its substrate specificity, it can also be used to study other non-canonical secondary messengers too.

In the current investigation, we report the first three-dimensional crystal structure of ExoY at 2.2 Å resolutions. Moreover, using isothermal calorimetric (ITC) and molecular dynamic (MD) simulation studies, we also provide some interesting insight into its catalytic mechanism and substrate specificity.

2. Materials and methods

2.1. Cloning, expression, and purification

The gene ExoY from *Pseudomonas aeruginosa PA01 2192* strain was PCR amplified using the primers 5′-AGA ATT CAT GCG TAT CGA CGG TCA TCG-3′ & 5′-TTA AGC TTT CAG ACC TTA CGT TGG AAA AAG TCG-3′ and was cloned into *Eco*RI/*Hin*dIII site of the pET 28b (+) vector. This resulted in the formation of pET 28b (+)-exoY having 6xHis-tag at the N-terminus of protein ExoY (6xHis-exoY).

For purification of ExoY, the expression plasmid was transformed into *Escherichia coli* BL21 pLyS. The overnight culture was used to inoculate Luria-Bertani (LB) media. The cells were grown at 37 °C to an optical density at 600 nm (OD_{600}) of 0.6. Recombinant protein expression was induced by 0.2 mM Isopropyl-b-D-thiogalactoside (IPTG) at 18 °C for 10 h. Cultures were then centrifuged at 6000*g* for 8 min and the pellet was resuspended in ice-cold lysis buffer (50 mM Phosphate Buffer pH 8, 150 mM NaCl, 10% Glycerol, 1 mM DTT and 1× protease inhibitor cocktail) and followed by sonication. The cell lysate was then centrifuged at 20000*g* for 40 min. The supernatant solution was then allowed to bind to Ni-nitrilotriacetic acid (NTA) agarose beads prepacked column, pre-equilibrated with equilibration buffer (50 mM Phosphate buffer pH 8, 150 mM NaCl, 10% Glycerol, 10 mM Imidazole). The column was then washed with a 50 mL of wash buffer (50 mM Phosphate buffer pH 8, 150 mM NaCl, 10% Glycerol) containing gradient imidazole concentration followed by specific elution of the protein complex with 250 mM imidazole concentration. The purity of the final product was more than 95% estimated by SDS-PAGE. The eluted proteins from Ni-NTA column were dialyzed against 25 mM Tris, 150 mM NaCl, 20 mM Na_2SO_4, 1 mM TCEP, pH 8.2 and loaded onto a Hi-load 16/60 Superdex 200 (GE Healthcare) gel filtration column pre-equilibrated with the same buffer with a flow rate of 1 mL/min and the collected fractions were analyzed by SDS-PAGE analysis. DLS profile of purified protein samples was obtained using a Malvern-Zetasizer Nano ZS DLS instrument.

2.2. Protease sensitivity analysis

The protein sample was dialyzed in 10 mM HEPES (pH-7.8) and 150 mM NaCl, 1 mM TCEP. Proteases α-chymotrypsin, Papain, Thermolysin, Glutamyl endoproteinase and Elastase from Proti-Ace Kit of Hampton research was diluted to 0.02 μg/μL by Proti-Ace dilution buffer from an initial stock of 1 μg/μL of protease in deionized water. 1 μL of protease from the diluted stock (0.02 μg/μL) was used for 10 μg of protein. Proteolytic digestion was carried out at 22 °C for different time points and SDS-PAGE sample lysis buffer was used to stop the protease activity. Finally, the products obtained after digestion were analyzed by SDS-PAGE.

2.3. MS/MS sequence analysis

Protease digested fragments of ExoY were separated by SDS-PAGE. The gel was stained with coomassie R-250 and destained with distilled water. The bands were excised from SDS-PAGE and In-Gel trypsin digestion was carried out using trypsin gold from Promega. The “In-Gel trypsin digestion and MS/MS analysis” protocol provided by Promega was followed. Only exceptions to the protocol are: the proteins were eluted in 40 mM ammonium bicarbonate, 10% acetonitrile and 0.3% trifluoroacetic acid buffer. During MS/MS analysis CHCA was used as matrix and prominent peaks were identified by MS after trypsin digestion. Peptides corresponding to the prominent peaks were further fragmented by laser and precise peptide fragments were sequenced using MSDB database of MASCOT search engine, using GPS explorer Software (version 3.6).