Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Molecular mechanism for the inhibition of DXO by adenosine 3′,5′-bisphosphate

Ji-Sook Yun [a, 1], Je-Hyun Yoon [b, 1], Young Jun Choi [a], Young Jin Son [c, 2], Sunghwan Kim [c, 3], Liang Tong [d, *], Jeong Ho Chang [a, d, **]

[a] Department of Biology Education, Kyungpook National University, Daegu 41566, South Korea
[b] Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, USA
[c] New Drug Development Center, Daegu-Gyeongbuk Medical Innovation Foundation, Daegu 41061, South Korea
[d] Department of Biological Sciences, Columbia University, New York, NY 10027, USA

ARTICLE INFO

Article history:
Received 10 August 2018
Accepted 22 August 2018
Available online xxx

Keywords:
5′-3′ exoribonuclease
DXO
Adenosine 3′,5′-bisphosphate
Crystal
Nuclease inhibitor

ABSTRACT

The decapping exoribonuclease DXO functions in pre-mRNA capping quality control, and shows multiple biochemical activities such as decapping, deNADding, pyrophosphohydrolase, and 5′-3′ exoribonuclease activities. Previous studies revealed the molecular mechanisms of DXO based on the structures in complexes with a product, substrate mimic, cap analogue, and 3′-NADP$^+$. Despite several reports on the substrate-specific reaction mechanism, the inhibitory mechanism of DXO remains elusive. Here, we demonstrate that adenosine 3′, 5′-bisphosphate (pAp), a known inhibitor of the 5′-3′ exoribonuclease Xrn1, inhibits the nuclease activity of DXO based on the results of structural and biochemical experiments. We determined the crystal structure of the DXO−pAp-Mg^{2+} complex at 1.8 Å resolution. In comparison with the DXO−RNA product complex, the position of pAp is well superimposed with the first nucleotide of the product RNA in the vicinity of two magnesium ions. Furthermore, biochemical assays showed that the inhibition by pAp is comparable between Xrn1 and DXO. Collectively, these structural and biochemical studies reveal that pAp inhibits the activities of DXO by occupying the active site to act as a competitive inhibitor.

1. Introduction

In eukaryotes, expression of a particular gene is tightly regulated in a coordinated manner via several interconnected processes, including mRNA synthesis and processing in the nucleus, mRNA export via nuclear pore complex, mRNA translation, and eventually mRNA degradation [1,2]. In these processes, the degradative events of mRNA are important determinants of gene expression, which are driven by diverse integral components of RNA metabolism [3]. Thus, the mRNA degradative pathways comprise various sets of enzymes such as Dcp1/2, Xrn1, exosomes, and Ccr4-Caf1 with important biochemical activities, including decapping, exonucleolysis, and deadenylation [4–6]. The mechanisms of both the 5′-3′ and 3′-5′ exoribonuclease reaction are well studied based on three-dimensional structures [7–9].

Although the mRNA capping process was previously thought to be devoid of any quality control mechanism, 5′-end capping surveillance mechanisms in yeast and mammalian cells are now known to help discriminate incomplete capped mRNAs and then degrade them in a two-step process involving the decapping and 5′-3′ exoribonuclease activities of DXO [10–12]. Therefore, these surveillance pathways involve the function of DXO in mRNA quality control for ensuring RNA fidelity. DXO was originally designated as Rai1/Ydr370c and Dom3Z in fungal species and mammals, respectively [13]. We previously determined the detailed distinct substrate specificity and biochemical activities among Rai1,

Abbreviations: DXO, decapping and exoribonuclease protein; NAD$^+$, nicotinamide dinucleotide; NMN, nicotinamide mononucleotide; m7GDP, 7-methylguanosine diphosphate; pAp, 3′, 5′-bisphosphate; pApS, 3′-phosphoadenosine 5′-phosphosulfate.

* Corresponding author.
** Corresponding author. Department of Biology Education, Kyungpook National University, Daegu 41566, South Korea.
E-mail addresses: ltong@columbia.edu (L. Tong), jhcbio@knu.ac.kr (J.H. Chang).

[1] These authors contributed equally to this work.
[2] Present address: Department of New Drug Discovery, Samhyun Inc., Daegu 41061, South Korea.
[3] Present address: R&D center, Polus Inc., 32 Songdogwahak-ro, Yeonsu-gu, Incheon 21984, South Korea.

https://doi.org/10.1016/j.bbrc.2018.08.135
0006-291X/

Ydr370c (Dxo1), and DXO proteins in 5′-3′ exoribonuclease and decapping activities based on their crystal structures in complex with their substrate mimic or product [10,12,14].

Recently, 5′ end nicotinamide dinucleotide (NAD^+)-capped mRNAs were found in human cells rather than the m7G cap, which could promote decay via a DXO-mediated deNADding mechanism [15,16]. The 5′ end NAD^+ was initially found in bacteria and can initiate selective mRNA decay by NudC, which hydrolyzes the NAD^+ to generate nicotinamide mononucleotide (NMN) and 5′ monophosphorylated RNA, which could be degraded by 5′-3′ exoribonucleases [17,18]. As similar to NudC, DXO can remove the NAD^+-capped RNA by both deNADding and degrade RNA using its 5′-3′ exoribonuclease activity in human cells [15]. The crystal structure of DXO in complex with 3′ phosphate NAD^+ revealed the deNADding mechanism in which the scissile phosphate position is shared with that of the substrate mimic RNA pU(S)6 [15].

Adenosine 3′, 5′ bisphosphate (pAp) was initially reported as an inhibitor of the 5′-3′ exoribonuclease activity of Xrn1 and Rat1 [19]. In *Saccharomyces cerevisiae*, pAp is produced from 3′-phosphoadenosine 5′-phosphosulfate (pApS) by Met16p, and is then converted to AMP and inorganic phosphate by Hal2 during sulfate assimilation [20]. Lithium inhibits Hal2 or Met22 to induce pAp accumulation, resulting in toxic effects. An elevated level of pAp was shown to reduce the activity of Xrn1 or Rat1 as well as Oligoribonuclease (Orn) in *Escherichia coli* and small fragment nuclease (Sfn) in humans that inhibits RNA metabolism such as mRNA turnover and transcription [21]. Inhibitory aptamers of DXO containing a 5′-GGATCCC-3′ motif were recently reported using a microplate-based microcolumn device (MEDUSA), which is an improved version of the aptamer screening method, and five candidates were evaluated by an *in vitro* 5′-3′ exoribonuclease activity assay [22].

To understand the inhibitory mechanism of DXO, in the present study we determined the crystal structure of DXO in complex with pAp and Mg^{2+} at 1.8 Å resolution and we examined the effects of pAp on the 5′-3′ exoribonuclease activity of DXO, using Xrn1 for comparison. In addition, we compared the nuclease activity and pAp-binding affinity between DXO and Xrn1.

2. Materials and methods

2.1. Protein expression, purification, and crystallization

Protein expression, purification, and crystallization of mammalian DXO were carried out as described previously [10,23]. In brief, E234A mutant protein and the wild-type enzyme were purified by Ni-NTA and gel filtration chromatography using the same protocol. Free enzyme crystals of DXO were obtained with the sitting-drop vapor diffusion method at 20 °C, using a reservoir solution containing 20% (w/v) PEG 3350. The pAp-Mg^{2+} complex was obtained by soaking the free enzyme crystals with 100 mM pAp (Sigma A5763) and 10 mM $MgCl_2$ for 60 min in the presence of 15% ethylene glycol. Crystals were flash-frozen in liquid nitrogen for diffraction analysis and data collection at 100 K.

2.2. Data collection and structure determination

X-ray diffraction data were collected at the National Synchrotron Light Source (NSLS) beamline X29A. The diffraction images were processed and scaled with the HKL package [24]. The crystals belong to space group $P2_1$, with the following cell parameters: a = 50.0 Å, b = 87.7 Å, c = 53.9 Å, and β = 112.2°. There is one molecule of DXO in the crystallographic asymmetric unit. Structure refinement was carried out with the CNS program [25]. The atomic model was built with the program Coot [26]. The crystallographic information is summarized in Table 1.

2.3. Exonuclease assays with fluorescently labeled RNA

The 3′-FAM-labeled 30-mer RNA with 5′-end monophosphate [27] and the equivalent single-stranded DNA oligos were purchased from Integrated DNA Technologies (IDT). Exonuclease assays were performed at 37 °C for 30 min with reaction mixtures containing 30 mM Tris (pH 8.0), 50 mM NH_4Cl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 25 μg/ml bovine serum albumin, 2 μM 3′-FAM-labeled oligo, 0.1–1 μM DXO, and 0.01–0.1 μM Xrn1. The products were fractionated by 5% denaturing polyacrylamide gel electrophoresis and visualized on an ultraviolet illuminator. Assays were repeated at least three times to ensure reproducibility.

2.4. Surface plasmon resonance analysis

The molecular interactions were determined by surface plasmon resonance (SPR) binding analysis as described previously [28,29]. Real-time protein inhibitor interactions were examined on a BIAcore instrument (BIAcore T200). DXO and Xrn1 were immobilized on a CM5 sensor chip using the amine-coupling method (GE Healthcare Life Sciences) according to the manufacturer's instructions. In brief, native DXO (25 μg/ml) or Xrn1 (25 μg/ml) was immobilized on a single N-hydroxysuccinimide-activated flow cell, and a reference flow cell was prepared by activating the surface. Remaining activated groups on each flow cell were blocked by ethanolamine HCl. The chip was then washed with 0.1 M NaOH to remove any non-covalently bound proteins. Finally, the system was primed with the running buffer comprising 50 mM phosphate buffer, pH 7.8, 150 mM NaCl, 5 mM $MgCl_2$, 0.05% P20, and 0.1 mg/ml bovine serum albumin. A range of concentrations (0.5, 1, 2, 4, 8 mM) of pAp and GTP were prepared in the running buffer and injected at a 30 μl/min flow rate. The sensorgram data were processed by subtracting the signals from the reference cell and a blank injection of the running buffer.

3. Results

3.1. pAp inhibits the 5′-3′ exoribonuclease activity of DXO

On the basis of the 5′-3′ exoribonuclease activity of DXO, we compared the nuclease activity between DXO and Xrn1. The processive 5′-3′ exoribonuclease Xrn1 showed almost 20-fold higher nuclease activity than that of the distributive 5′-3′ exoribonuclease DXO (Fig. 1A). We next assessed whether pAp can inhibit the nuclease activity of DXO. The 5′-3′ exoribonuclease activity of DXO at 0.5 μM was inhibited by pAp starting from 0.5 mM, and 20 mM of pAp completely blocked the activity (Fig. 1B). To compare the potency of pAp inhibition between DXO and Xrn1, we performed a nuclease activity assay of Xrn1 in the presence of pAp. The activity of Xrn1 at 0.02 μM was reduced by pAp at concentrations greater than 0.5 mM and was completely inhibited by 10 mM pAp (Fig. 1B). The activity of DXO was completely inhibited by an approximately 40,000-fold molar ratio of pAp, and loss of Xrn1 activity was detected with an approximately 500,000-fold molar ratio of pAp. Based on these results, the inhibitory potency of pAp toward the nuclease activity seemed to be slightly higher for DXO than for Xrn1. However, direct comparison of pAp-mediated inhibition may be controversial because more DXO could facilitate the formation of pAp complex in the assay, thereby requiring less pAp for complete inhibition. Moreover, in the presence of pAp, the DXO products got smaller (Fig. 1B). On the other hand, Xrn1 still showed no intermediate. Furthermore, DXO and Xrn1 are distributive and processive nucleases, respectively.

Please cite this article in press as: J.-S. Yun, et al., Molecular mechanism for the inhibition of DXO by adenosine 3′,5′-bisphosphate, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.08.135