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Crystal analysis of binding structures of inhibitory compounds to viral RNase domains

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1 Introduction

Influenza viruses cause acute respiratory infection in humans that occasionally progresses to a severe pulmonary condition. Even seasonal epidemics account for 300,000 or more deaths per a year all over the world. Recently, the emergence of highly pathogenic avian and swine influenza viruses has become a global threat to humans. While several anti-influenza drugs are currently approved, their effectiveness for pandemic viruses may be limited due to drug resistance. Therefore, the development of additional antiviral agents against influenza virus infection is needed.

The currently available anti-influenza drugs target one of two viral proteins; M2 protein and neuraminidase. M2 protein is embedded in the lipid membrane of the viral envelope and functions as an ion channel to pump protons into the viral particles. Amantadine and Rimantadine block the function of M2 protein by combining at the center of the channel or the side domain of this enzyme. Neuraminidase is a kind of spike protein sticking out on the viral particle surface. Neuraminidase causes the hydrolysis of neuraminic acid of glycan of host cell. Zanamivir, Oseltamivir, Peramivir, and Laninamivir have been used as neuraminidase inhibitors. Emergence of drug-resistant viruses has been reported for the above approved drugs. Since an RNA virus easily acquires amino acid mutations, the emergence rate of drug-resistant viruses is high. A drug-resistant virus is a serious issue in infectious diseases because a chemotherapeutic approach is restricted. Accordingly, development of novel antiviral drugs that act on a target different from the currently approved drugs is needed.

In this study, we have developed a new class of anti-influenza virus agent inhibiting the endo-nuclease activity of influenza polymerase. The endo-nuclease activity is functionalized at the N-terminal region of domain A of polymerase (PAn). Therefore, we obtained purified PAn using the expression system with *E coli* and, then, performed chemical screening for PAn inhibitor from compound stock in our research team. Several chemical agents to block the function of PAn were found by the screening. In the present crystallographic study, the complex structure of PAn and one of the active agents was clarified.

2 Experiment

An expression plasmid vector, pET50(b), was employed for expressing the target protein, PAn, conjugated with 6xHis-fused Nus-tag at the N-terminal side. The *E. coli* strain, Rosetta, transformed with this plasmid was incubated at 37 °C. The protein expression was induced by adding 1mM isopropyl β-D-thiogalactoside at an OD_{600} value of 0.9 and incubated at 20 °C for 48 hours after induction. A cell pellet corresponding to 2.0 L of culture was resuspended in 50 mM Tris-HCl at pH 8.0, 250 mM NaCl, 1% Triton X-114. Bacteria cell membrane was disrupted by sonication. After removing unnecessary disrupted fragments from lysate with centrifuge, the expressed protein was obtained from the supernant.

The target PAn was purified with HiTrap Ni affinity column with an elution buffer containing 500 mM imidazole. Eluted protein fraction was dialyzed overnight against the buffer containing 50 mM Tris-HCl at pH 8.0, 250 mM NaCl. The dialyzed PAn was incubated with HRV 3C protease for 24 hours at 4 °C to cleavage the Nus-tag attached at the N-terminus. Protein was again purified by Ni-NTA to remove the uncleaved protein and HRV 3C protease. PAn protein was further purified by anion exchange chromatography. Finally, PAn protein was purified by gel filtration with the running buffer of 10 mM Tris-HCl at pH 8.0, 150 mM NaCl.

Crystals of PAn were grown by vapor diffusion in hanging drops containing 1.0 μL of 9.9 mg/mL protein containing 4.0mM $MnCl_2$ and well solution (100 mM MES [pH 5.8], 1.1M ammonium sulfate, 0.1M potassium chloride and 9% (v/v) trehalose) at 18 °C. Crystals were soaked with well solution containing 0.5 μL of 50 mM inhibitory compound in DMSO for 2.0 hr. The crystal was cryo-protected by a brief immersion in well solution containing 22.5 % (v/v) glycerol followed by flash-cooling and storage in liquid N2. X-ray data were collected at 100 K and a wavelength at the NE3A station of PF. The data were processed using HKL2000.

Table 1. X-ray data collection and refinement statistics

Space group	P41 21 2
Cell constant	66.8, 66.8, 126.3
a, b, c (Å)	90.0, 90.0, 90.0
Wavelength (Å)	1.00
Resolution (Å)	50.0 – 1.90
Observations	310070
Unique reflections	23157
Rmerge	0.168
Completeness (%)	98.1
Av. I/σ	12.7
Data refinement	
Resolution Range	32.27-1.90
No. of reflections used	22653
Rwork / Rfree	0.2041 / 0.2383

3 Results and Discussion

The crystal structure PAn bound to an inhibitory compound was obtained at a 1.9 Å resolution at pH 5.8. The PAn crystal was in the P41 21 2 space group with one molecule per asymmetric unit (Table 1).

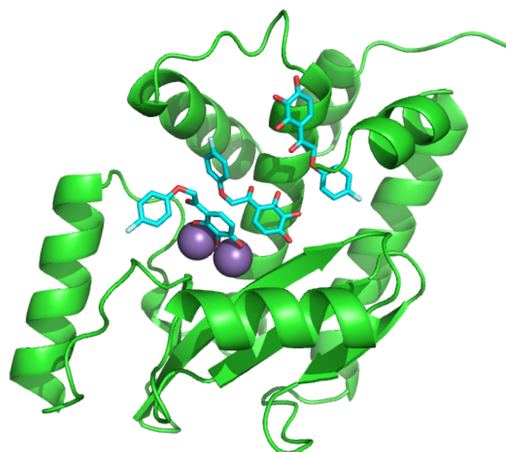

Figure 1: Whole structure of PAn bound to an inhibitor compound.

It was shown in the crystal strucrure that the PAn consists of five helices and one sheet (Fig. 2). This structure is consistent with the previous report on the same protein. The complex of PAn and the inhibitory compound was prepared by soaking technique. In the crystal analysis, three compound molecules are present at the binding pocket of the target protein.

The endo-nuclease of influenza virus exerts its enzymatic activity by incorporating divalent metal ions at the reaction site. In present, there were 30 entries for the crystal structure of PAn in Protein Data Bank. We surveyed the number of divalent metal ions observed at the endo-nuclease domain through all of these 30 crystal structures. Two metal ions were observed in many of the crystal structures. The presence of Mn^{2+} ions was was observed in many structures. Mn^{2+} ion is often used in protein crystallization, because the coordination force to the active site becomes strong with change from Mg and Mn. A single metal ion is observed in some structures that was obtained in the early stage of the study. Hence, it had been controversial previously how many metal ions were required at the reaction site to exert its enzymatic activity. Recently it will be a common consensus that the presence of two divalent metal ions is essential for endo-nuclease activity and that two metal ions act cooperatively with facilitating nucleophilic binding of a substrate and stabilizing the transition state for the enzymatic reaction. A theoretical study on the two metal nuclease enzymatic reaction suggested that there is a difference in role of those two metal ions. One of the ions is stably bound to the active site, while the other is slightly irregular with changing the coordination bond.

Figure 2 is a magnified image of the compound binding site. One inhibitory compound makes a contact with two Mn^{2+} ions. The compound includes a polyphenol structure. The crystal structure clearly indicates that three OH groups are adequately attached to Mn^{2+} ions. A phenyl group connected to fluoride has a hydrophobic contact with the inside of the binding pocket.

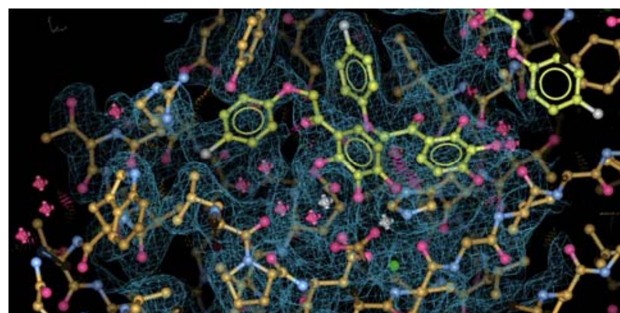

Figure 2: Magnified view of the binding site of the inhibitory compound to the target protein.

The crystallographic study suggested that the inhibitory compound found in our chemical screening will inhibit PAn enzymatic activity by bound to the metal ions and by allosteric effect. This information is helpful to enhance the activity by modifying the chemical structure of the compound.

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