

Crystal Structures of Norwalk Virus Polymerase Complexes Provide a Structural Basis for Understanding Enzymatic Catalysis and a Novel Mode of Inhibition

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Introduction

Norwalk virus (NV) is the archetype species of the Norovirus genus within the Caliciviridae family and is a major cause of gastroenteritis outbreaks in developed countries. NV also belongs in the superfamily of positive-strand RNA viruses that is responsible for such serious and widespread human and animal diseases as polio, hepatitis C and foot-and-mouth disease. Unfortunately, effective treatments are not currently available for many important diseases

caused by NV and related RNA viruses. Because of its critical importance to the process of genome replication in all positive-strand RNA viruses, the virally encoded RNA-dependent RNA polymerase (RdRP) is one of the key targets for the development of novel antiviral therapeutics.

Molecular structural information on RdRP complexes is critical for the development of more effective antiviral agents. High-resolution X-ray crystallographic structures of RdRP complexes provide the detailed molecular structural information that is required to understand how the RdRP normally functions and can be inhibited. With this goal in mind, we have determined 1.8 Å-resolution crystal structures of NV RdRP complexes formed in the presence of divalent metal cations, nucleoside triphosphate substrates and inhibitors, as well as an RNA primer-template oligonucleotide [1].

Method

An enzymatically active form of NV polymerase was expressed in *Escherichia coli* and purified by affinity and ion-exchange chromatography. Crystals of NV polymerase were grown in the presence of Mn^{2+} , a short self-complementary RNA oligonucleotide (5'-UGCCCGG-3') and either the natural substrate CTP or the inhibitor 5-nitrocytidine-5'-triphosphate (NCT). Crystals were grown by the hanging drop vapour diffusion technique under solution conditions with salt and pH near physiological levels. A single crystal grown in the presence of NCT (0.2 x 0.1 x 0.05 mm) was flash-cooled at 100 K under nitrogen gas and transported to CLS beamline 08ID-1. A complete data set consisting of 240 images (1 sec.

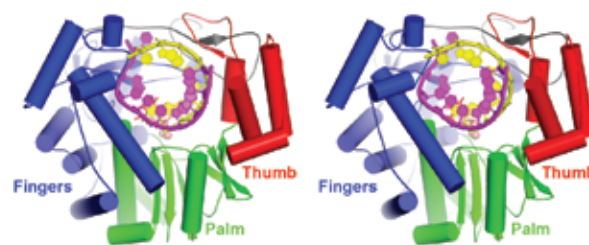


Figure 1: Stereoscopic view of the crystal structure of the Norwalk virus RdRP:RNA:NCT complex. The RNA duplex (primer strand coloured yellow and template strand coloured purple) lies in the active site cleft. The polymerase structure is analogous to a cupped right hand, with portions of the structure corresponding to the fingers, palm and thumb. The nucleotide and metal cations are hidden behind the RNA duplex, towards the back of the structure in this view.

exposure/image, 0.5° rotation/image) was measured using the Marmosaic CCD detector with X-rays at a wavelength of 0.97934 Å. The data were processed using XDS ($R_{sym} = 0.08$, 40-1.8 Å resolution), and the structure was solved using the molecular replacement method (PHASER) with the previously determined structure of unliganded NV polymerase as a search model (1SH0). The structure was refined using Refmac and molecular models were fit to electron density maps using Coot ($R_{cryst} = 0.20$, $R_{free} = 0.24$). A larger, isomorphous crystal of NV polymerase containing Mn^{2+} , RNA and the natural

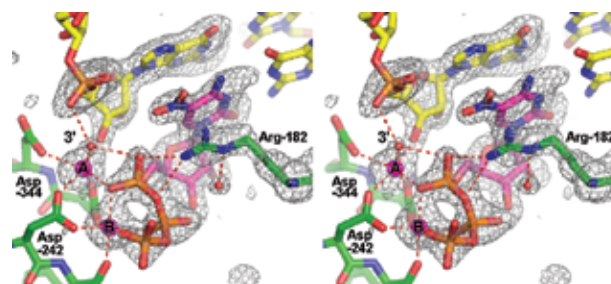


Figure 2: Stereoscopic view of the active site of the Norwalk virus RdRP:RNA:NCT complex. A refined-omit electron density map (3 σ contour level drawn as light grey lines) is superimposed on a stick representation of the molecular structure. The carbon atoms of the protein are coloured green, NCT magenta and RNA yellow. Mn^{2+} ions are coloured magenta and labelled "A" and "B". Hydrogen bonds and coordination bonds are drawn as red, dashed lines.

substrate CTP was also grown (0.3 x 0.15 x 0.08 mm). Data of similar quality to that obtained for the NCT complex were measured using an ADSC Quantum-315 CCD detector at the Advanced Light Source beamline 8.3.1. Data from this second crystal was processed using the HKL suite ($R_{\text{sym}} = 0.05$, 40-1.8 Å resolution). The structure was fitted to electron density maps and refined starting from the coordinates of the refined structure of the NCT complex ($R_{\text{cryst}} = 0.20$, $R_{\text{free}} = 0.23$).

The structures of the NCT and CTP complexes of NV polymerase reveal an RNA primer-template duplex, nucleoside triphosphate and two divalent metal cations bound in the active site cleft (Figure 1). RNA binding induces a large conformational change in the structure of the polymerase that results in the displacement of the C-terminal tail from the active site cleft, as well as the movement of a helix and several loops to accommodate the bound RNA.

These structures also reveal for the first time the detailed arrangement of the enzyme active site and its interactions with metal ions, substrates, inhibitors and water molecules (Figure 2). Comparisons between the structure of the complex formed with a natural nucleotide substrate (CTP) and a potent inhibitor (NCT) provide insights into the normal enzyme mechanism, as well as a novel mechanism of inhibition.

Discussion

The packing arrangement of NV polymerase molecules in the crystals of the NCT and CTP ternary complexes appears to arrest the normal reaction cycle of NV polymerase at the point immediately prior to nucleotidyl transfer, because there is insufficient space in the crystal lattice to accommodate an elongated RNA oligonucleotide. The fortuitous molecular packing arrangement in the NV RdRP:RNA:NTP crystal form has allowed us to trap a critical intermediate in the reaction cycle without introducing distortions through chemical modifications.

Comparisons between the structures of the NCT and CTP complexes suggest a novel mechanism of inhibition that may be generally exploited for the design of novel antiviral nucleoside analogues. The location of the negatively charged nitro group between the α -phosphate and the 3'-OH nucleophile of the RNA primer is expected to destabilize the transition state of the nucleotidyl transfer reaction. The arrangement of residues at the enzyme active site and base-pairing interactions with the template strand also act to fix the position of the nucleotide base, thus keeping the nitro group close to the negatively charged transition state.

Conclusion

The molecular structures of NV RdRP:RNA:NTP complexes reveal for the first time structural features underlying the enzymatic reaction mechanisms of RdRPs that may be

exploited for developing novel antiviral inhibitors. We have now established an experimental system for determining the structures of RdRP complexes formed with a wide range of substrates and inhibitors. In combination site-directed mutagenesis studies on NV RdRP, future studies are expected to provide additional information on the mechanisms of catalysis and inhibition that will be necessary for the design of more effective antiviral therapeutics.

Reference

1. Zamyatkin, D.F., Parra, F., Alonso, J.M.M., Harki, D.A., Peterson, B.R., Grochulski, P., Ng, K.K.S. (2008) Structural insights into mechanisms of catalysis and inhibition in Norwalk Virus polymerase. *J. Biol. Chem.* 283:7705-7712. Doi:10.1074/jbc.M709563200.

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