Crystallization and preliminary crystallographic study of human coronavirus NL63 main protease in complex with an inhibitor

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Human coronavirus NL63 mainly infects younger children and causes cough, fever, rhinorrhea, bronchiolitis and croup. It encodes two polyprotein precursors required for genome replication and transcription. Each polyprotein undergoes extensive proteolytic processing, resulting in functional subunits. This process is mainly mediated by its genome-encoded main protease, which is an attractive target for antiviral drug design. In this study, the main protease of human coronavirus NL63 was crystallized in complex with a Michael acceptor. The complex crystals diffracted to 2.85 Å resolution and belonged to space group P41212, with unit-cell parameters a = b = 87.2, c = 212.1 Å. Two molecules were identified per asymmetric unit.

1. Introduction

Since the global outbreak of severe acute respiratory syndrome (SARS) in 2003, which was caused by SARS coronavirus (SARS-CoV), human CoVs have been in the spotlight. Shortly thereafter in 2004, another human CoV, named HCoV-NL63, was isolated from the nasopharyngeal aspirate of a seven-month-old child with coryza, conjunctivitis and fever hospitalized in Amsterdam (van der Hoek et al., 2004). It has been shown that HCoV-NL63 mainly infects younger children and immunocompromised persons and causes mild upper respiratory symptoms such as cough, fever and rhinorrhea or more serious lower respiratory tract involvement such as bronchiolitis and croup (Abdul-Rasool & Fielding, 2010). This CoV can be detected in as high as 1.0–9.3% of respiratory tract infections in children worldwide (Vabret et al., 2005; van der Hoek et al., 2006; Fielding, 2011).

Similar to other coronaviruses, HCoV-NL63 contains a single-stranded positive-sense polyadenylated RNA genome that encodes two large polyproteins (pp1a and pp1ab) which are processed into 16 nonstructural proteins (nspl–16) by two virus-encoded proteases. Nsp5, also named main protease (Mpro), is responsible for 11 out of 15 cleavage sites, thus playing a pivotal role in this digestion process, and is indispensable for viral replication (van der Hoek et al., 2004; Pyrc et al., 2004, 2007). The absence of its cellular homologue in humans makes it an ideal target for antiviral drug design (Yang et al., 2005; Anand et al., 2005). To date, several crystal structures of other coronavirus main proteases have been solved (Yang et al., 2003, 2005). The sequence similarity of HCoV-NL63 protease to those of porcine transmissible gastroenteritis virus (TGEV) and SARS-CoV is 60 and 44%, respectively. Based on the structural analysis of these main proteases, the idea of designing wide-spectrum inhibitors against CoVs has been proposed. Here, we report the crystallization and preliminary crystallographic study of human coronavirus NL63 main protease in complex with a designed Michael acceptor inhibitor named N3.
2. Materials and methods

2.1. Protein expression and purification

The coding sequence for HCoV-NL63 main protease was subcloned into pGEX-6P-1 plasmid and verified by sequencing. The recombinant plasmid was then transformed into Escherichia coli strain BL21 (DE3) for protein expression. Cultures were grown in LB medium containing 0.1 mg ml\(^{-1}\) ampicillin at 310 K until the optical density at 600 nm reached 0.6. Isopropyl \(\beta\)-D-1-thiogalactopyranoside was added to a final concentration of 0.5 mM and the cultures were induced to express HCoV-NL63 main protease at 289 K for 16 h. Thereafter, centrifugation was used to harvest the cells and the bacterial pellets were resuspended in PBS (140 mM NaCl, 10 mM Na\(_2\)HPO\(_4\), 2.7 mM KCl, 1.8 mM KH\(_2\)PO\(_4\) pH 7.3) supplemented with 1 mM DTT, 10% glycerol. After sonication at 277 K, the lysate of the bacteria was centrifuged at 12 000 \(g\) for 50 min at 277 K and the precipitate was discarded. The supernatant was loaded onto a disposable column containing glutathione Sepharose 4B affinity resin (Pharmacia) for purifying the GST-tagged HCoV-NL63 main protease. The fusion protein was then subjected to on-column cleavage using commercial PreScission protease (Pharmacia) at 277 K for 18 h. The protease was added to a final concentration of 0.25 mg ml\(^{-1}\) and the buffer used for proteolysis was PBS. The resulting protein of interest was further purified by anion-exchange chromatography using a HiTrap Q column (GE Healthcare) in a linear gradient from 25 to 250 mM NaCl with 20 mM Tris–HCl pH 8.0, 10% glycerol, 1 mM DTT and reached greater than 95% purity as determined by SDS–PAGE analysis (Fig. 1a).

Table 1

<table>
<thead>
<tr>
<th>Data-collection and processing statistics.</th>
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<td>Values in parentheses are for the highest resolution shell.</td>
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- Wavelength (\(\AA\)) 1.0000
- Resolution (\(\AA\)) 50.0–2.85 (2.90–2.85)
- Space group P4\(_2\)2,2
- Unit-cell parameters (\(\AA^3\)) 201.89, 201.89, 211.1
- \(\alpha = \beta = \gamma = 90^\circ\)
- \(\alpha = \beta = \gamma = 90^\circ\)
- No. of observed reflections 283898
- No. of unique reflections 19967
- Matthews coefficient (\(A^3 Da^{-1}\)) 2.06
- Solvent content (%) 59.8
- Average \(I/\sigma(I)\) 21.5 (6.1)
- Completeness (%) 100 (100)
- Multiplicity 14.2 (12.6)
- \(R_{\text{merge}}\) 14.4 (46.6)
- \(R_{\text{merge,l}}\) 14.9 (46.6)
- \(R_{\text{merge,s}}\) 4.0 (13.7)

\(\dagger R_{\text{merge}} = \sum_{i}[I(hkl) - \langle I(hkl)\rangle]/\sum_{i} I(hkl), \) where \(I(hkl)\) is the intensity of the \(i\)th observation of reflection \(hkl\) and \(\langle I(hkl)\rangle\) is the average intensity. \(\ddagger R_{\text{merge}} = \sum_{i} [\langle N(hkl)\rangle]/[\langle N(hkl)\rangle] - 1)^2 \sum_{i} [I(hkl) - \langle I(hkl)\rangle]/\sum_{i} I(hkl). \) § \(R_{\text{merge}} = \sum_{i} [I(hkl) - 1]^2 \sum_{i} [I(hkl) - \langle I(hkl)\rangle]/\sum_{i} I(hkl). \)

2.2. Crystallization

The purified protein was immediately supplemented with 10% DMSO and concentrated to 1 mg ml\(^{-1}\). The previously reported inhibitor N3 (Yang et al., 2005), dissolved in 100% DMSO to a final concentration of 10 mM as a stock, was added to purified protein at a molar ratio between 3:1 and 5:1. After mixing at 277 K for 4 h, the protein complex was centrifuged at 12 000\(g\) for 10 min and exchanged into a buffer consisting of 10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT using Thermo iCON concentrators. The final protein was concentrated to 10 mg ml\(^{-1}\) for crystallization. The method described by Bittle & Curry (2005) was used to screen for crystallization conditions for the protein complex. Generally speaking, the reservoir solution was prepared by mixing the original crystallization condition [0.1 M HEPES pH 5.5, 10% (w/v) polyethylene glycol 8000, 4% (v/v) ethylene glycol] for the apo form (PDB entry 3f10; C. P. Chuck & K. B. Wong, unpublished work) and the commercial Crystal Screen and Crystal Screen 2 kits (Hampton Research, Laguna Niguel, California, USA) in a ratio of 80:20. The hanging-drop vapour-diffusion method was then used to screen for initial crystallization conditions. Crystallization drops were carefully set up by mixing 1.0 \(\mu\)l protein solution with 1.0 \(\mu\)l of the reservoir solution above and were then left in 16-well crystallization plates at 291 K to reach equilibrium. Crystals with typical dimensions of 0.1 \(\times\) 0.05 \(\times\) 0.03 mm appeared after about 48 h using condition No. 11 of Crystal Screen, which consisted of 0.1 M sodium citrate tribasic dihydrate pH 5.6, 1.0 M ammonium phosphate monobasic. These crystals were used for subsequent diffraction and data collection (Fig. 1b).

Figure 1

Purification and crystallization of HCoV-NL63 main protease. (a) SDS–PAGE analysis of purified HCoV-NL63 main protease. The molecular masses of the marker and HCoV-NL63 main protease are indicated in kDa. (b) Typical complex crystals of HCoV-NL63 main protease with inhibitor N3 grown by the hanging-drop method. These crystals with typical dimensions of 0.1 \(\times\) 0.05 \(\times\) 0.03 mm were used for subsequent diffraction and data collection.
2.3. X-ray data collection and processing

The crystal was cryoprotected in a solution consisting of 0.1 M HEPES pH 5.5, 10%(w/v) polyethylene glycol 8000, 4%(v/v) ethylene glycol, 20% glycerol and then mounted in a nylon loop and flash-cooled in a nitrogen stream at 100 K using a MAR165 charge-coupled device detector on beamline 1W2B of the Beijing Synchrotron Radiation Facility (BSRF) with a wavelength of 1.0000 Å. The crystals diffracted to 2.85 Å resolution (Fig. 2). All intensity data were indexed, integrated and scaled with the HKL-2000 package (Otwi-nowski & Minor, 1997). The Rmerge and completeness in the outer resolution shell were less than 50% and greater than 90%, respectively. The diffraction data set was firstly indexed in space group \( P_4 \). Subsequent calculation of the self-rotation function with MOLREP (Vagin & Teplyakov, 2010) using diffraction data between 10 and 3 Å resolution indicated an additional twofold symmetry axis (Fig. 3). The data set was then processed as \( P_422 \) and evaluated using phenix.xtriage (Adams et al., 2010); no twinning or pseudo-translational symmetry were detected. Further aided by the systematic absences, a twofold screw axis along the \( a \) axis and a fourfold screw axis along the \( c \) axis were identified. The following molecular-replacement search over all alternative subgroups of \( P_422 \) helped to determine the space group as \( P_{41212} \) with a TFZ score of 30.5. Based on the molecular weight of the protomer, the Matthews coefficient (Matthews, 1968) was calculated to be 3.06 Å\(^3\) Da\(^{-1}\) and the solvent content was 59.8%, with two molecules per asymmetric unit. A final diffraction data set was collected to 2.85 Å resolution and the related data-collection and processing statistics are summarized in Table 1.

3. Results and discussion

Initially, we crystallized apo-form HCoV-NL63 main protease based on the reported crystallization condition (PDB entry 3tlo) and tried soaking it with the inhibitor N3. However, most of the crystals cracked. Although the remaining crystals, which seemed to be intact, were able to produce high-resolution diffraction patterns, the collected data images could not be indexed. We then turned to an alternative method to co-crystallize the protease with the inhibitor N3 (Birtley & Curry, 2005). The crystals of the protease complex belonged to space group \( P_{41212} \), with unit-cell parameters \( a = b = 87.2, c = 212.1 \) Å and two molecules per asymmetric unit, corresponding to a Matthews coefficient and solvent content of 3.06 Å\(^3\) Da\(^{-1}\) and 59.8%, respectively. Further structural and functional analysis of the HCoV-NL63 main protease in complex with the Michael acceptor N3 will lead to better design and optimization of this lead drug against HCoV-NL63-associated diseases.
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References

