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Crystal structure of the papain-like protease of MERS coronavirus reveals unusual, potentially druggable active-site features

Jian Lei [a,c], Jeroen R. Mesters [a,c], Christian Drosten [b,c], Stefan Anemüller [a,c], Qingjun Ma [a,c], Rolf Hilgenfeld [a,c,*]

[a] Institute of Biochemistry, Center for Structural and Cell Biology in Medicine, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany
[b] Institute of Virology, University of Bonn Medical School, 53127 Bonn, Germany
[c] German Center for Infection Research (DZIF), Germany

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ABSTRACT

The Middle-East Respiratory Syndrome coronavirus (MERS-CoV) causes severe acute pneumonia and renal failure. The MERS-CoV papain-like protease (PL^{pro}) is a potential target for the development of antiviral drugs. To facilitate these efforts, we determined the three-dimensional structure of the enzyme by X-ray crystallography. The molecule consists of a ubiquitin-like domain and a catalytic core domain. The catalytic domain displays an extended right-hand fold with a zinc ribbon and embraces a solvent-exposed substrate-binding region. The overall structure of the MERS-CoV PL^{pro} is similar to that of the corresponding SARS-CoV enzyme, but the architecture of the oxyanion hole and of the S3 as well as the S5 specificity sites differ from the latter. These differences are the likely reason for reduced *in vitro* peptide hydrolysis and deubiquitinating activities of the MERS-CoV PL^{pro}, compared to the homologous enzyme from the SARS coronavirus. Introduction of a side-chain capable of oxyanion stabilization through the Leu106Trp mutation greatly enhances the *in vitro* catalytic activity of the MERS-CoV PL^{pro}. The unique features observed in the crystal structure of the MERS-CoV PL^{pro} should allow the design of antivirals that would not interfere with host ubiquitin-specific proteases.

1. Introduction

Ten years after the outbreak of severe acute respiratory syndrome (SARS) of 2002/2003 (Hilgenfeld and Peiris, 2013), another highly pathogenic coronavirus, Middle-East Respiratory Syndrome coronavirus (MERS-CoV), has been recognized to infect humans (Zaki et al., 2012; de Groot et al., 2013). Accumulating evidence suggests camels to act as a zoonotic source of the virus (Reusken et al., 2013; Haagmans et al., 2014; Meyer et al., 2013). Limited human-to-human transmission of the virus has been described (Assiri et al., 2013). As of June 11, 2014, 683 cases of MERS have been reported, with 204 deaths (http://www.who.int). The clinical symptoms of MERS include severe pneumonia and sometimes acute renal failure (Eckerle et al., 2013). However, the majority of MERS patients had/has comorbidities, such as diabetes, lung disease, or chronic renal disease (Perlman, 2013).

SARS-CoV and MERS-CoV belong to the genus *Betacoronavirus* but pertain to highly distinct phylogenetic clades termed b and c, respectively (de Groot et al., 2013). In case of SARS-CoV, the best-characterized potential antiviral drug targets are the two viral proteases, the main protease (M^{pro}, also called 3C-like protease, $3CL^{pro}$) (Hilgenfeld and Peiris, 2013; Anand et al., 2003; Yang et al., 2003, 2005; Xu et al., 2005; Lu et al., 2006; Verschueren et al., 2008; Zhu et al., 2011; Kilianski et al., 2013) and the papain-like protease (PL^{pro}) (Hilgenfeld and Peiris, 2013; Kilianski et al., 2013; Barretto et al., 2005; Ratia et al., 2006, 2008; Baez-Santos et al., 2014). The latter enzyme exists in all coronaviruses (Woo et al., 2010) and has been shown to be responsible for releasing non-structural proteins (Nsp) 1, 2, and 3 from the N-terminal part of polyproteins 1a and 1ab. The three cleavage sites contain the sequence motif LXGG↓XX. In addition, the SARS-CoV PL^{pro} has been shown to have deubiquitinating and interferon antagonism activities, thereby interfering with the host innate immune response (Barretto et al., 2005; Lindner et al., 2005; Devaraj et al., 2007; Frieman et al., 2009). Specifically, it can prevent the activation of IRF3 (interferon-regulatory factor 3) and antagonize the NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) pathway, but the

* Corresponding author at: Institute of Biochemistry, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany. Tel.: +49 451 500 4060; fax: +49 451 500 4068.
E-mail address: hilgenfeld@biochem.uni-luebeck.de (R. Hilgenfeld).

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detailed mechanisms involved are still unclear. Very recently, the MERS-CoV PL^{pro} has been reported to also have proteolytic, deubiquitinating, and deISG15ylating activities in HEK293T cells (Yang et al., 2013; Mielech et al., 2014) (ISG15 = interferon-stimulated gene 15); it therefore also acts as an interferon antagonist through blocking the IRF3 pathway. Interestingly, these reports differ in their finding that the interferon-antagonizing activity of the MERS-CoV PL^{pro} is either independent of (Yang et al., 2013) or dependent on (Mielech et al., 2014) its proteolytic activity.

In spite of the accumulating knowledge on the essential roles of the coronavirus PL^{pro} in virus replication and evasion of the host-cell innate immune response (Devaraj et al., 2007; Frieman et al., 2009; Yang et al., 2013; Mielech et al., 2014; Lindner et al., 2007; Clementz et al., 2010), the three-dimensional structures of only two of these enzymes have been reported so far, i.e., that of the PL^{pro} from SARS-CoV (Ratia et al., 2006) and that of the PL1^{pro} from Transmissible Gastroenteritis Virus (TGEV) (Wojdyla et al., 2010). Here we present the crystal structure of the MERS-CoV PL^{pro} at 2.50 Å resolution, in order to unravel the structural basis of the activities of the enzyme and facilitate structure-based drug design efforts. In addition, we report the *in vitro* hydrolytic activities of the enzyme towards two synthetic peptide substrates and a fluorogenic ubiquitin derivative.

2. Materials and methods

2.1. Recombinant production of MERS-CoV papain-like protease (PL^{pro})

The PL^{pro} of MERS-CoV (strain 2c EMC/2012; GenBank: AFV09327.1) comprises 320 amino-acid residues, corresponding to Gln1482 – Asp1801 of pp1a, and is part of non-structural protein 3. In the interest of an easy description, we renumber Gln1482 into Gln1 here. A gene coding for the PL^{pro} was amplified by the polymerase chain reaction (PCR) using the forward primer 5′-CTAGCTAGC-cagttaacaatcgaagtcttagtg-3′ and the reverse primer 5′-CCGCTCGAGttaatcgctactgtatttttggccggg-3′. The resulting PCR product was digested with restriction enzymes NheI and XhoI for ligation into pET-28a (Novagen). Cloning was designed to include an N-terminal hexahistidine (His_6) tag and a thrombin cleavage site. The recombinant plasmid was used to transform *Escherichia coli* strain Tuner (DE3) pLacI (Novagen). Transformed cells were grown at 37 °C overnight in LB medium, supplemented with kanamycin (50 μg/mL) and chloramphenicol (34 μg/mL). The culture was used to inoculate LB medium the day after. When the OD_{600} of the culture reached 0.6–0.8, overexpression of the PL^{pro} gene was induced for 20 h with the addition of isopropyl-D-thiogalactoside (IPTG, final concentration 0.5 mM) at 20 °C. Subsequently, the culture was harvested by centrifugation for 30 min at ~7300×*g* and 4 °C. Cells were resuspended in 30 mL buffer A (20 mM Tris–HCl, 500 mM NaCl, 10 mM imidazole, pH 8.8, 10 mM β-mercaptoethanol (BME)) and lysed by sonication on ice. The lysate was centrifuged for 1 h at ~48,000×*g* and 4 °C to remove the cell debris. The supernatant was applied to a HisTrap™ nickel column (GE Healthcare) and the His-tagged protein was eluted with buffer B (20 mM Tris–HCl, 500 mM NaCl, 500 mM imidazole, pH 8.8, 10 mM BME) using standard protocols. The target protein was processed overnight by thrombin (Sigma) cleavage at 4 °C to remove the His-tag. Left with six extra residues (GSHMAS) at the N-terminus after this processing step, the PL^{pro} was further purified by gel filtration (HiLoad™ 16/60 Superdex 200 column, GE Healthcare) using buffer C (20 mM Tris–HCl, 150 mM NaCl, pH 8.8, 10 mM BME).

2.2. Crystallization and diffraction-data collection

Purified PL^{pro} was concentrated to ~11 mg/ml in buffer C. Crystallization was performed at 18 °C by using a Phoenix crystallization robot (Art Robbins) employing the sitting-drop vapor-diffusion method, with mixing 0.25 μl of protein and 0.25 μl of reservoir to equilibrate against 75 μl reservoir solution. The following commercially available screens were used: SaltRx™, PEG/Ion™ 1 & 2 Screen, Index™, and PEG Rx™ 1 & 2 (Hampton Research). Crystals were observed under condition 19 of Index™. Optimized crystals were subsequently obtained within one day using 0.056 M NaH_2PO_4, 1.344 M K_2HPO_4, pH 8.0, and 15% glycerol as reservoir, with mixing 2 μl of protein and 2.5 μl of reservoir to equilibrate against 500 μl reservoir solution.

Crystals were flash-cooled in a 100-K nitrogen-gas stream. A dataset to 2.50 Å resolution was collected using synchrotron radiation at wavelength 0.98 Å at beamline P11 of DESY, Hamburg. Diffraction data were processed with the program *XDS* (Kabsch, 2010). The space group was determined as *C2*, with unit-cell parameters a = 100.89 Å, b = 47.67 Å, c = 88.43 Å, β = 122.35°. Diffraction data statistics are given in Table 1.

2.3. Phase determination, model building and refinement

The structure of the MERS-CoV PL^{pro} was solved by molecular replacement using the program *BALBES* (Long et al., 2008). The program selected molecule A of the SARS-CoV PL^{pro} (PDB: **2FE8**, Ratia et al., 2006) as the most suitable search model. The resulting model for the MERS-CoV PL^{pro} was inspected and rebuilt using *Coot* (Emsley et al., 2010), and refined using *autoBUSTER* (Bricogne et al., 2011). The final refinement statistics are presented in Table 1. Atomic coordinates and structure factors have been deposited in the PDB with accession code **4P16**. All figures except Fig. 3 and

Table 1
Data collection and refinement statistics.

	MERS-CoV PL^{pro}
Data collection statistics	
Space group	C2
Unit-cell dimensions (Å, °)	a = 100.89, b = 47.67, c = 88.43 β = 122.35
Wavelength (Å)	0.98
V_m (Å^3/Da)	2.53
Solvent content (%)	51.34
Resolution range (Å)	42.62–2.50 (2.64–2.50)
Number of unique reflections	12337
R_{merge}	0.059 (0.472)
R_{pim}[1]	0.025 (0.194)
Completeness (%)	99.0 (98.3)
Mean I/σ (I)	19.2 (3.9)
Multiplicity	6.6 (6.8)
Refinement statistics	
R_{cryst} (%)[2]	18.7 (23.6)
R_{free} (%)[2]	23.4 (30.3)
No. of atoms	
Protein	2462
Ligand/ion	1
Water	94
Clashscore[3]	2
r.m.s.deviation in bond lengths (Å)	0.01
r.m.s.deviation in bond angles (°)	1.13
Average *B*-factor for all atoms (Å^2)	61
Ramachandran plot	
Residues in favored regions (%)	96.8
Residues in additionally allowed regions (%)	3.2
Residues in outlier regions (%)	0

[1] R_{pim} (Weiss and Hilgenfeld, 1997).

[2] $R_{cryst} = \sum_{hkl}|F_o(hkl) - F_c(hkl)|/\sum_{hkl} F_o(hkl)$. R_{free} was calculated for a test set of reflections (4.9%) omitted from the refinement.

[3] Clashscore is defined as the number of clashes calculated for the model per 1000 atoms (including hydrogens) of the model. Hydrogens were added by *MolProbity* (Chen et al., 2010).