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X9A, X29

PUBLICATION

I.C. Lorenz, J. Marcotrigiano, T.G. Dentzer, and C.M. Rice, "Structure of the Catalytic Domain of the Hepatitis C Virus NS2-3 Protease," *Nature* **442**, 831-835 (2006).

FUNDING

The National Inst. of Health; the Greenberg Medical Research Inst.; the Swiss National Science Foundation; the Roche Research Foundation; the Swiss Foundation for Medical-Biological Stipends; Life Sciences Research Foundation

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Crystal Structure of the Catalytic Domain of the Hepatitis C Virus NS2-3 Protease

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Hepatitis C virus (HCV) is an important human pathogen leading to cirrhosis and liver cancer. The viral RNA genome encodes a polyprotein that is processed into 10 distinct proteins by action of host cellular and viral proteases. Recently, we have determined the crystal structure of the catalytic domain of the HCV NS2-3 protease, which mediates one cleavage reaction in the viral polyprotein essential for viral replication. NS2 contains a dimeric cysteine protease with a novel fold. Interestingly, the dimer contains a pair of composite active sites, with both monomers contributing residues to each active site. This unusual feature may enable the virus to regulate proteolytic processing and thus the onset of RNA replication. Moreover, the structure may serve as a basis for the development of new antiviral therapies.

An estimated 120 million people are infected worldwide with HCV. Chronic infection is common and causes severe liver damage and hepatocellular carcinoma. A vaccine has not yet been developed, and only 55-60% of the patients respond to the currently available therapies. Therefore, approaches to identify novel drug targets are sorely needed.

The single-stranded, positive-sense RNA genome contains one open reading frame, which codes for ten viral proteins. Three proteins are structural components of the virus particle, whereas the remainder is involved in viral replication and host interactions. Synthesis of the HCV proteins occurs by translation of the viral genome into a polyprotein precursor, which is processed into individual proteins by two host cellular and two viral proteases (**Figure 1**). One of them, the HCV NS2-3 protease, mediates cleavage of the viral polyprotein at the

junction between nonstructural proteins (NS) 2 and 3. This step is required for replication of the viral RNA both *in vivo* and *in vitro*.

We have recently determined the crystal structure of the catalytic domain of the NS2-3 protease, which spans the carboxy-terminal half of NS2 (NS2^{pro}, residues 94-217). The functional unit of NS2^{pro} appears to be a dimer, with each monomer consisting of an amino-terminal alpha-helical subdomain

and a carboxy-terminal subdomain forming an antiparallel beta sheet (**Figure 2a and b**). The two subdomains are connected by an extended linker, resulting in an exchange of the carboxy-terminal subdomains.

Early mutagenesis experiments led to the identification of a putative catalytic triad consisting of amino acid residues histidine 143, glutamate 163, and cysteine 184. In the crystal structure, these residues form an active site with a geometry similar to those of other viral and cellular cysteine proteases. Surprisingly, each active site consists of residues from both monomers: histidine 143 and glutamate 163 are contributed by one monomer, whereas the nucleophilic cysteine 184 originates from the other chain (**Figure 3a and b**). The dimeric form of the protease suggests a mechanism for regulated polyprotein processing and viral RNA replication.

The NS2^{pro} structure corresponds to the post-



Authors (from left to right) Joe Marcotrigiano, Charlie Rice, lab mascot Sadie, Thomas Dentzer, and Ivo Lorenz

cleavage form of the protease. The carboxy-terminal leucine 217 remains coordinated in the active site after cleavage, forming hydrogen bonds with the adjacent atoms of the catalytic cysteine and histidine residues (**Figure 3b**). Binding of leucine 217 to the active site has two implications: (I) the protease becomes inaccessible for other substrates, and (II) NS2 is 'locked' as a dimer, which may have other functions in the viral life cycle after proteolytic processing.

To demonstrate that NS2 can form dimers with composite active sites

in vivo, we used two mutant NS2-3 polypeptides, either containing a histidine 143 to alanine or a cysteine 184 to alanine point mutation in the NS2 active site. Each of these NS2-3 variants expressed individually cannot undergo cleavage at the NS2/3 junction. However, if a composite active site can form, then mixing of the two NS2-3 mutants should lead to the reconstitution of a functional active site, resulting in partial proteolytic processing. This was indeed the case when these mutants were analyzed in a series of experiments in tissue culture. Thus, we were able to demon-

strate the *in vivo* relevance of the mechanism we proposed based on the crystal structure.

Proteolysis through formation of a composite active site has never been observed in a cysteine or serine protease before. However, these features are reminiscent of human immunodeficiency virus (HIV), which encodes a dimeric aspartic protease with a single active site at the dimer interface. Thus, HCV and HIV may have evolved similar strategies to control the timing of events during their viral life cycles.

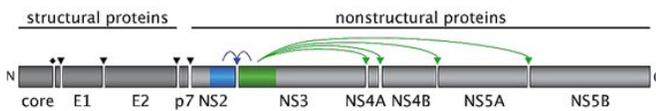


Figure 1. The hepatitis C virus polyprotein. Schematic representation of the HCV polyprotein containing the structural proteins (core, envelope proteins E1 and E2), a small hydrophobic protein (p7), and the nonstructural (NS) proteins (NS2 to NS5B). Cleavages in the structural region and at the amino-terminus of NS2 occur by action of the host signal peptidase (arrowheads) and signal peptide peptidase (diamond). The NS2-3 protease, which consists of residues 94-217 of NS2 (blue) and residues 1-181 of NS3 (green), cleaves at the NS2/NS3 junction (blue arrow). All cleavages downstream of NS3 are mediated by the NS3-4A protease (green arrows). N, amino-terminus; C, carboxy-terminus.

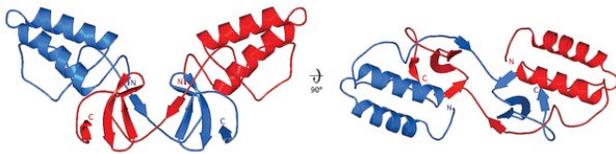


Figure 2. Structure of the NS2 protease domain. **a**, Ribbon diagram showing the structure of the NS2^{pro} dimer, with one monomer in blue, the other in red. **b**, Ribbon diagram of the NS2^{pro} dimer rotated 90° around the horizontal axis in **a**. The amino- and carboxy-termini are labeled.

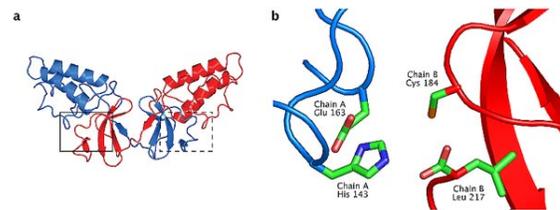


Figure 3. The active site of NS2. **a**, Location of the two active sites in the NS2^{pro} dimer, shown within the boxed regions. **b**, Close-up view of the NS2^{pro} active site in the solid-lined box in **a**. The amino acid residues His 143, Glu 163, Cys 184, and Leu 217 are shown as ball-and-stick drawings. The active site is composed of His 143 and Glu 163 from one molecule of the dimer (chain A, drawn in blue), and Cys 184 from the other molecule (chain B, drawn in red). The C-terminal residue, Leu 217, originates from the same chain as Cys 184.