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Review

# Towards the design of antiviral inhibitors against flaviviruses: The case for the multifunctional NS3 protein from Dengue virus as a target

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ABSTRACT

New treatments are urgently needed to combat the increasing number of dengue fever cases in endemic countries as well as amongst a large number of travellers from non-endemic countries. Of the 10 virus encoded proteins, NS3 (non-structural 3) and NS5 carry out all the enzymatic activities needed for polyprotein processing and genome replication, and are considered to be amenable to antiviral inhibition by analogy with successes for similar targets in human immunodeficiency virus and hepatitis C virus. The multifunctional NS3 protein of flavivirus forms a non-covalent complex with the NS2B cofactor and contains the serine-protease activity domain at its N-terminus that is responsible for proteolytic processing of the viral polyprotein and a ATPase/helicase and RNA triphosphatase at its C-terminal end that are essential for RNA replication. In addition, NS3 seems to be also involved in virus assembly. This review covers the recent biochemical and structural advances on the NS2B-NS3 protease-helicase and presents an outlook for the development of small molecules as antiviral drugs targeting this fascinating multifunctional protein.



**Contents**



*Abbreviations:* DENV, Dengue virus; DHF, dengue hemorrhagic fever; DENV3, dengue virus serotype 3; NS3, non-structural protein 3; NS5, non-structural protein 5; NS2B, non structural protein 2B; NS2A, non-structural protein 2A; RdRp, RNA-dependent RNA polymerase; FL NS3, full-length NS3; nt, nucleotide; ss, single strand; ds, double strand.

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## 1. Introduction

Several members of the *Flavivirus genus* [Tick-Borne encephalitis (TBE), Yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV) and Dengue virus (DENV)] are important human pathogens that are transmitted by an arthropod (tick or mosquito). While prophylactic vaccines exist for YFV (Theiler and Smith, 1937) and JEV (Oya and Kurane, 2007), no vaccines against DENV have reached the market yet, despite several decades of intensive efforts. It is estimated that 50–100 million cases of dengue fever occur in the tropical and sub-tropical regions of the world, and the infected cases exhibit a broad spectrum of clinical symptoms, ranging from being fully asymptomatic to causing life-threatening conditions like dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). Current treatments are mostly symptomatic, and they make use of analgesics for the containment of fever and fluid replacement to address the vascular leakage in DHF and DSS patients (Halstead, 1988, 2007; Fink et al., 2006). The resurgence of dengue and the very rapid manner in which the related WNV spread in the United States of America has led to renewed efforts in research activities towards finding safe vaccines and chemotherapeutics. Additionally, the recent determination of complete genomic sequences for *Aedes Aegypti*, the principal dengue virus vector, may open new perspectives for a better vector control using insecticides that are less harmful to the environment (Nene et al., 2007; Boonserm et al., 2006). Other approaches such as the use of genetically modified sterile male mosquitoes to control the vector population may require detailed environmental impact studies (Phuc et al., 2007).

The dengue diseases are caused by the four antigenically distinct dengue virus serotypes, DENV 1–4. All available evidence suggests that the two virus encoded non-structural proteins NS3 and NS5 which contain the key enzymatic activities required for RNA replication are sufficiently conserved within the four serotypes to permit the design of compounds that will be active against all dengue virus strains and also other related flaviviruses such as YFV, WNV and JEV (Li et al., 2005; Keller et al., 2006). As members of the *Flaviviridae* family, it is also possible to make interesting comparisons with the ongoing efforts to develop drugs against hepatitis C virus (HCV) although Dengue provokes an acute self-limiting illness, while HCV provokes a chronic disease that can potentially lead to hepatocarcinoma. Given the short period of viremia, the question that is often posed is—what benefit would an antiviral compound against dengue have in treating the patient? Epidemiological studies have indicated that serious dengue diseases are often associated with higher levels of circulating virus (Libraty et al., 2002; Gubler et al., 1981), therefore if dengue fever is diagnosed at an early stage through the identification of the viral genome by RT-PCR or the presence of circulating NS1 proteins in the blood, an antiviral treatment could be given that would limit the viral load in the patient, and hence reduce the severity of the disease (Keller et al., 2006). Second, in the case of epidemic outbreaks, one could also envision prophylactic treatments targeting clusters of fever patients identified through decision tree algorithms by using simple clinical and hematological parameters (Tanner et al., 2008).

The goal to find novel antiviral compounds directed at dengue enzyme targets that are essential for viral replication required detailed 3D structural studies of the two multifunctional proteins NS3 and NS5. Here, we review the recent determination of 3D structures of the major replicative enzymes from flaviviruses and focus on the NS3 protein. The 618 amino acid residues long protein contains a serine-protease domain at its N-terminus that requires formation of a non-covalent complex with the NS2B membrane-bound cofactor for this activity, and an ATP-driven helicase and RNA triphosphatase at its C-terminal end (Fig. 1). Interestingly, the NS3 appears to be also involved in virus assembly independently of its known enzymatic functions outlined above (Khromykh et al., 2001; Patkar and Kuhn, 2008).

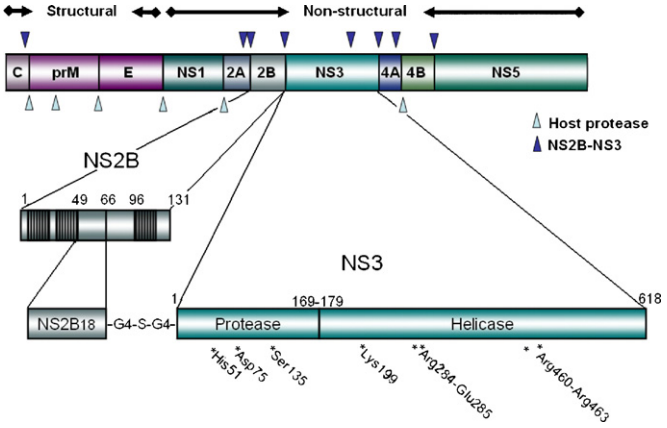


**Fig. 1.** Schematic representation of the flaviviviral polyprotein, with the cleavage sites processed by NS2B-NS3pro indicated by arrows. Also shown is the partition of the various functional domains along the primary sequence of NS2B-NS3. Predicted membrane associated regions within the NS2B proteins are represented as filled boxes. Evolutionary conserved residues for NS3 enzymatic activities are indicated.

## 2. Overview of the Dengue virus life cycle

The dengue viral particle contains a lipid bilayer surrounding a capsid that packages the positive-strand RNA genome of 10.7 kb (for a review see Lindenbach et al., 2007; Kuhn and Strauss, 2003). The viral RNA contains a type I methyl-guanosine cap structure at its 5′-end but is devoid of a poly-adenylate tail (Ray et al., 2006; Egloff et al., 2002; Cleaves and Dubin, 1979; Wengler et al., 1978). After attachment to the cell surface (Chu et al., 2005; Fink et al., 2006), viral entry proceeds through the endocytic pathway and upon acidification in the endosome, major structural changes occur in the surface envelope glycoprotein E, a class I viral fusion glycoprotein (Lescar et al., 2001; Roussel et al., 2006), leading to the merging between the host and virus lipid membranes (Modis et al., 2004; Bressanelli et al., 2004) and release of the viral genomic RNA into the cytoplasm of the host cell (see Fig. 2 for an overview of the life cycle). The genomic viral RNA serves as mRNA and can thus be directly translated – as a single open reading frame – by the host cell translation machinery into a single polyprotein precursor of approximately 370 kDa. This polyprotein is cleaved co- and post-translationally into three structural (C-prM-E) and seven non-structural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5). Both the viral NS2B-NS3 enzyme (on the cytoplasmic side of the ER membrane) and cellular proteases (in the endoplasmic reticulum) take part in this process which involves both *cis*- and *trans*-cleavage events (Lindenbach et al., 2007; Bera et al., 2007). Once the viral polymerase NS5 has been synthesized and released from the polyprotein precursor, the viral RNA can be transcribed, starting from the 3′-end, into the complementary minus strand RNA, which is then transcribed back into a positive strand. A dsRNA transient intermediate is thus formed during this process that must be separated into its individual strands in order to give access to the NS5 polymerase for further rounds of viral genome replication.

Very elegant work by Gamarnik and others (Filomatori et al., 2006; You et al., 2001; You and Padmanabhan, 1999) have detailed the structural requirements for the RNA molecule that ensure viral RNA synthesis. These include a promoter region located at the 5′-end of the genome to which the NS5 polymerase directly binds and long range RNA–RNA interactions between stem-loop forming

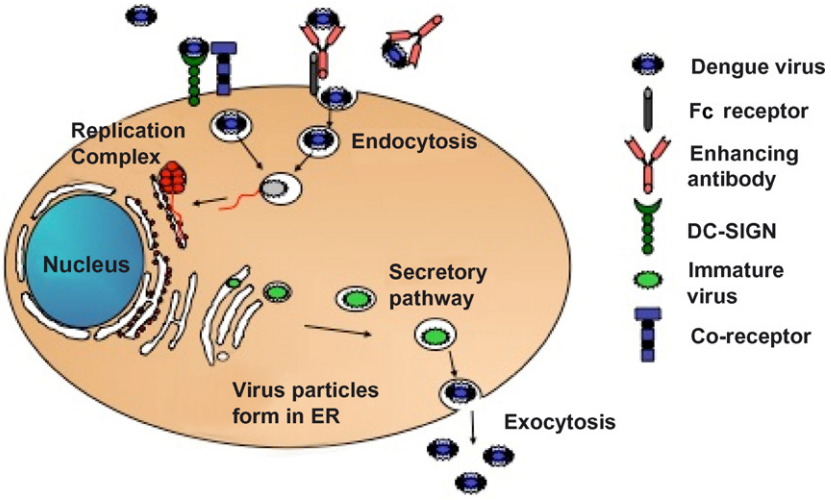


**Fig. 2.** Overview of the Dengue virus life cycle. The figure adapted from Fink et al. (2006) illustrates the multiple ways in which the virus can attach to the cell, followed by endocytosis, membrane fusion and nucleocapsid release. The plus strand RNA is the template for polyprotein synthesis and via a replicative dsRNA, forms the template for further RNA synthesis in the cytoplasm. The NS2B-NS3 function in polyprotein processing and RNA replication is critical to viral the viral life cycle.

motifs located at the 5′ and 3′-end of the viral genome, leading to its cyclization. The NS3 protein which physically associates with the NS5 polymerase (Yon et al., 2005; Johansson et al., 2001; Chen et al., 1997; Kapoor et al., 1995) appears to play an essential role in the viral life cycle since mutagenesis studies demonstrated that impairment of either its proteolytic activity or its NTPase/helicase activity led to a defective genome unable to produce infectious viral particles (Matusan et al., 2001). In the following section we review recent biochemical and structural data that have been published on this multifunctional enzyme

## 3. The NS2B-NS3 protease

The virus encoded serine protease lies within the N-terminal 180 amino acid residues of the 618 amino acid residues long multifunctional NS3 protein (Fig. 1). Heterologously expressed NS3 protease domain (NS3 pro) or NS 3 full-length (NS3 FL) has the tendency to form aggregates, and is inactive as a protease. The central hydrophilic portion (spanning residues 49–95) of the integral membrane protein NS2B is required to form the active protease (Arias et al., 1993; Falgout et al., 1991, 1993; Li et al., 1999; Yusof et al., 2000; Leung et al., 2001; Li et al., 2005; Fig. 1). Although crystallographic structures have been reported for NS3 pro that was obtained after refolding (Murthy et al., 1999), they did not provide any insights on how the essential cofactor, NS2B, activated the protease (see Table 1 for a list of structures of flaviviral NS3 and its domains).

The expression of soluble and active protease was achieved when the hydrophilic portion of the NS2B viral cofactor spanning residues 49–95 (hereafter named CF40) of either WNV or DENV2 was fused to amino-acids 1–169 of the NS3 protein via a flexible ($Gly_4$-Ser-$Gly_4$) linker (Leung et al., 2001). Extending this work, Li et al. (2005) cloned and expressed the protease from all four dengue serotypes (DENV 1–4 CF40- $Gly_4$-Ser-$Gly_4$-NS3pro) and adapted the *in vitro* assay described by Yusof et al. (2000) to screen a tetrapeptide library containing more than 130,000 substrates. The tetrapetide benzoyl-norleucine (P4)-lysine (P3)-arginine (P2)-arginine (P1)-ACMC (Bz-Nle-Lys-Arg-Arg-ACMC) was identified as the optimal substrate with the steady state kinetics parameter $k_{cat}/K_m$ of 51,800 $M^{-1}$ $s^{-1}$ which is >150-fold more sensitive than other published peptides. The sensitivity enabled miniaturization of assay for high-throughput screening (Keller et al., 2006). Also this ideal tetrapeptide sequence also formed the basis for the peptidomimetic approach for finding potent substrate-based inhibitors (Yin et al., 2006a,b).

Starting with the tetrapeptide aldehyde, Bz-Nle-Lys-Arg-Arg-H, which was found to be a competitive inhibitor of both the protease domain and the full-length NS3 protein with its cofactor (CF40- $Gly_4$-Ser-$Gly_4$-NS3FL) with $K_i$ of 5.8 μM and 7.0 μM respectively, a structure–activity relationship (SAR) study was carried out to find more potent inhibitors. The study underlined the relative importance of the various side chains in the binding pocket and also highlighted such features as the possibility to replace the P1 charged residue with a neutral group (Yin et al., 2006b), thus opening the possibility of developing compounds that extend these finding using strategies that have been used to overcome the shallow binding pocket of the HCV protease.

A breakthrough in understanding the interaction between NS3 and NS2B was made using the tetrapeptide aldehyde inhibitor NS3 and NS2B (Erbel et al., 2006) when structural studies revealed that upon substrate binding, the central hydrophilic portion of the NS2B cofactor refolds to form an important component of the protease catalytic site. Two structures were described at high resolution by Erbel et al. (2006) that would be critical for finding drugs that can target flaviviral protease: the apo-enzyme from DENV2 and a complex formed between the WNV NS2B-NS3Pro enzyme and tetrapeptide aldehyde inhibitor Bz-Nle-Lys-Arg-Arg-H,. In both structures, the N-terminal part of the NS2B cofactor (residues 49–66) forms a β-strand which is inserted into the N-terminal β-barrel of the protease concealing hydrophobic residues from the solvent and providing stabilization to this domain (Fig. 3). This explains the observed strong tendency for NS3 pro and FL NS3 to aggregate when the strand contributed by NS2B is absent in synthetic constructs. In this respect the N-terminal of NS2B has a chaperone-like role in stabilizing NS3. On the other hand the conformation of the C-terminal part of the NS2B cofactor appears totally different between the apo- and inhibitor-bound enzyme: a large rearrangement brings residues 66–88 of NS2B in close proximity to the substrate-like inhibitor, forming a belt that braces the second chymotrypsin-like β-barrel. Amino-acids Arg78-Leu87 of the NS2B cofactor affect the formation of the active site by contributing a β-hairpin that inserts into the C-terminal β-barrel thus reorienting residues for substrate recognition. Using NMR experiments in solution, the authors could demonstrate that this C-terminal domain is disordered in the absence of the substrate-like inhibitor but becomes ordered upon binding (Erbel et al., 2006).

**Table 1**
Published crystal structures of helicases (Ns3hel) or proteases (NS2B-NS3pro) catalytic domains from flaviviruses

| Structure | Flavivirus | PDB code | Resolution (Å) | Reference |
|---|---|---|---|---|
| NS2B-NS3pro | WNV | 2GGV | 1.8 | Aleshin et al. (2007) |
| NS2B-NS3pro + aprotinin/BPTI | WNV | 2IJO | 2.3 | Aleshin et al. (2007) |
| NS2B-NS3pro | DENV2 | 2FOM | 1.5 | Erbel et al. (2006) |
| NS2B-NS3pro + Bz-Nle-Lys-Arg-Arg-H | WNV | 2FP7 | 1.7 | Erbel et al. (2006) |
| NS3hel | YFV | 1YKS | 1.8 | Wu et al. (2005) |
| NS3hel + ADP | YFV | 1YMF | 2.6 | Wu et al. (2005) |
| NS3hel | DENV2 | 2BMF | 2.4 | Xu et al. (2005) |
| NS3hel | MVE | 2V8O | 1.9 | Mancini et al. (2007) |
| NS3hel | KUN/WNV | 2QEQ | 3.1 | Mastrangelo et al. (2007) |
| NS3hel | Kokobera | 2V6I | 2.1 | Speroni et al. (2007) |
| Ns3hel | JEV | 2Z83 | 1.8 | Yamashita et al. (2008) |
| NS2B18-NS3FL[a] | DENV4 | 2VBC | 3.1 | Luo et al. (2008) |

[a] In this case, the full-length protease-helicase was crystallized.

Thus, the conformation adopted by the C-terminal segment of NS2B in the apo-enzyme crystal structure is probably only one amongst those that are accessible in solutions and appears dictated by crystal packing forces. The dual role of the 40 amino acid residues long NS2B cofactor region to both stabilize the protein and form the active site of the protease is indeed unique to flaviviruses and sets it apart from HCV NS3 protease which requires only a 12 amino acid sequence from NS4A to form the active protein.

The unprecedented way in which the NS2B cofactor region forms a belt around the protease domain was recently confirmed in a second structure that was reported for the WNV enzyme as a complex with the aprotinin/BPTI inhibitor (Aleshin et al., 2007). The aprotinin occupies all of the specificity pockets of the protease and induces a fully formed oxyanion hole, which allowed Aleshin et al. (2007) to provide a complete view of the enzyme substrate Michaelis complex for a flavivirus protease. These structures open up new opportunities for discovering flavivirus specific drugs that could function by interfering with protein–protein interactions that are needed for the activation of the protease in addition to active site directed competitive inhibitors. Johnston et al. (2007) reported the search for small molecular weight inhibitors of flaviviral NS3 protease. The high through-put screening with WNV NS2B-NS3 protease and the work-up of the hits from the primary screen that was described by the authors is typical of the process carried out in the non-academic settings with libraries that consists of more than a million compounds. Interestingly Johnston et al., were able to select a common scaffold from their primary screen that was further improved by targeted chemical modification during lead optimization to identify a class of [5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl)] compound that is suggested to function by blocking the NS2B binding pocket within NS3 thereby preventing the productive interaction between the two proteins that is needed for the protease activity. The 3D structures of the two-component NS2B-NS3 protease from WNV and DENV together with the advances in assembling sensitive and robust miniaturized assays using optimized substrates makes the flaviviral protease an attractive antiviral target that has the potential to deliver novel, potent and selective inhibitors.

## 4. The NS3 NTPase/helicase

The C-terminal region of NS3 forms the RNA helicase domain (Figs. 2, 4 and 5). Like many other cellular enzymes modifying the topology of nucleic acids, it is an ATP-driven molecular motor. The precise role of the helicase domain during the viral life cycle however, is not known with certainty: it is generally thought to separate into individual strands the dsRNA intermediate that is transiently formed during the polymerization reaction catalyzed by the NS5

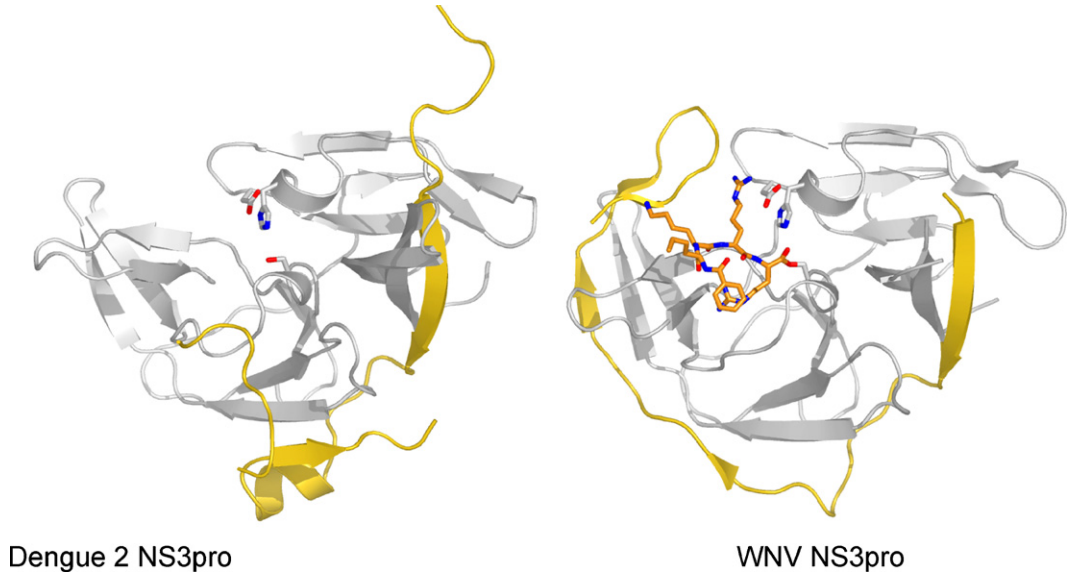


**Fig. 3.** Schematic representation of the NS2B-NS3 protease. (Left) The apo-enzyme from DENV2 with the NS2B cofactor in yellow and the catalytic triad represented as sticks. (Right) Complex of WNV NS2B-NS3 protease with the KKRR tetrapeptide. The N-terminal part of the NS2B cofactor (49–66) is sufficient to stabilize the enzyme (Erbel et al., 2006).

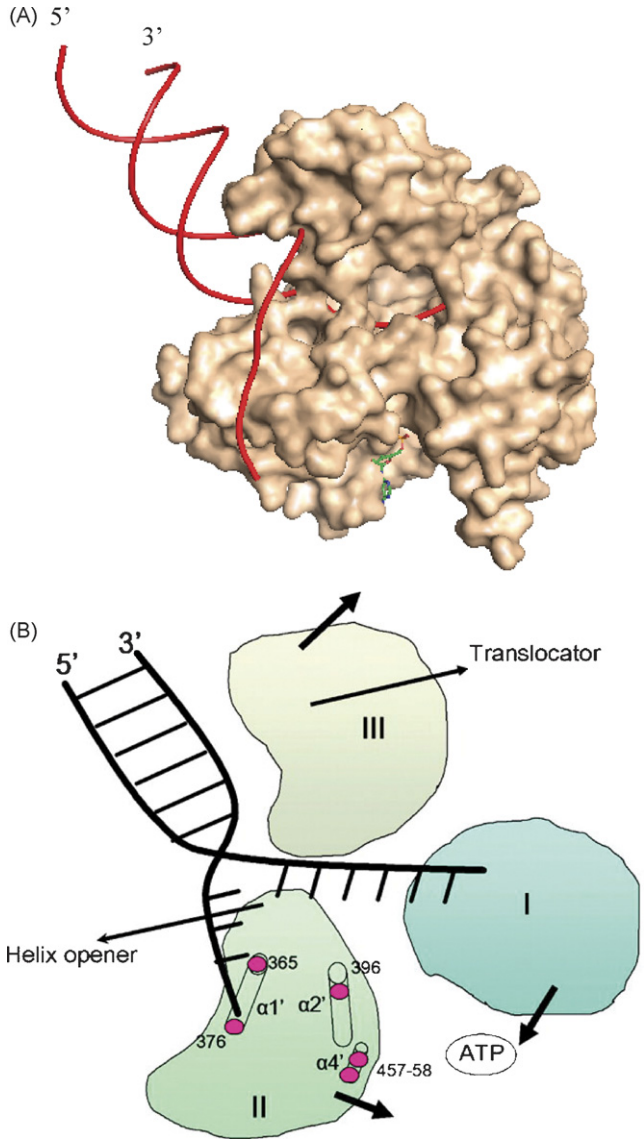


**Fig. 4.** Surface representation of the NS3 ATPase/helicase structure with a dsRNA substrate. (A) We propose that the enzyme translocates along the 3′ overhang of the duplex which is buried in the tunnel that separates subdomain 3 (top) with subdomains 1 and 2 (bottom) which form the ATP binding site. (B) The schematic model for Den NS3 helicase translocation and unwinding activity. The thick arrows indicate the possible hinge interdomain motion during the RNA translocation and unwinding (Sampath et al., 2006).

viral polymerase (Malet et al., 2007; Yap et al., 2007a,b), thus facilitating further rounds of genome replication and transcription. It could also disrupt secondary structures formed by the ssRNA template or displace other host or viral proteins that are bound to viral RNA in order to facilitate its replication. The ATPase activity of NS3 is stimulated by the presence of RNA highlighting the extensive cross-talk between the various catalytic centers of the NS3 enzyme.

In back-to-back papers, structures were first reported for the NS3 helicase/nucleoside triphosphatase catalytic domains from DENV2 and YFV (Xu et al., 2005; Wu et al., 2005). Subsequently a number of NS3 structures for other flavivirus helicases have been published (Table 1). Each of these proteins comprises three subdomains of approximately 140 residues. All seven amino-acid sequence motifs including motif I (also known as Walker A or phosphate binding loop or P-loop) which have been identified for the members of the SF2 superfamily of helicases (Gorbalenya and Koonin, 1993) are located within subdomains 1 and 2. In spite of a very low sequence identity, both subdomains adopt the same α/β-fold that was first seen in the RecA protein (Story and Steitz, 1992). The ATP binding site is housed between these two subdomains. The ATP binding mode was initially visualized through the soaking of ATP (even though only ADP could be located in the binding site with low occupancy as shown by very high crystallographic temperature factors) and model building by comparison with other ATP-driven DNA helicase. Both methods concurred to propose that the ATP was primarily held through its triphosphate moiety via contacts mediated through residues emanating from the P-loop and the DEAH motif II (or Walker B motif). In the ATP binding pocket, a Mg ion (which can be substituted by a Mn ion for the enzymatic reaction) is coordinated in a octahedral manner by the γ- and β-phosphate oxygen atoms from ATP, two equatorial water molecules and oxygen atoms from residues Glu-285 (motif II) and Thr 200 (motif I) (Xu et al., 2005; Luo et al., 2008). Interestingly, no aromatic residues from the protein participate in stacking the base moiety as seen in several other ATP binding sites: the base essentially bulges out from the site and can adopt several conformations, an observation consistent with the observed lack of discrimination between various nucleotides.

We have recently obtained high resolution structures for the DENV4 helicase bound to ADP and AMPNP which show that the ribose 3′-OH makes direct contacts with the protein while the 2′-OH makes a contact mediated by a water molecule. Atomic details for these structures will be reported in a separate manuscript (Luo et al., 2008, in preparation). Whereas the ATP binding domain is structurally well conserved compared to other SF2 helicases including the one from HCV, subdomain 3 of the flavivirus helicase is the most divergent (Xu et al., 2005). It is tempting to try to reconcile this observation with the structural divergence that is observed between the respective polymerase enzymes and the way they associate with NS3 subdomain 3 for the formation of the replication complex (Johansson et al., 2001; Kapoor et al., 1995). A conspicuous structural feature is the groove located at the interface between the three subdomains. Based on previous pioneering work on the NS3 helicase from HCV (Kim et al., 1998) this groove was proposed to house a single stranded nucleic acid substrate of approximately 6–8 ribonucleotides that would form the 3′ overhang needed by the enzyme to translocate along the substrate during the unwinding reaction. Using site-directed mutagenesis on the full-length NS3 enzyme, Sampath et al. (2006) examined the effect on the ATPase, helicase and RTPase activities of Ala substitutions in subdomain 2. Since an excellent correlation was found between ATPase and RTPase activities for the various mutants studied, the two reactions were proposed to share the same site confirming previous studies (Benarroch et al., 2004; Bartelma and Padmanabhan, 2002). Mutants studied could be partitioned into three categories: those that affect either the helicase or the ATPase (and hence also the RTPase activity) and those which affect both the activities (e.g. Lys396Ala mutation). The bad news for drug design was the existence of strong residual helicase activities that were ATPase independent (e.g. the double mutant Arg457–458Ala and the single mutants Arg460Ala and Arg463Ala retain more than 60% of the wild-type helicase activity; Sampath et al., 2006; Xu et al., 2006). Considering the paucity of interactions provided by the sugar and base moieties in the ATP binding site, this observation does not provide strong encouragement to target the ATP binding site for the design of competitive inhibitors. The NS3 enzyme cycles between different stages representing NTP binding, hydrolysis and product release:

$$E \rightarrow E + NTP \rightarrow E + NDP + Pi \rightarrow E + NDP \rightarrow E$$

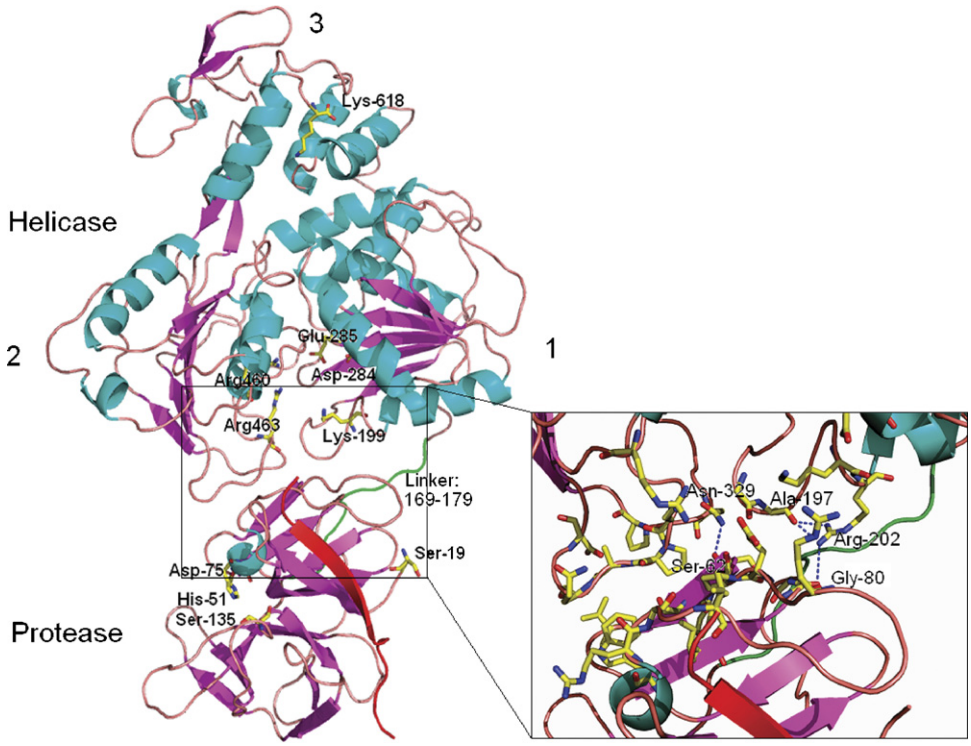


**Fig. 5.** Ribbon representation of the FLNS3 structure. The three subdomains of NS3hel are numbered. The NS2B cofactor is colored in red. The region linking the protease and helicase (residues 169–179) is colored in green. Key residues for NS3 enzymatic activities are shown as sticks and labeled. N-terminal residues are labeled and an expanded view of the interface between the protease and helicase domains is also shown (Luo et al., 2008).

These reactions must be accompanied by conformational changes in the helicase including in its RNA binding site(s) leading to changes in its affinity for the RNA that are necessary for unidirectional translocation. These conformational changes also drive the unwinding in a stepwise fashion. A working model can be proposed for the ATP-coupled reaction performed by NS3 (Fig. 4) with the duplex RNA located in the concave surface formed between subdomains 2 and 3 and the enzyme progressing along the 3′ overhang. Interestingly, Arg-376 and Lys-396 belong to helix α1′ and α2′ of domain II respectively (Fig. 4B). The question is whether NS3 comprises two spatially distinct RNA binding sites. The region at the interface of domain 2 and 3 and especially a β-hairpin with exposed hydrophobic residues that projects from subdomain 2 could act as the "helix opener" by disrupting hydrogen bonds at the fork either in a passive (through thermal fraying) or active manner. The basic concave face between domains II and III would act as "the translocator" by binding dsRNA ahead of the fork (Fig. 4B). The pocket next to Ile-365 (which completely abolished the helicase activity when mutated to Ala) appears as an attractive region for the design of small molecules that would lock the helicase in a non-functional conformation (Sampath et al., 2006). Important mechanistic question still remain as for the number of base pairs separated at each step and the amount of ATP needed for the reaction. A key issue in the near future is to also define precisely the various structural states assumed by the NS3 enzyme along the reaction pathway. Such knowledge should undoubtedly assist the design of "allosteric inhibitors" aimed at hindering such structural transitions.

## 5. The full-length NS3 protease-helicase

Why has evolution led to the fusion we observe today between the protease and ATPase/helicase domains for *flaviviridae* NS3 enzymes? While this question cannot be answered with certainty, some arguments appear plausible: Colocalization of these two activities (and furthermore with the NS5 RNA polymerizing activity through non-covalent contacts) is probably beneficial for an efficient coupling of polyprotein proteolysis, release of individual proteins and immediate initiation of viral RNA replication. Regulation of the protease activity via *cis* interactions might also be at play, as was first shown for the HCV protease-helicase (Yao et al., 1999). However, to find a full answer to the question, structural studies of the whole "replication complex" of a flavivirus will be required (Westaway et al., 1999). Such studies will likely involve a combination of structural techniques giving information at various – complementary – resolution as published recently for the flock house virus which is also a plus strand RNA virus (Kopek et al., 2007).

The observation that the protease domain had a strong influence on both its ATPase by decreasing the rate of ATP hydrolysis and helicase activities by increasing the unwinding reaction by a factor of ~30-fold, provided the impetus for the structure determination of the full-length NS3 enzyme using X-ray crystallography. Another intriguing question was the relative orientation adopted between the protease and helicase domains and the assessment of possible interdomain flexibility in solution between these domains. This question was addressed using a construct encompassing the 18 residues that form the N-terminal β-strand of NS2B fused via a $Gly_4$ Ser $Gly_4$ linker to the complete NS3 protease-helicase domain spanning residues 1–618. The crystallographic structure determined to a resolution of 3.1 Å (Luo et al., 2008) revealed a relative orientation between the protease and helicase domains that was drastically different compared to the homologous engineered single-chained NS3-NS4A molecule from HCV, which was caught in the act of *cis*-cleavage at the NS3-NS4A junction (Fig. 5). The protease domain partially occludes access to the ATP

binding site, giving the molecule a rather elongated shape that was in agreement with an envelope determined *ab-initio* using small angle X-ray scattering in solution. Measurement of the affinity for ADP and ATP performed using Fluorescence Correlation Spectrometry in the presence of $Mg^{2+}$ showed that the affinity of the FLNS3 for nucleotides was approximately 10 fold higher than for the isolated helicase domain (comprising residues 178–618). This effect was attributed to electrostatic effects brought about by a basic patch located at the surface of the protease domain which could participate in RNA binding, explaining the higher helicase activity of the full-length enzyme compared to the isolated helicase domain that was also shown biochemically (Yon et al., 2005). Furthermore recent studies showed that NS3 helicase and NS3 protease-helicase from WNV could unwind both DNA and RNA, but when NS2B is present the full-length NS3 protease-helicase can unwind only the RNA substrate (Chernov et al., 2008). Therefore the co-localization of the protease and helicase in flavivirus NS3 not only increases the affinity for ATP but it also restricts the substrate specificity to RNA when the NS2B cofactor is present thus making the protein a more efficient RNA helicase.

## 6. Possible role played by NS3 other than its known enzymatic functions

Apart from its well-documented enzymatic roles, the NS3 protein of YFV has been proposed to participate in viral assembly (Patkar and Kuhn, 2008). The substitution of an evolutionary conserved Trp residue in an exposed loop of subdomain 2 leads to the release of non-infectious viral particles even though RNA replication itself is not affected by this point mutation (in YFV Tr349Ala). Interestingly the same subdomain 2 was proposed to interact with the NS2A protein and this interaction might be required for genome packaging. Thus, in addition to its known enzymatic functions, the NS3 protein appears to be involved in the assembly of an infectious flaviviral particle, through its interactions with NS2A and presumably host cell proteins (Patkar and Kuhn, 2008; Khromykh et al., 2001). Interestingly, an involvement of the NS3 helicase domain from HCV in the assembly of infectious intracellular particle has also been demonstrated (Ma et al., 2008).

## 7. Conclusion

In summary, the stage is now set for a more informed structure-based drug discovery program targeting the replicase enzymes from Dengue virus. It is interesting to draw a parallel with a similar undertaking targeting HCV enzymes started about 10 years ago with several compounds directed against the protease and polymerase now in clinical trials and neither helicase nor ATPase inhibitor having reached this stage. In the case of the Dengue virus, additional considerations regarding the cost of synthesis of a drug that should be affordable to poor patients in developing countries brings in an additional complexity for such development (Keller et al., 2006). The structures of the active protease, helicase and FL NS3 from DENV suggest that a compound that would hamper the dynamics of this multifunctional enzyme seems attractive. Such a compound could for instance target the entrance of the RNA binding tunnel (towards the 5′-end of the RNA substrate) which is lined by two mobile helices or prevent protein–protein interactions between NS3 and NS2B. A challenging aspect to finding potent and selective antiviral that function through limiting the dynamics of multifunctional NS3 protein is the design of appropriate assays that will capture druggable sites that are relevant *in vivo* in the viral replication complex.

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