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Structural features of Zika virus non-structural proteins 3 and -5 and its individual domains in solution as well as insights into NS3 inhibition

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ABSTRACT

Zika virus (ZIKV) has emerged as a pathogen of major health concern. The virus relies on its non-structural protein 5 (NS5) including a methyl-transferase (MTase) and a RNA-dependent RNA polymerase (RdRp) for capping and synthesis of the viral RNA and the nonstructural protein 3 (NS3) with its protease and helicase domain for polyprotein possessing, unwinding dsRNA proceeding replication, and NTPase/RTPase activities. In this study we present for the first time insights into the overall structure of the entire French Polynesia ZIKV NS3 in solution. The protein is elongated and flexible in solution. Solution studies of the individual protease- and helicase domains show the compactness of the two monomeric enzymes as well as the contribution of the 10-residues linker region to the flexibility of the entire NS3. We show also the solution X-ray scattering data of the French Polynesia ZIKV NS5, which is dimeric in solution and switches to oligomers in a concentration-dependent manner. The solution shapes of the MTase and RdRp domains are described. The dimer arrangement of ZIKV NS5 is discussed in terms of its importance for MTase-RdRp communication and concerted interaction with its flexible and monomeric counterpart NS3 during viral replication and capping. The comparison of ZIKV NS3 and -NS5 solution data with the related DENV nonstructural proteins shed light into the similarities and diversities of these classes of enzymes. Finally, the effect of ATPase inhibitors to the enzymatic active ZIKV NS3 and the individual helicase are provided.

1. Introduction

Zika virus (ZIKV) is an ongoing global public health emergency with 70 countries and territories reporting evidence of ZIKV transmission. First isolated in 1947 from a sentinel rhesus monkey in Uganda (Dick et al., 1952), ZIKV circulated enzoonotically within Africa and equatorial Asia as two distinct lineages: the African and Asian (Haddow et al., 2012). Prior to 2007, only 14 sporadic human infections, confined to Africa and equatorial Asia, were documented (Musso and Gubler, 2016). Since then, three outbreaks of ZIKV belonging to the Asian lineage have ensued: in 2007, on Yap island within the Federated States of Micronesia, in 2013 and 2014, within the French Polynesian islands and most notably, the current large outbreak in Brazil in 2015 (Maurer-Stroh et al., 2016). Unlike the Yap island outbreak which was characterised by cases with relatively mild dengue-like symptoms (Duffy et al., 2009), the outbreaks in French Polynesia and Brazil coincided with an unusual rate of cerebral congenital anomalies, including microcephaly (ECDC, 2016).

ZIKV belongs to the Flavivirus genus which contains important human pathogens such as dengue (DENV), yellow fever (YFV), West Nile (WNV), Japanese encephalitis (JEV) and tick-borne encephalitis (TBEV) viruses (Pierson and Diamond, 2013). Its single-stranded RNA genome encodes a single polyprotein, which, by analogy to other flaviviruses, is assumed to be cleaved by host-cell proteases and the viral NS2B/NS3 protease into three structural (C, prM/M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). Like for other flaviviruses, replication of the ZIKV genome takes place in the ER-membrane associated replication complexes (RC), including NS- and host proteins (Davidson, 2009),

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and with NS3 and NS5 residing in the functional center. NS3 possesses protease activity (with cofactor NS2B) for polyprotein cleavage, helicase activity for unwinding dsRNA proceeding replication, and NTPase/RTPase activities essential for genome capping (Li et al., 2014). The N-terminal residues 1-169 of the 71 kDa ZIKV NS3 (618 residues) form the protease, which is connected by a 10-residues linker to the helicase (Supplementary Fig. S1A). The protease requires the central domain of NS2B as a cofactor for proper folding (Lei et al., 2016). The helicase (residues 179–618) possesses NTPase-dependent RNA helicase activity, which may unwind the double-stranded RNA during RNA replication using the energy supplied by the NTP hydrolysis (Jain et al., 2016; Lindenbach et al., 2007; Luo et al., 2015; Warrener et al., 1993).

The flavivirus NS5 harbors multiple enzymatic activities: RNA-dependent RNA polymerase (RdRp) activity required for genome replication and methyltransferase (MTase)/guanylyltransferase (GTase) activities for genome capping (Egloff et al., 2002; Issur et al., 2009). The cap, a 7-methylguanosine (m7G) moiety linked to the first nucleotide of the transcript via a 5′–5′ triphosphate bridge, has the roles to protect viral mRNA from degradation by 5′ exoribonucleases, to confer stability to mRNAs, and to ensure their efficient recognition by eukaryotic translation initiation factor 4E (eIF4E) for translation (Decroly et al., 2012; Liu and Kiledjian, 2006). The RNA guanylyltransferase (GTase) function is attributed to the N-terminal segment of the MTase (Supplementary Fig. S1B) and it has been described that NS3 stimulates this activity (Issur et al., 2009). Most recently, the existence of a dimeric- (Klema et al., 2016) and monomeric state of DENV NS5 inside the RC during *de novo* initiation and asymmetric RNA replication, respectively, has been proposed (Tay et al., 2016).

The MTase forms the N-terminal domain (residues 1-274 in ZIKV) of NS5 and contains the N-terminal subdomain with the GTP-binding pocket, the core MTase subdomain, which is responsible for AdoMet-binding and catalytic activity, and the C-terminal subdomain (Egloff et al., 2002; Yap et al., 2010; Zhao et al., 2015b). The MTase from ZIKV is connected via a 10-residues linker to the C-terminal RdRp (Supplementary Fig. S1B), composed of the residues 274 to 903, forming the subdomains called the *fingers*, *palm*, and *thumb* (Davidson, 2009; Yap et al., 2007; Zhao et al., 2015b).

In case of ZIKV, the atomic structures of the protease domain from the Brazilian ZIKV (Lei et al., 2016) and East African strain MR 766 ZIKV (Zhang et al., 2016b), the helicase from the French Polynesia (Jain et al., 2016) as well as the East African strain MR 766 (Tian et al., 2016) and MTase from East African strain MR 766 (Coloma et al., 2016; Coutard et al., 2016; Zhang et al., 2016a) were determined. However, the atomic structures of the entire NS3 and NS5 or of the individual RdRp domain are not described yet. Although while writing this manuscript the crystal structure of full-length NS5 from East African strain MR 766 (PDB ID: 5TFR) (Longenecker et al., to be published) was deposited in the protein databank. Furthermore, the mechanism of how the MTase and RdRp domains inside the ZIKV NS5 and the protease and helicase in NS3 communicate as well as how the catalytic entities are arranged relative to each other in solution are still elusive. In addition, the oligomeric state of the individual ZIKV NS3 and NS5 and their arrangement during the RC-formation are unknown.

Here we present for the first time the low resolution solution structure of the ZIKV NS3 ($NS2B_{18}NS3$) from French Polynesia as well as its individual protease and helicase domains, and reveal the flexibility of NS3 in solution. A systematic small-angle X-ray scattering (SAXS) approach was used to study the solution shape and flexibility of the MTase and RdRp domains as well as structural traits of the entire NS5 from French Polynesia ZIKV. Individual MTase and RdRp domains of ZIKV NS5 are monomeric and flexible in solution. The ZIKV NS5 forms a dimer, which is similar to the DENV type II dimer and/or East African ZIKV NS5 dimer, and different oligomers at higher concentrations in solution. These phenomena will be discussed in context with the variety of cell loci, the stoichiometry of NS3:NS5 and the arrangement of these ensemble during RNA replication. Furthermore, enzymatic studies unravel the interaction and effects of resveratrol- and quercetin-binding in ZIKV NS3.

2. Materials and methods

2.1. Purification of full-length NS5 and single domains of NS5 from Zika virus

The full-length NS5 gene from French Polynesia ZIKV (strain H/PF/2013; GenBank: KJ776791) was synthesized (Integrated DNA Technologies, Singapore) and used as the template for subsequent molecular cloning (Table 1). To transfer the NS5 gene into the plasmid, the gene encoding NS5 was amplified using the forward primer 5′-ATT AGG ATC CGG GGG TGG AAC AGG AGA GAC-3′ and reverse primer 5′-TAC GAA GCT TCT ACA GCA CTC CAG GTG TAG ACC-3′, with the incorporation of restriction sites *Bam*HI and *Hin*dIII (underlined). Following digestion with both restriction enzymes, the PCR product was ligated into the modified pET32b vector, as described before (Luo et al., 2008). The vector was kindly provided by Prof. Luo Dahai (Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore).

To obtain ZIKV MTase and RdRp with linker (residues 265-274), the genes encoding residues 1 to 274 and 265 to 903, which correspond to the MTase (ZIKV $NS5_{1-274}$) and RdRp (ZIKV $NS5_{265-903}$), were amplified with the following primer pairs: forward and reverse primers for MTase 5′-ATT AGG ATC CGG GGG TGG AAC AGG AGA GAC-3′ and 5′-ACA TAA GCT TCT AGT TGG GAG CTT CAG CGC AG-3′, respectively; forward and reverse primers for RdRp 5′-ATT AGG ATC CGC TGT GGT AAG CTG CGC T-3′ and 5′-TAC GAA GCT TCT ACA GCA CTC CAG GTG TAG ACC-3′, respectively. The restriction sites *Bam*HI and *Hin*dIII (underlined) were incorporated to these forward and reverse primers, respectively. Following digestion with both restriction enzymes, the PCR product was ligated into the modified pET32b vector.

The plasmids for all three constructs were transformed into *E. coli* BL21 CodonPlus (DE3)-RIL cells (Stratagene, USA) for protein production and purification. The cells were lysed on ice by sonication for 3 × 1 min in buffer A (50 mM Tris/HCl, pH 7.5, 500 mM NaCl, 5% glycerol, 0.8 mM DTT and 2 mM Pefabloc^{SC} (BIOMOL)) for full-length NS5, as well as for the MTase and RdRp. The cell lysate was centrifuged at 12 500×*g* for 25 min, the resulting supernatant was filtered (0.45 μm; Millipore), and subsequently incubated with Ni-NTA Agarose (Qiagen) for 1 h at 4 °C. The Trx-His_6-tagged proteins were eluted with an imidazole gradient from 20 mM to 500 mM in buffer A. Fractions containing the recombinant proteins, identified by SDS-PAGE, were pooled and dialyzed overnight with thrombin in buffer B (50 mM Tris/HCl, pH 7.5, 300 mM NaCl, 5%

Table 1
Lists of proteins used in SAXS experiments.

	Strain	Plasmid	Residue no.	GenBank
ZIKV NS5	H/PF/2013	pET32b	1–903	KJ776791
ZIKV MTase	H/PF/2013	pET32b	1–274	KJ776791
ZIKV RdRp	H/PF/2013	pET32b	265–903	KJ776791
ZIKV $NS2B_{18}NS3$	H/PF/2013	pET32b	NS2B 49 - 68 NS3 1 - 617	KJ776791
ZIKV $NS2B_{48}$ Protease	H/PF/2013	pET32b	NS2B 49 - 97 NS3 1 - 181	KJ776791
ZIKV Helicase	H/PF/2013	pET32b	NS3 169 - 617	KJ776791