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Thermodynamic stability of domain III from the envelope protein of flaviviruses and its improvement by molecular design

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The *Flavivirus* genus includes widespread and severe human pathogens like the four serotypes of dengue virus (DENV1 to DENV4), yellow fever virus, Japanese encephalitis virus and West Nile virus. Domain III (ED3) of the viral envelope protein interacts with cell receptors and contains epitopes recognized by virus neutralizing antibodies. Its structural, antigenic and immunogenic properties have been thoroughly studied contrary to its physicochemical properties. Here, the ED3 domains of the above pathogenic flaviviruses were produced in the periplasm of *Escherichia coli*. Their thermodynamic stabilities were measured and compared in experiments of unfolding equilibriums, induced with chemicals or heat and monitored through protein fluorescence. A designed ED3 domain, with the consensus sequence of DENV strains from all serotypes, was highly stable. The low stability of the ED3 domain from DENV3 was increased by three changes of residues in the protein core without affecting its reactivity towards DENV-infected human serums. Additional changes showed that the stability of ED3 varied with the DENV3 genotype. The T_m of ED3 was higher than 69°C for all the tested viruses and reached 86°C for the consensus ED3. The latter, deprived of its disulfide bond by mutations, was predominantly unfolded at 20°C. These results will help better understand and design the properties of ED3 for its use as diagnostic, vaccine or therapeutic tools.

Keywords: antigenicity/consensus sequence/envelope protein/flavivirus/protein stability

Introduction

The *Flavivirus* genus includes several human pathogens such as the yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV) and the four serotypes of dengue virus (DENV1 to DENV4). These viruses are widely distributed in the world, result in millions of human infections annually, and can cause deadly encephalitis and haemorrhagic fever. Here, we focus on these mosquito-borne flaviviruses but important studies have also been performed on tick-borne flaviviruses (Weaver and Reisen, 2010).

The flaviviruses are enveloped RNA viruses. The structures of the whole virus and of its envelope (E) protein have been solved by electron cryo-microscopy and X-ray crystallography for a number of them (Kuhn *et al.*, 2002; Modis *et al.*, 2003, 2005; Mukhopadhyay *et al.*, 2003; Kanai *et al.*, 2006; Nybakken *et al.*, 2006; Luca *et al.*, 2012). Ninety dimers of the E protein cover the surface of the virus. Each E protein monomer comprises three ectodomains, ED1–ED3, and a transmembrane segment. ED2 includes the dimerization interface, glycosylation sites and the peptide of fusion with the cellular membrane. ED3 is continuous and comprises residues 296–400 of the E protein (DENV1 numbering). Its fold is compact, immunoglobulin-like and stabilized by a disulfide bond between residues Cys302 and Cys333. The solution structures of recombinant ED3 domains have been solved by NMR methods for YFV, JEV, WNV, DENV2 and DENV4 (Wu *et al.*, 2003a; Volk *et al.*, 2004; Volk *et al.*, 2007; Huang *et al.*, 2008; Volk *et al.*, 2009). The structure of the isolated ED3 domain is close to its structure in the crystals of the E protein.

The ED3 domain participates in the interaction between the virus and primary or secondary cell receptors, including heparan sulfates and ribosomal protein SA (Chen *et al.*, 1996; Thullier *et al.*, 2001; Hung *et al.*, 2004; Lee *et al.*, 2006; Pattnaik *et al.*, 2007; Huerta *et al.*, 2008; Hershkovitz *et al.*, 2009; Kaufmann and Rossmann, 2011; Watterson *et al.*, 2012; Zidane *et al.*, 2013). Consistently, recombinant ED3 domains inhibit infectivity of the cognate virus for DENV1, DENV2, WNV and JEV and some authors have proposed to use ED3 as a basis for therapeutic molecules (Hung *et al.*, 2004; Jaiswal *et al.*, 2004; Chu *et al.*, 2005; Chin *et al.*, 2007; Chavez *et al.*, 2010; Li *et al.*, 2012). Mutations in the ED3 domains of YFV and DENV2 affect their cell tropism and virulence (Hahn *et al.*, 1987; Jennings *et al.*, 1994; Pryor *et al.*, 2001).

In mice, the ED3 domain is highly antigenic and recognized by monoclonal antibodies (mAbs) that neutralize the virus, strongly block its adsorption to cells and protect against viral infection (Crill and Roehrig, 2001; Brien *et al.*, 2010). ED3 contains type-, subcomplex- and complex-specific epitopes (Roehrig, 2003; Diamond *et al.*, 2008; Chavez *et al.*, 2010; Guzman *et al.*, 2010). The crystal structures of several complexes between the ED3 domain and either the Fab or Fv fragment of neutralizing monoclonal antibodies have been solved to high resolution (Nybakken *et al.*, 2005; Lok *et al.*, 2008; Austin *et al.*, 2012; Cockburn *et al.*, 2012; Midgley *et al.*, 2012).

In humans, only a small proportion of the IgG population in DENV2-, DENV3- or WNV-infected serums targets ED3,

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it appears to play a minor role in the immune response to the infection, and it does not correlate with its clinical outcome (Oliphant *et al.*, 2007; Crill *et al.*, 2009; Wahala *et al.*, 2009). However, one group has shown that the titer in IgG to ED3 in DENV2 infected serums correlates with the neutralization of DENV2 (Crill *et al.*, 2009). Moreover, human mAbs (hmAb) to the ED3 of DENV potently neutralize DENV infection (Beltramello *et al.*, 2010).

Several studies have shown that the ED3 domain is a useful antigen for flavivirus serologic diagnosis since antibodies to ED3 are less cross-reactive than those against ED1 or ED2 (Chavez *et al.*, 2010). ED3 domains have been used as antigen in indirect IgM or IgG enzyme-linked immunosorbent assay (ELISA) to detect infection by DENV, WNV, JEV and several tick-borne flaviviruses (Simmons *et al.*, 1998b; Beasley *et al.*, 2004; Holbrook *et al.*, 2004; Shukla *et al.*, 2009; Batra *et al.*, 2011). A single polypeptide that includes the ED3 domains of the four DENV serotypes, has been used as an antigen for detecting infection by any of the four DENV serotypes (Batra *et al.*, 2010b; 2011). An ED3 domain (ED3.DENVc) with the consensus sequence of ED3 domains from viral strains of the four DENV serotypes is recognized by immune ascitic fluids from mice infected by DENV1, DENV2 or DENV3 but not DENV4 (Danecek *et al.*, 2010; Bowen *et al.*, 2012).

ED3 has been considered as a potential immunogen for subunit vaccines against flaviviruses (Chavez *et al.*, 2010; Guzman *et al.*, 2010). Many studies have shown that the isolated ED3 domain is immunogenic in mice and elicits neutralizing and often protective antibodies, e.g. to JEV, WNV and the four DENV serotypes (Simmons *et al.*, 1998a; Wu *et al.*, 2003b; Alka *et al.*, 2007; Chu *et al.*, 2007; Zhang *et al.*, 2007; Babu *et al.*, 2008; Verma *et al.*, 2009; Batra *et al.*, 2010a; Guzman *et al.*, 2010). The ED3 domain from DENV2 (ED3.DENV2) elicits neutralizing antibodies and partial protection in monkey against the cognate virus (Guzman *et al.*, 2010). Multivalent ED3 domains, i.e. single polypeptides including the ED3 domains from several serotypes of DENV, elicit neutralizing and protective antibodies in mice, e.g. simultaneously to DENV2 and DENV4 or to the four DENV serotypes (Khanam *et al.*, 2006; Chen *et al.*, 2007; Etemad *et al.*, 2008). A consensus ED3.DENV domain elicits protective antibodies to all four DENV serotypes (Leng *et al.*, 2009; Chiang *et al.*, 2011). Recombinant or synthetic genes coding for a single ED3 domain or several ED3.DENV domains in tandem have been inserted in the genome of infective non-pathogenic viruses and shown to elicit neutralizing antibodies in mice, e.g. using adenovirus or measle vaccine virus as vectors (Brandler *et al.*, 2007; 2010; Khanam *et al.*, 2009).

Thus, the ED3 domains have been thoroughly studied from the viewpoints of structure, function, antigenicity and immunogenicity. However, very few studies of their physicochemical properties have been reported (Yu *et al.*, 2004). Here, we report the production of the isolated ED3 domains from several pathogenic flaviviruses in *Escherichia coli*. We measured and compared their thermodynamic stabilities in experiments of unfolding equilibriums, induced with chemical denaturants or heat, and monitored through the protein intrinsic fluorescence. In particular, we measured the stability of the ED3.DENVc domain that derived from the four serotypes of DENV by consensus (Danecek *et al.*, 2010). We increased the stability of the least stable ED3 domain, ED3.DENV3, by molecular design without modifying its antigenicity and analyzed whether this stability varied with the genotype within the serotype. Finally, we abolished the disulfide bond of an ED3 domain by mutation and determined its folding state at 20°C. We discuss our results in relation with the biology of the flaviviruses and the potential applications of the ED3 domains.

Materials and methods

Media, reagents and buffers

The culture medium 2-YT, NuPAGE Novex Bis-Tris 4–12% gradient gels, MES-SDS Running Buffer, sample buffer and See Blue Plus 2 Prestained Standards were purchased from Invitrogen; IPTG from Euromedex; phosphate-buffered saline (PBS), MES hydrate, Tween 20, 4-nitrophenyl phosphate (pNPP), polymyxin B sulfate, hen egg-white lysozyme and goat antibodies to human IgMs from Sigma-Aldrich; bovine serum albumin from Roche; low-fat milk powder from Regilait; Maxisorp ELISA plates from NUNC; the fast-flow Ni-NTA resin from Qiagen; the Quick change II site-directed mutagenesis kit from Agilent Technologies; and ultrapure urea and Guanidine-HCl (GuHCl) from MP Biochemicals. Ampicillin was used at 200 $\mu g\ ml^{-1}$ and chloramphenicol at 34 $\mu g\ ml^{-1}$. The solutions of 10 M urea and 8 M GuHCl were prepared daily and their pH was adjusted as described (Pace, 1986). The concentrations of denaturant, urea or GuHCl, were measured with a refractometer and a precision of 0.01 M.

Buffer A contained 20 mM Tris-HCl, 500 mM NaCl; buffer B: 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0; buffer C: 20 mM Tris-HCl, 100 mM NaCl; buffer D: 50 mM Tris-HCl, 150 mM NaCl, pH 7.9; buffer E: 30 mM MES, 150 mM NaCl, pH 5.5; buffer F: 0.1% Tween 20 in PBS; buffer G: 5% (w/v) low-fat milk powder in buffer F; buffer H: 1% (w/v) low-fat milk powder in buffer F; and buffer J: 10% diethanolamine, pH 9.8, 10 mM $MgSO_4$, 20 μM $ZnCl_2$. The pH of buffers A and C was distant by ≥ 1 unit from the protein pI.

Bacterial, plasmid and viral strains

The *E.coli* strain BL21(DE3, pLysS), plasmid pET20b+ (Novagen) and the plasmids encoding the ED3-H6 and H6-ED3-PhoA hybrids (Brandler *et al.*, 2007; Lisova *et al.*, 2007; Zidane *et al.*, 2013) have been described. The origin of the viral ED3 domains and the corresponding segment of the envelope protein are indicated in Table I.

Clinical samples

A first set of human serums (Group 1) was collected within the normal activity of the National Reference Center (NRC) for Arboviruses, Institut Pasteur de la Guyane, French Guiana. These serums were collected from patients who displayed clinical symptoms of dengue and whose infection by DENV was confirmed by laboratory methods. A second set of human serums (Group 2) was collected in the context of a clinical study (DENFRAME project) that was performed in French Guiana (Dussart *et al.*, 2012). These serums were collected from patients who displayed clinical symptoms of dengue but were diagnosed as negative for DENV infection.

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Table I. Viral origins of the ED3-H6 domains. The last column gives the residues of the viral E protein present in the ED3-H6 domains. The ED3 domains of WNV and JEV have three extra residues. The codons in the recombinant genes were synonymous but not necessarily identical with those in the original viral genomes.

Virus	Strain	Genbank no	E residues
DENV1	FGA/89	AF226687	296–400
DENV2	Jamaica/N.1409	M20558	296–400
DENV3	PaH881/88	AF349753	294–398
DENV4	ThD4–0113–76	AY618949	296–400
YFV	17D vaccine strain	X03700	294–398
YFV	Asibi	AY640589	294–398
WNV	IS-98-STD	AF481864	299–406
JEV	SA14	U14163	298–405

In the following, we designate these serums as DENV-uninfected serums. Both Group 1 and Group 2 serums consisted of paired blood samples collected during the acute and convalescent phases of infection. The samples were characterized by standard diagnostic methods, as described in details elsewhere; in particular, the DENV serotype that was responsible for the disease was identified by reverse transcriptase polymerase chain reaction (RT-PCR) (Dussart *et al.*, 2012). All the serums of group 1 were positive for the presence of anti-DENV IgM in an IgM antibody capture ELISA (MAC-ELISA) that used mouse brain extracts as antigens. The constitution of the above human serum collections and their use for the present study were approved by the Institutional Review Board of Institut Pasteur and a regional ethical committee (Comité de Protection des Personnes, Ile-de-France 1).

Genetic constructions

The genes coding for the ED3.YFV(Asibi), ED3.DENVc and ED3.DENV3(st) domains were chemically synthesized by GenScript (USA) after optimization of the codon usage for *E.coli*. They were inserted between the NcoI and XhoI of plasmid pET20b+, under control of the *pelB* signal sequence as previously described (Lisova *et al.*, 2007). Mutations were introduced by site-directed mutagenesis with synthetic oligonucleotides and the Quick change II kit. The changes of nucleotides that resulted in the change C302V of amino acid residue in ED3.DENVc, introduced a second NcoI site at codons 300–302, 5′ACC-ATG-GTT3′. The *pelB* signal sequence of the double mutant ED3.DENVc(C302V, C333A) was precisely deleted by inserting a synthetic double-stranded DNA cassette between the NdeI site, coding for the initiator Met, and the NcoI sites at codons 300–302. This cassette was formed by annealing two synthetic oligonucleotides: 5′TATGGGCATGAGCTATAC3′ and 5′CATGGTATA GCTCATGCCCA′. All the genetic constructions were verified by DNA sequencing.

Production and purification of the hybrids

The productions of the ED3-H6 and H6-ED3-PhoA hybrids in the periplasmic space of *E.coli* and their purification from periplasmic extracts through their His-tag were performed essentially as described (Brandler *et al.*, 2007; Lisova *et al.*, 2007; Zidane *et al.*, 2013). The cysteine-less domain ED3.DENVc(C302V, C333A)-H6 was produced in the cytoplasm of strain BL21(DE3, pLysS). The producing bacteria were grown at 24°C in 2-YT broth, supplemented with ampicillin and chloramphenicol during a preculture and with ampicillin alone during the main culture. The expression of the Cys-less ED3-H6 domain was induced during the exponential phase of growth by the addition of 1.0 mM IPTG and the bacteria further grown overnight in the same conditions. The subsequent steps were performed at 4°C. One volume of culture was cooled on ice and then centrifuged to pellet the bacteria. The bacterial pellet was resuspended in 1/20th volume of 1 mg ml^{-1} lysozyme, 5 mM imidazole in buffer A and the cells were disrupted by sonication. The insoluble materials were pelleted by centrifugation, washed with 1/20th volume of 5 mM imidazole in buffer A and pelleted again. The subsequent steps were performed at room temperature to avoid crystallization of urea. The insolubles were resuspended in 1/20th volume of buffer B. The suspension was mildly agitated until it became translucent, and then centrifuged 20 min at 15000 *g* to remove the cell debris. The supernatant contained the solubilized proteins.

The Cys-less ED3-H6 domain was then separated from the other proteins by affinity chromatography on a column of NiNTA resin (0.5 ml l^{-1} of culture), and refolded within the column before elution. The column was equilibrated with 6 volumes of buffer B, loaded with the preparation of solubilized proteins and then washed with 20 volumes of buffer C to remove urea and the unbound proteins. The subsequent steps were performed at 4°C. The outlet of the column was capped, one volume of buffer C was added and the reaction of refolding was left to run for several hours. The column was washed with 10 volumes of 5 mM imidazole in buffer A to remove the weakly bound proteins. The Cys-less ED3-H6 domain was then eluted by a step gradient of imidazole in buffer A. The fractions of purification were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions. The purest fractions were pooled, snap-frozen and kept at −80°C. They were homogeneous at >98%. The yield of purified ED3.DENVc(C302V, C333A) domain was about 3 mg l^{-1} of bacterial culture.

Fluorescence measurements

The fluorescence spectra were recorded with a FP-6300 spectrofluorometer (Jasco) in a quartz cuvette of 10×2 mm^2 path lengths, between 310 and 370 nm with excitation at 278 nm in buffer D. The slit width was equal to 2.5 nm for the excitation light and 5 nm for the emission. The increment of wavelength was equal to 0.5 nm. The fluorescence signal for the protein was obtained by subtraction of the signal for the solvent alone.

Unfolding with urea or GuHCl

Unfolding with urea or GuHCl was performed as described (Pace, 1986). Each reaction mixture (1 ml) contained purified protein (10 μg ml^{-1}) and varying concentrations of urea (0–8 M) or GuHCl (0–6 M) in buffer D. Control reactions were prepared by replacing the protein with buffer. The mixtures were incubated for 14 h at 20°C to enable the reaction of unfolding to reach equilibrium. To test the reversibility of the unfolding reaction, a protein sample was denatured in 8 M urea or 6 M GuHCl and buffer D for 4 h. The denatured protein was diluted with buffer D to reach varying concentrations of denaturant. The diluted mixtures were then incubated

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for 14 h at 20°C as above. The concentration of denaturant was measured in each reaction mixture after completion of the experiments. The equilibriums of unfolding were monitored with the intrinsic fluorescence of proteins as described in the previous paragraph.

Thermal unfolding

Unfolding with heat was performed with a Quantamaster spectrofluorometer (Photon Technology International), equipped with a computer-operated thermoelectric cuvette holder. The signal for the protein was obtained by subtraction of the signal for the solvent alone, recorded in a separate experiment. The purified protein (80 μg ml^{-1}) in buffer D or E was melted in a quartz cuvette of $10 \times 10\ mm^2$ path-lengths with a constant heating rate of 0.25°C min^{-1} between 45 and 90°C. The intrinsic fluorescence was excited at 278 nm and the emission was recorded at 330 and 350 nm. The excitation and emission slit widths were equal to 1 and 10 nm, respectively.

Thermal precipitation

The periplasmic extracts were divided into identical portions, which were simultaneously incubated at 4°C, 59°C and 69°C for 30 min. The samples were cooled for ten minutes on ice, the protein precipitates were eliminated by centrifugation and the supernatants were analyzed by SDS-PAGE in reducing conditions.

MAC-ELISA

The capture ELISAs were performed in 96-well flat-bottom microtiter plates with a volume of 100 μl/well. The plates were sensitized with antibodies to human IgMs as follows. A goat antibody to human IgMs (1.0 μg ml^{-1} in PBS) was loaded in wells of the plate. The plate was incubated overnight at 4°C for the reaction of adsorption. The wells were washed with buffer F (three times), blocked with buffer G for 1 h at 37°C and then washed as above. The serums and recombinant antigens were diluted in buffer H. The serums were diluted 100-fold. Wells were loaded with the diluted serums or with buffer H as a blank sample, and the plate was incubated for 1 h at 37°C for the reaction of antibody capture. The wells were washed as above and then loaded with the solution of recombinant antigen. The plate was incubated for 1 h at 37°C for the binding reaction. The wells were washed as above and the bound antigen, $(ED3\text{-}PhoA)_2$, was detected by addition of 5 mM pNPP in buffer J and measurement of A_{405nm} after 3 h at 25°C.

Analysis of the sequence, structure and experiment data

The values of the theoretical molecular mass (MM_{th}) and extinction coefficient ϵ_{280nm} of the proteins were computed from the amino acid sequences with the sub-program PepStat of the EMBOSS software suite (Rice *et al.*, 2000). The atomic coordinates of the proteins were retrieved from the RCSB protein data bank (www.rcsb.org). The solvent accessible surface areas (ASA) were computed with the access routine of the What If software package and a probe radius of 1.4 Å (Vriend, 1990). The structures were drawn with RasMol version 2.7.3 (www.rasmol.org).

The equilibriums of unfolding were analyzed with a two-state system, exactly as described (Ould-Abeih *et al.*, 2012). The curve fits were performed with Kaleidagraph (Synergy Software), which gives Pearson's coefficient of correlation, R_P. Semi-parametric receiver operating characteristics (ROC) curves were computed with the LABROC4 program (Metz *et al.*, 1998) as implemented in the Web based calculator JLABROC4 by J. Eng (http://www.jrocfit.org). These curves plot the true positive fraction of samples as a function of their false-positive fraction when the value of the threshold varies. The area under the curve (AUC) is an unbiased measure of the assay accuracy. The standard error SE on a sum of two terms A and B was calculated from the equation of error propagation: $SE(A+B)^2 = SE(A)^2 + SE(B)^2$.

Results

Fluorescence properties of recombinant ED3 domains

The isolated ED3 domains from several pathogenic flaviviruses were produced in the periplasmic space of *E. coli* where the formation of their disulfide bond is enzymically catalyzed, and purified through their hexahistidine tag. Each ED3 domain includes a tryptophan residue, which is conserved in all flaviviruses, unique in the mosquito-borne flaviviruses (position 391 in the E protein of DENV1) and partially buried in the available crystal structures (see Introduction). The intrinsic fluorescence of the ED3 domains was excited at the wavelength of 278 nm. For all the ED3 domains that we studied here, the value of the fluorescence intensity F decreased and the wavelength λ_{max} of its maximum increased when going from the native state (N) to the unfolded state (U) in 8 M urea or 6 M guanidinium-HCl (GuHCl) as previously reported for the ED3 domain from DENV1 (ED3.DENV1) (Monsellier and Bedouelle, 2005). More precisely, we found $340\ nm < \lambda_{max} < 343.3\ nm$ for the N state and $347\ nm < \lambda_{max} < 350.5\ nm$ for the U state at pH 7.9, 20°C.

Stability of ED3 domains as measured with chemical denaturants

We measured the thermodynamic stability of ED3 domains by analyzing their unfolding equilibriums, induced with urea and monitored through their fluorescence intensity F. The signal was measured at the emission wavelength for which the difference between the F values for states N and U was maximal. The profiles of unfolding showed a single cooperative transition when the concentration of denaturant x varied (Fig. 1). We, therefore, modeled them by a two-state system, N and U. The fitting of this model to the experimental data allowed us to determine three characteristic parameters (only two of which are independent): the difference in free energy $\Delta G(H_2O)$ between states N and U in the absence of denaturant, which is the protein stability; its coefficient of dependence m towards the concentration of denaturant, which measures the cooperativity of unfolding; and the concentration $x_{1/2}$ of denaturant for half-unfolding, which measures the protein resistance to unfolding (Table II).

The unfolding profile of ED3.DENV2, induced with urea, was incomplete because the cooperative transition happened at too high a value of x. We, therefore, induced its unfolding with GuHCl, a stronger denaturant. We performed both types of experiments, with urea and GuHCl, for ED3.DENV1 because we wanted to rank the stabilities of the different ED3 domains (Table II) and the $\Delta G(H_2O)$ values are not

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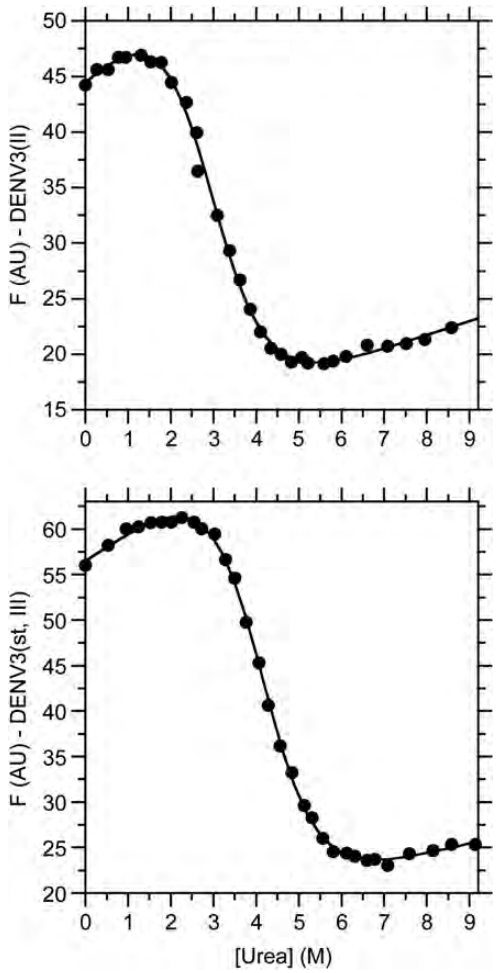

Fig. 1. Unfolding equilibria of ED3.DENV3 derivatives, induced with urea at 20°C in buffer D and monitored through their intrinsic fluorescence. The proteins were excited at 278 nm and their fluorescence intensity F recorded at 332.5 nm for ED3.DENV3(II) (top part) and 337 nm for ED3.DENV3(st, III) (bottom part), in arbitrary units (AU). The solid lines correspond to the fitting of a two-state model to the experimental data points ($R_P = 0.99879$ and 0.99967, respectively).

necessarily identical when obtained with urea or GuHCl (Monera *et al.*, 1994). We checked that its unfolding equilibriums were reversible when induced with GuHCl, as they were with urea (Monsellier and Bedouelle, 2005).

We found the following decreasing order for the stabilities $\Delta G(H_2O)$ of the ED3 domains: WNV = DENV2 > DENV4 > DENV1 > JEV > YFV(17D) > YFV(Asibi) > DENV3. The values of $x_{1/2}$ gave a slightly different order because of the slight variations in the values of m and the relation $\Delta G(H_2O) = mx_{1/2}$. For proteins of identical lengths and close structures, the variations of m are usually due to differences in the residual interactions that exist in the unfolded state and are favored by the existence of a disulfide bond (Monsellier and Bedouelle, 2006).

Stability of ED3.DENV1 at pH 5.5

The envelope protein of flaviviruses undergoes a transition from a dimeric to a trimeric quaternary structure when it goes from a neutral to a low pH, as in the endosomes, and fuses with the cell membrane. The structure of the ED3 domain is maintained during this transition but its position relative to ED1 and ED2 is modified (Modis *et al.*, 2004; Nayak *et al.*, 2009). We compared the stabilities of the ED3.DENV1 domain at pH 7.9 and 5.5 to reveal a potential change in the endosomes. We found a significant difference in stability between the two pHs, $\Delta\Delta G(H_2O) = 0.9 \pm 0.2$ kcal mol^{-1}, due to a smaller cooperativity of unfolding at pH 5.5, $\Delta m = 0.19 \pm 0.03$ kcal mol^{-1} M^{-1} (Table II).

Table II. Thermodynamic parameters for the unfolding equilibriums of ED3 domains, induced with chemical denaturants at 20°C and monitored through the intrinsic fluorescence intensity of the protein. The fluorescence was excited at 278 nm. Mean and SE value in at least three independent experiments.

ED3 origin	Denat.	$\Delta G(H_2O)$ (kcal mol^{-1})	m (kcal mol^{-1} M^{-1})	$x_{1/2}$ (M)
DENV1	Urea	5.21 ± 0.10	1.04 ± 0.01	5.02 ± 0.03
DENV1 (pH5.5)	Urea	4.34 ± 0.20	0.85 ± 0.03	5.13 ± 0.06
DENV3(II)	Urea	2.78 ± 0.14	0.95 ± 0.03	2.91 ± 0.07
DENV4	Urea	5.54 ± 0.08	1.32 ± 0.02	4.20 ± 0.01
YFV(Asibi)	Urea	3.92 ± 0.24	1.21 ± 0.06	3.23 ± 0.06
YFV(17D)	Urea	4.86 ± 0.15	1.17 ± 0.03	4.17 ± 0.03
WNV	Urea	6.05 ± 0.30	1.47 ± 0.07	4.12 ± 0.02
JEV	Urea	5.10 ± 0.23	1.39 ± 0.06	3.65 ± 0.04
DENV3(st, II)	Urea	3.73 ± 0.12	1.00 ± 0.03	3.75 ± 0.03
DENV3(st, II, L303T)	Urea	5.28 ± 0.47	1.24 ± 0.12	4.28 ± 0.08
DENV3(st, II, K385N)	Urea	3.72 ± 0.27	0.97 ± 0.06	3.83 ± 0.06
DENV3(st, III)	Urea	4.37 ± 0.21	1.05 ± 0.05	4.17 ± 0.03
DENV1	GuHCl	4.24 ± 0.19	2.87 ± 0.14	1.48 ± 0.05
DENV2	GuHCl	5.07 ± 0.16	2.69 ± 0.09	1.89 ± 0.01
DENVc	GuHCl	5.17 ± 0.33	2.28 ± 0.12	2.26 ± 0.03

Denat, nature of the denaturant.

Stability of a consensus ED3 domain

We designed, optimized for *E.coli*, and chemically synthetized a recombinant gene that encoded the consensus sequence for the ED3 domains from viral strains of the four DENV serotypes (Danecek *et al.*, 2010). We produced and purified this consensus domain, which we named ED3.DENVc, as described for the parental ED3 domains and measured its stability by monitoring its unfolding equilibriums induced with GuHCl. Its yield of production and purification was equal to 7 mg l^{-1} of culture in flask. Its stability was identical to that of ED3.DENV2, i.e. greater or equal to the stabilities of the individual ED3.DENV domains from the four serotypes. The resistance $x_{1/2}$ to denaturation of ED3.DENVc was significatively greater than those of ED3.DENV1 and ED3.DENV2, but its cooperativity m of unfolding was slightly lower (Table II).

Design of a stabilized ED3.DENV3 domain

The ED3.DENV3 domain was the least stable of all the flaviviral ED3 domains tested. This poor stability could be a disadvantage for its uses. We, therefore, sought to increase its stability without compromising its antigenicity. Our design consisted in changing buried residues of ED3.DENV3 into those of ED3.DENVc, while keeping unchanged its solvent exposed residues. Therefore, we considered only those residues of ED3.DENV3 whose side chains had solvent ASA lower than 10 Å^2 and which were different in ED3.DENVc. This search restricted the changes to three positions, 320,

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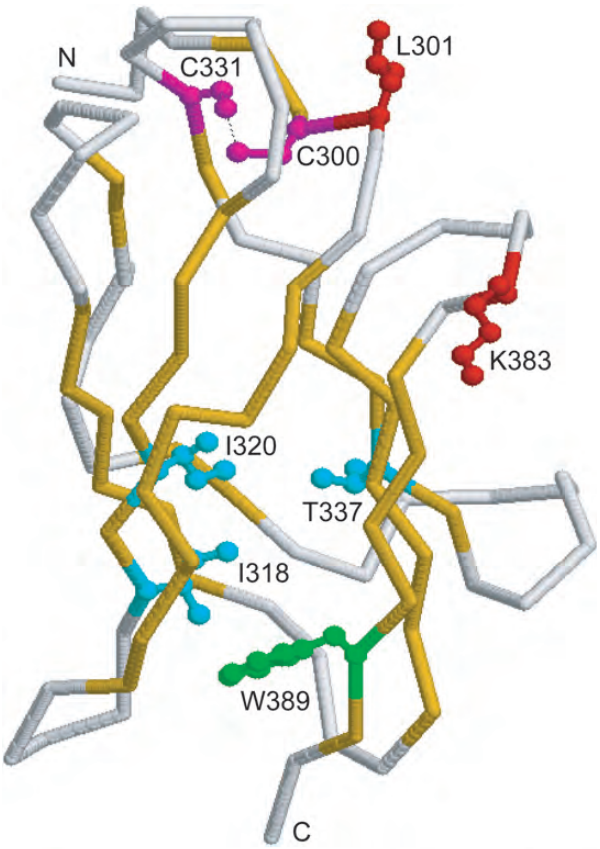
322 and 339 (with the DENV1 numbering; Fig. 2). We, therefore, introduced three changes I320V, I322V and T339I in ED3.DENV3 at the genetic level. The resultant derivative, which we named ED3.DENV3(st), was more stable than the parental ED3.DENV3, with $\Delta\Delta G(H_2O) = 0.95 \pm 0.18$ kcal mol^{-1}. This stabilization was due to a greater resistance to denaturation, with $\Delta x_{1/2} = 0.84$ M urea, without any change in the cooperativity m of unfolding.

Variation of stability between genotypes within a DENV serotype

The sequence of our parental ED3.DENV3 domain was identical to that of the ED3 domain from the prototypic strain Philippines/H87/1956, which belongs to the Asian genotype II of DENV3. Therefore, we renamed ED3.DENV3(II) and ED3.DENV3(st, II) our parental ED3.DENV3 domain and its stabilized derivative to indicate their genotype. The ED3 domain from strain H87 is not recognized by human serums that were infected and sampled during the Havana (Cuba) epidemics of 2001–02 (Zulueta *et al.*, 2006). We observed that the sequence of the ED3.DEN3 domains from strains of the Asian genotype II and from strains of the Latin-American genotype III differ only by two changes, L303T and K385N (DENV1 numbering), of residues whose side chains are exposed to the solvent and, for some of them, by an additional change, A331V (Fig. 2) (Modis *et al.*, 2005; Wahala *et al.*, 2010). These residues are, respectively, Thr303, Ala331 and Ser385 in ED3.DENVc. On the basis of the above, we constructed an additional derivative of ED3.DENV3(II) that derived from ED3.DENV3(st, II) by the two changes of residues L303T and K385N and that we named ED3.DENV3(st, III). We also constructed two intermediate domains that carried the single changes L303T or K385N and that we named ED3.DENV3(st, II, L303T) and ED3.DENV3(st, II, K385N).




```
                                        MGMSYT 300
MCTGKFKIEK EVAETQHGTV VVKVKYEGED 330
APCKIPFEIQ DLQGKTHNGR LITANPIVTN 360
KDSPVNIEAE PPFGESYIVI GVGDSALKLN 390
WFKKGSSIGK LEHHHHHH
```

Fig. 2. Comparison between the structure of ED3.DENV3(II) and the sequence of ED3.DENVc. The structure of ED3.DENV3(II) is represented by the chain of its C_α atoms. The β-sheets are colored in yellow. Relevant amino acid side chains are in ball-and-stick representation. The residues are numbered according to the E protein of DENV3, as in the PDB file 1UZG. Add two to obtain the DENV1 numbering. The sequence of ED3.DENVc is numbered according to the E protein of DENV1. The color codes of the residues are identical in the structure and sequence.

The ED3.DENV3(st, III) domain was stabler than ED3.DENV3(st, II), with $\Delta\Delta G(H_2O) = 0.64 \pm 0.25$ kcal mol^{-1}. The main contribution to the difference in stability came again from the resistance to denaturation, with $\Delta x_{1/2} = 0.42 \pm 0.05$ M urea. Overall, ED3.DENV3(st, III) was 1.59 ± 0.26 kcal mol^{-1} more stable than ED3.DENV3(II) (Table II). ED3.DENV3(st, II, L303T) was 2.50 ± 0.49 kcal mol^{-1} more stable than ED3.DENV3(II) with $\Delta x_{1/2} = 1.37 \pm 0.11$ M urea. Interestingly, mutation K385N was neutral in the structural context of ED3.DENV3(st, II) and destabilizing in the context of ED3.DENV3(st, II, L303T). The effect of mutation K385N therefore depended on the side chain in position 303. Such a context effect is quite common (Monsellier and Bedouelle, 2006).

Unfolding with heat

The experiments of chemical unfolding, induced with urea or GuHCl, allowed us to determine and compare the stabilities of ED3 domains from various flaviviruses. However, heat rather than chemical unfolding is important for applications. Therefore, we analyzed the unfolding equilibriums of several ED3 domains, induced with heat and monitored through their fluorescence intensity.

The profiles of heat-induced unfolding showed a single cooperative transition for all the analyzed ED3 domains (Fig. 3). We checked that these equilibriums were reversible (not shown) and modeled them again with a two-state system, N and U. The model depended on three parameters: the temperature T_m at which the variation of free energy $\Delta G(T)$ between states N and U was nil; the variation of enthalpy ΔH_m between states N and U at temperature T_m, and the variation of caloric capacity ΔC_p between states N and U. We reduced this number to two characteristic parameters by empirically predicting $\Delta C_p = 1.678$ kcal mol^{-1} K^{-1} as described (Myers *et al.*, 1995)

The results (Table III) showed that the ED3.DENV1 and ED3.DENVc domains had close values of ΔH_m, 113.33 and 118.49 kcal mol^{-1}, respectively, and therefore close cooperativities of unfolding. In contrast, T_m was greater by 7.8 ± 0.2°C for ED3.DENVc than for ED3.DENV1. The T_m value was remarkably high for these two proteins, 86.17°C for ED3.DENVc and 78.37°C for ED3.DENV1. The ED3.DENV3(II) domain had both T_m and ΔH_m values lower than those of ED3.DENV1, resulting in a lower stability. The T_m value of ED3.DENV3(II) was nevertheless quite high, 70.39°C. ED3.DENV3(II) and ED3.DENV3(st, III) had close

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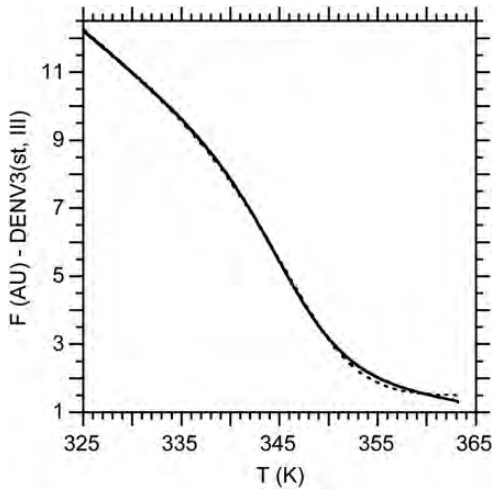

Fig. 3. Unfolding equilibria of the ED3.DENV3(st, III) domain, induced with heat in buffer D and monitored through its intrinsic fluorescence. The protein was excited at 278 nm and its fluorescence intensity F recorded at 330 nm in arbitrary units (AU). The solid line corresponds to the 3172 experimental data points and the dashed line to the fitting of a two-state model with $\Delta C_p = 1.678$ kcal mol^{-1} K^{-1} ($R_P = 0.99993$).

Table III. Thermodynamic parameters for the unfolding equilibriums of ED3 domains, induced with heat and monitored through the intrinsic fluorescence intensity of the protein. The fluorescence was excited at 278 nm and its intensity recorded at 330 nm. SE in the curve fit.

ED3 origin	T_m (K)	ΔH_m (kcal mol^{-1})	R_P
DENV1	351.52 ± 0.17	113.3 ± 4.9	0.99966
DENV1 (pH5.5)	350.32 ± 0.04	74.1 ± 0.6	0.99996
DENV3(II)	343.54 ± 0.21	67.7 ± 1.6	0.99980
DENV3(st, III)	347.28 ± 0.03	61.0 ± 0.1	0.99993
DENVc	359.32 ± 0.18	118.5 ± 1.8	0.99985

values of ΔH_m, 67.7 and 61.0 kcal mol^{-1}, respectively. In contrast, T_m was greater by 3.74°C for ED3.DENV3(st, III) than for ED3.DENV3(II). Finally, we found a slight destabilization of ED3.DENV1 at pH 5.5 relative to pH 7.9, mainly due to a lower cooperativity of unfolding, with heat as with urea (see above). It was not possible to calculate the values of $\Delta G(H_2O)$ at 20°C from the values of T_m and ΔH_m and to compare them with those obtained by unfolding with urea or GuHCl because of a too long extrapolation.

A consensus ED3 domain without disulfide-bond

We tested whether the ED3.DENVc domain, which had one of the highest thermodynamic stability ($\Delta G(H_2O) = 5.2$ kcal mol^{-1} at 20°C), could fold in a stable state in the absence of its disulfide bond. We constructed three double mutants of ED3.DENVc, carrying the couples of changes (C302A, C333V), (C302V, C333A) and (C302A, C333A), on the basis of published studies on immunoglobulin variable fragments (Proba *et al.*, 1998). The three double mutants were produced in very small quantities in the periplasm of *E.coli*, in comparison with the parental ED3.DENVc domain. We further studied the double mutant ED3.DENVc(C302A, C333V), which was produced slightly better than the other two mutants. We constructed, at the genetic level, a derivative of ED3.DENVc(C302A, C333V) that was devoid of signal peptide for a cytoplasmic expression. An analysis of cellular extracts by SDS-PAGE showed that the double mutant was produced predominantly in the insoluble fraction and in very low quantities in the soluble fraction (not shown). The insoluble fraction was solubilized with 8 M urea and loaded on a nickel ion column. The ED3 domain was refolded within the column by washing out the urea, and then eluted with imidazole. The purified protein was highly homogeneous and its yield equal to several milligrams of protein per liter of culture in flask. An analysis of the unfolding equilibriums of the purified refolded ED3.DENVc(C302A, C333V) mutant, induced with urea at 20°C and monitored through the ratio F_{330}/F_{350} of the intrinsic fluorescence intensities at 330 and 350 nm, showed a profile that could be approached by an exponential decay function, with a decay constant of 1.56 M^{-1}, and therefore a half-decay at 0.44 M urea (Fig. 4). These results showed that the ED3.DENVc(C302A, C333V) mutant, devoid of disulfide bond, was in equilibrium between a predominant fraction of unfolded state and a minority fraction of folded state even in the absence of urea. They were consistent with the presence of this domain as inclusion bodies in the cytoplasm and our observation that it slowly precipitated at 4°C.

Thermoprecipitation of periplasmic extracts

The unfolding equilibriums, induced with heat, showed that the values of T_m were high for some ED3 domains. We tested whether it would be possible to obtain partially purified preparations of the ED3 domains by a differential heat-induced precipitation of the bulk of proteins in the periplasmic extracts. Identical aliquots of such extracts were treated at 4°C, 59°C and 69°C for 30 min and cooled on ice. The protein precipitate was removed by centrifugation and the content of the supernatant analyzed by SDS-PAGE (not shown). We observed that the tested ED3 domains, from DENV1, DENVc and DENV3(st, III), resisted to the treatment at 69°C and that the heat-treated periplasmic extracts were highly enriched in recombinant ED3 domain. The

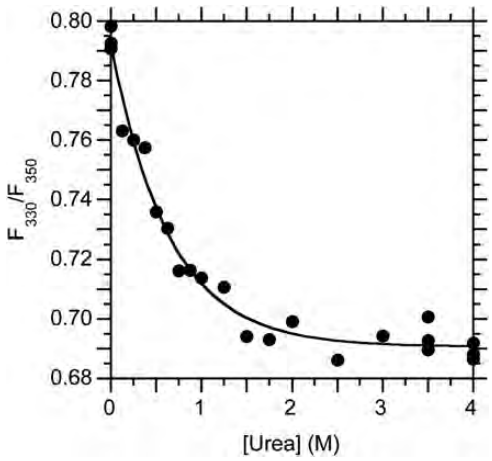

Fig. 4. Unfolding equilibria of the ED3.DENVc(C302V, C333A) domain, induced with urea at 20°C in buffer D and monitored through its intrinsic fluorescence. The protein was excited at 278 nm and its fluorescence intensities F_{330} and F_{350} recorded at 330 and 350 nm. The urea concentration is given along the x axis and the ratio F_{330}/F_{350} along the y axis. The solid line corresponds to the fitting of an exponential decay function to the experimental data points ($R_P = 0.990$).

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results of these thermoprecipitation experiments confirmed the high resistance to heat of these three ED3 domains and could lead to simple methods of partial purification.

Antigenicity of the ED3.DENV3 derivatives

Since the engineering of the ED3.DENV3 domain aimed at increasing its stability without modifying its antigenicity, it was important to test this assumption. We constructed three dimeric hybrids (H6-ED3-PhoA)$_2$ between ED3 and *E.coli* alkaline phosphatase to assay and compare the recognitions of the engineered ED3 domains by anti-DENV IgM in human serums. We assayed 6 serums of patients infected with DENV1, 5 with DENV2, 21 with DENV3, 5 with DENV4 and 6 non-infected serums by an IgM antibody capture ELISA (MAC-ELISA), using the dimeric hybrids as antigens. We focused our analysis on the IgMs because they are the first antibodies to appear after a flaviviral infection. The distribution of the MAC-ELISA signals were very similar for the antigens derived from DENV3(II), DENV3(st, II) and DENV3(st, III) (Fig. 5). We quantified the capacity of the three dimeric hybrids to distinguish either between DENV3-infected serums and non-infected serums or between DENV3-infected serums and the pool of serums that were non-infected or infected by DENV1, DENV2 or DENV4, by using ROC curves. AUC is a measure of the precision of the test. There were not significant differences between the precisions of the test when it was performed with any of the three dimeric hybrids, given the errors on the AUC values. Nevertheless, we observed that the signal was higher on average when the antigen was one of the stabilized ED3.DENV3 domains. In conclusion, we increased the stability of the ED3.DENV3 domain without modifying its antigenicity in a MAC-ELISA.

Discussion

A better knowledge of the biophysical properties of the ED3 domains is needed to clarify their role in antigenicity, immunogenicity and function, and to improve their applications. In particular, more data are needed on the relations between folding and either antigenicity or immunogenicity.

Periplasmic expression and folding state

Recombinant ED3 domains are generally produced in the cytoplasm of *E.coli* in the form of either insoluble aggregates or soluble fusion proteins, e.g. between ED3 and the maltose-binding protein. However, the disulfide bonds do not form in the reducing medium of the cytoplasm and therefore must be established *in vitro*. Here, we exported the isolated ED3 domains in the periplasmic space of *E.coli* where the formation of disulfide bonds is efficiently catalyzed *in vivo* by the Dsb system (Kadokura *et al.*, 2003). In these conditions, we have previously shown that the experimental mass of the ED3.DENV1 domain is equal to the theoretical mass of a domain whose disulfide bond is correctly formed and whose N-terminal residue, resulting from the cleavage of the signal peptide, is Gly296 or Met-Gly296. An Ellman's test has shown that >94% of the molecules of the ED3 domain have a disulfide bond, for DENV1, WNV, JEV and YFV(17D) (Lisova *et al.*, 2007). The ED3.DENV1, ED3.DENV2 and ED3.DENV4 thus produced were crystallized in complex with the mouse neutralizing mAb4E11, whose

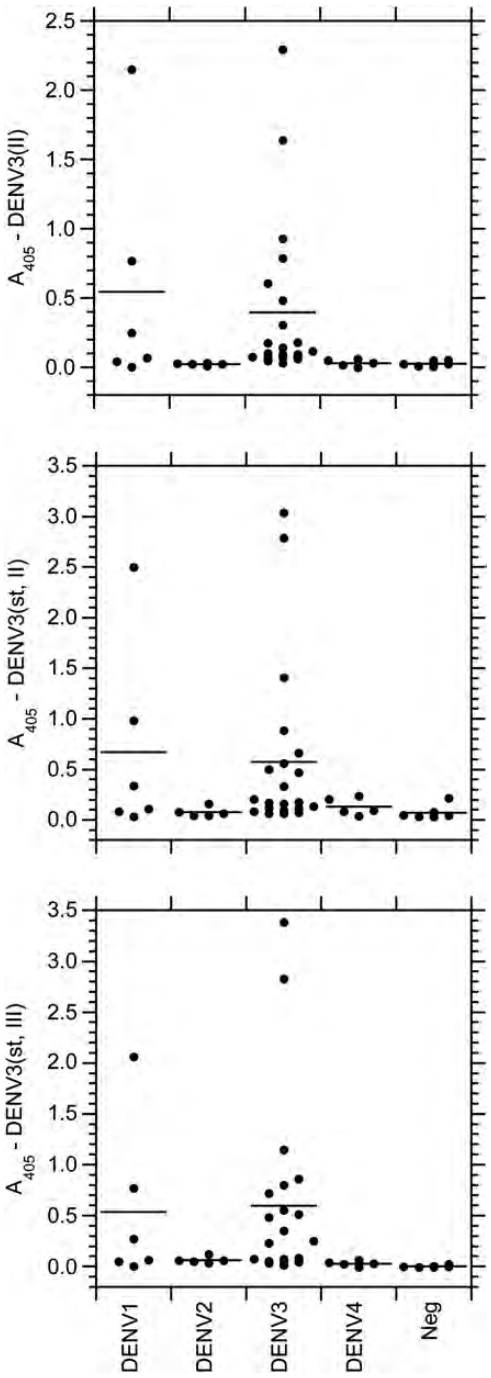

Fig. 5. MAC-ELISA performed with (H6-ED3-PhoA)$_2$ dimers on five categories of human serums. The graphs give the distribution of the serum-specific signals along the *y* axis for each category of serum. The horizontal lines correspond to the mean value of the serum-specific signals for each category of serums. DENV1 to DENV4, human serums infected by the corresponding virus; Neg, DENV-uninfected serums. Top, middle and bottom parts, MAC-ELISA performed with (H6-ED3-PhoA)$_2$ dimers from ED3.DENV3(II), ED3.DENV3(st, II) and ED3.DENV3(st, III), respectively.

epitope depends on the correct folding of the ED3 domain. The structures of the complexes at a resolution of 2 Å showed the presence of a disulfide bond and correct folding of the ED3 domain (Cockburn *et al.*, 2012). All the ED3 domains that we studied here, except one, showed a cooperative transition of unfolding by denaturants, with a shift of their λ_{max} of fluorescence emission from <343.3 nm to

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>347 nm, corresponding to an increased exposure of their unique Trp residue to solvent. The exception, ED3.DENVc(C302V, C333A), was devoid of disulfide bond, did not show any cooperative transition and was already more than half-unfolded in 0 M urea.

We tested the antigenicity of ED3.DENV3 and its various derivatives in the form of dimeric hybrids (ED3-PhoA)$_2$. The ED3 domain has one disulfide bond, the PhoA monomer has two disulfide bonds and PhoA is enzymically active only as a dimer. We have previously shown that the (ED3-PhoA)$_2$ dimers have both their ED3 and PhoA portions correctly folded and active when they are produced in the *E.coli* periplasm (Bedouelle *et al.*, 2008). These dimeric hybrids have been used to probe the interaction between ED3 domains and cell receptors by using the reinforced interaction due to an avidity effect, e.g. with the NKp44 receptor of natural killer cells or the ribosomal protein SA, which is also known as the high-affinity laminin receptor 1 (Hershkovitz *et al.*, 2009; Zidane *et al.*, 2013). In conclusion, producing the ED3 domains, either isolated or in fusion with PhoA, in the periplasm ensures their proper folding, the formation of the disulfide bonds and thus the reproducibility of the biophysical and functional results.

Validity of a two-state model of unfolding

The recombinant ED3 domains are in a monomeric state at high concentration, as shown by structural methods (see Introduction). They can be refolded *in vitro* from a fully denatured state to their native state (Nybakken *et al.*, 2005; Lok *et al.*, 2008; Austin *et al.*, 2012; Midgley *et al.*, 2012). Previous studies have shown that the profiles of unfolding for the ED3 domains from several flaviviruses, monitored through circular dichroism, fluorescence or gel filtration, present a single transition in every case (Yu *et al.*, 2004; Monsellier and Bedouelle, 2005). Here, we observed that the profiles of unfolding were cooperative, showed only one visible transition and were reversible. These profiles were approximated satisfactorily by functions that were derived from a two-state system of unfolding (Tables II and III). With regard to the experiments with chemical denaturants, the values of the cooperativity coefficient, m, were close to the values that could be predicted from their number of residues and disulfide bond, i.e. $1.218 < m < 1.270$ and $2.493 < m < 2.593$ for urea and GuHCl-induced unfolding, respectively (Myers *et al.*, 1995). The $\Delta G(H_2O)$ values were consistent with those reported for monomeric proteins having a similar molecular mass, and unfolding according to a two-state model (Kumar *et al.*, 2006). With regard to the experiments with heat, the values of T_m and ΔH_m were consistent with those reported for similar proteins, although those for DENVc were among the highest reported for proteins from mesophilic organisms (Kumar *et al.*, 2006). We concluded that our analysis of the unfolding profiles with a two-state model was pertinent.

Stability, structure and in vivo *functions*

The stability, disulfide bond and correct folding of the ED3 domain are important for its functions. For example, the epitopes of three neutralizing mouse mAbs in the E protein of DENV2 are inactivated by changing residue Cys333 into Ala at the genetic level and thus eliminating the disulfide bond between residues Cys302 and Cys333 (Roehrig *et al.*, 2004). The epitope of another neutralizing mAb in a recombinant ED3.DENV2 domain is inactivated by the reduction and carbamidomethylation of the domain Cys residues. However, the binding of this ED3.DENV2 domain to its receptors in a cell microsomal fraction is unaffected by the chemical treatment (Huerta *et al.*, 2008). Moreover, preparations of ED3 domains whose folding state is ill defined, can nevertheless detect antibodies to flaviviruses in infected human serums and elicit neutralizing antibodies in mice and monkeys (Simmons *et al.*, 1998b; Beasley *et al.*, 2004; Alka *et al.*, 2007; Shukla *et al.*, 2009; Wahala *et al.*, 2009). Thus, the folding of the ED3 domain seems important only for some of its functions.

The folding of most mosquito-borne flaviviruses depends on the side chain of Tyr326, which belongs to a loop between two β-strands, is buried in the ED3 structure and forms hydrogen bonds with the main chain nitrogen atoms of residues 303 and 304 (DENV1 numbering). This structure, named 'tyrosine corner', does not exist in the viruses of the YFV group where Tyr326 is replaced by Val326 (Zhang *et al.*, 2010). This structural difference could explain the lower stability of the ED3.YFV domains relative to those from other flaviviruses.

ED3.DENV3 was the least stable and least resistant to denaturation among the studied ED3 domains. This weak stability could explain the difficulties that we had to produce and crystallize this domain in complex with a monoclonal antibody (Cockburn *et al.*, 2012). It could be related to the higher dynamics of this domain. Indeed, we observed that more residues are missing in the crystal structure of the isolated ED3 domain for DENV3 than for DENV1, DENV2, DENV4 or WNV (Nybakken *et al.*, 2005; Lok *et al.*, 2008; Austin *et al.*, 2012; Cockburn *et al.*, 2012; Midgley *et al.*, 2012). This higher dynamics could be important for the interaction between the ED3 domain and cellular receptors.

Previously, differences in biophysical properties between various ED3 domains have been proposed to correlate with the higher tolerance of tick-borne flaviviruses to environmental conditions compared with mosquito-borne flaviviruses (Yu *et al.*, 2004). Among the small sample of mosquito-borne flaviviruses that we analyzed here, a high virulence of the parental virus in primary infections was associated with a low stability of the ED3 domain, e.g. the wild-type strain YFV(Asibi) versus the vaccine strain YFV(17D); or DENV1 and DENV3 versus DENV2 and DENV4. Indeed, DENV1 and DENV3 are more pathogenic than DENV2 and DENV4 for patients who had had not an immune priming from other serotypes (Fried *et al.*, 2010). Consistent with a proposed relation between lower stability and higher virulence, the interaction between ED3 and its cell receptors is strengthened at endosomal pH (Huerta *et al.*, 2008) and we found that ED3.DENV1 was less stable in buffer E (pH 5.5) than buffer D (pH 7.9) (Tables II and III).

Applications

We found that some ED3 domains had strong resistances to chemical denaturant- and heat-induced unfolding. These properties are important for potential applications, e.g. use in diagnostic assays, vaccines or therapy. ED3.DENV3 was the least stable and least resistant to denaturation among the studied ED3 domains. This weak stability could bias the experimental data on antigenicity and immunogenicity obtained

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with this domain. It could also be an impediment or a disadvantage for its applications. Here, we improved its stability without compromising its antigenicity. These results have potential applications for the diagnostic assays and recombinant vaccines that use ED3.DENV3 either alone or in fusion with the ED3 domains from other serotypes. It could be extended to protein domains from other pathogens. Previously, it has been reported that the binding and neutralization capacity of mouse mAbs to ED3.DENV3 are strongly influenced by naturally occuring mutations in DENV3 (Brien *et al.*, 2010; Wahala *et al.*, 2010). Here, the comparison between ED3.DENV3(st, II) and ED3.DENV3(st, III) showed that solvent-exposed residues could influence stability and that different genotypic strains of the same viral serotype may have different biophysical properties. These variabilities complicate the design of efficient diagnostic assays and vaccines against DENV.

As mentioned above, the ED3 domains of the four DENV serotypes are used both in diagnostic assays and vaccines. There is a clear interest in a unique antigen or immunogen that could detect an infection by anyone of the four DENVs or vaccinate against all of them. Two types of solutions have been proposed to this problem. Some authors have created recombinant molecules that link the ED3 domains of the four serotypes in a single chimeric polypeptide (see Introduction). Others have created a unique ED3 domain that has the consensus sequence of the four serotypes. They have reported that such a consensus domain indeed raises protective antibodies against the four DENV serotypes and is recognized by murine antibodies to at least three of the serotypes (Leng *et al.*, 2009; Danecek *et al.*, 2010; Chiang *et al.*, 2011; Bowen *et al.*, 2012). The consensus ED3.DENVc domain has the advantage of being highly stable and having a defined fold relative to the four ED3.DENV domain linked in a single recombinant polypeptide. The greater stability of a consensus protein relative to the individual proteins of organisms that live in the same physiological conditions has been reported previously and form the basis of a general method for increasing the stability of proteins (Steipe *et al.*, 1994; Steipe, 2004; Monsellier and Bedouelle, 2006). The present study shows that such an approach can be used to increase the stability not only of antibodies or enzymes, as previously reported, but also of antigens and immunogens, provided that the solvent-accessible residues are kept unchanged.

In conclusion, our results could contribute to a better understanding of the flavivirus biology and immunology and should be useful for the potential applications of the ED3 domains in the fields of diagnostics, vaccines and therapeutic molecules against this important class of viral pathogens.

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