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Refolding, crystallization and preliminary X-ray structural studies of the West Nile virus envelope (E) protein domain III

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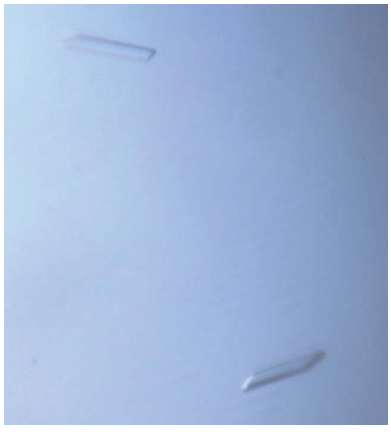

Domain III of the West Nile virus envelope protein, the putative receptor-binding domain, is a major virion-surface determinant for virulence. This protein was reported to be intrinsically unstable and has defied previous crystallization attempts. It has now been purified from inclusion bodies by protein refolding and was crystallized using the hanging-drop vapour-diffusion method at 291 K. The crystals belong to space group $P222_1$, with unit-cell parameters a = 52.6, b = 59.7, c = 95.0 Å. A complete data set was collected to 2.8 Å at 100 K with Cu $K\alpha$ X-rays from a rotating-anode generator.

1. Introduction

West Nile virus (WNV) is a member of the genus *Flavivirus* from the family *Flaviviridae* that infects a variety of mammalian and avian hosts, including human beings. It is an emerging virus in the Western hemisphere. The first outbreak in the United States was reported in 1999 in New York and subsequently spread across the country. In 2002, WNV was responsible for the largest outbreak of arthropod-borne encephalitis in the Western hemisphere. In 2003, more than 9800 human cases and 264 deaths were reported (Centers for Disease Control and Protection, 2003). There were still more than 2400 cases and 87 deaths in 2004 (Centers for Disease Control and Protection, 2004). Flaviviruses produce three structural proteins, with the envelope (E) protein being the major component of the virion surface. All flaviviral E proteins have three domains: I, II and III. It is known that domain III (ED3) of flaviviruses has important implications in virulence. In louping ill virus, dengue virus and WNV, single-amino-acid mutations in this domain disrupt the binding of neutralizing monoclonal antibodies (Jiang *et al.*, 1993; Gao *et al.*, 1994; Volk *et al.*, 2004; Modis *et al.*, 2005). Subsequently, ED3 was proposed to be the receptor-binding domain (Crill & Roehrig, 2001; Mandl *et al.*, 2000) and thus plays a central role in the receptor-mediated endocytotic entry mechanism of flaviviruses (Heinz & Allison, 2000). Comparative structural studies of ED3s might shed light on the general pathogenesis and virulence of this genus of viruses. Owing to their crucial roles in the viral life cycle, the ED3s are attractive targets for drug development. An atomic structure of the ED3 may aid the design of virus-entry inhibitors, for example, by virtual screening. Recently, the solution structure of the ED3 from the New York strain of WNV has been described (Volk *et al.*, 2004). However, structures determined by X-ray crystallography are often more desirable for guiding structure-based drug design and protein-engineering studies because the atomic positions of ordered surface side chains are generally better defined. In the past, the WNV ED3 has defied crystallization, presumably because the protein is intrinsically unstable, as revealed by biophysical comparisons with three other homologous flaviviral ED3s (Yu *et al.*, 2004). We have overcome the production and purification of recombinant WNV ED3 (WNV-rED3) by protein refolding and report here the successful crystallization of this protein.

Table 1
Data-processing statistics.

Values in parentheses are for the last resolution shell.

X-ray wavelength (Å)	1.5418
Temperature (K)	100
Space group	$P222_1$
Unit-cell parameters (Å)	a = 52.56, b = 59.69, c = 95.03
Resolution range (Å)	50.0–2.8
Total/unique reflections	58444/7238
R_{merge}	19.2 (54.5)
Data completeness (%)	92.7 (100)
Average $I/\sigma(I)$	9.9 (3.1)

2. Materials and methods

2.1. Protein expression

The DNA encoding WNV-rED3 (residues 298–415) was cloned into the pET11a vector (Novagen) and transformed into *Escherichia coli* BL21DE3 cells. The recombinant protein was overexpressed in bacteria grown in LB medium containing 100 µg ml^{-1} ampicillin at 310 K. When the culture density reached an OD_{600} of 0.5–0.7, IPTG was added to a final concentration of 1 m*M* to induce expression and the cells were grown for a further 4 h at 310 K. The proteins were expressed as inclusion bodies and prepared as previously described (Gao *et al.*, 1998). Cells were harvested by centrifugation and sonicated in lysis buffer (10 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 10 m*M* EDTA, 2 m*M* DTT, 0.5 m*M* PMSF, 100–400 µg ml^{-1} lysozyme, 10% glycerol, 20 µg ml^{-1} DNAse I). After centrifugation, the pellet was treated with Triton wash buffer (0.5% Triton X-100, 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 0.1% sodium azide, 10 m*M* EDTA, 2 m*M* DTT) and further washed with resuspension buffer (50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 10 m*M* EDTA, 2 m*M* DTT). The washed inclusion bodies were then dissolved in guanidine buffer (6 *M* guanidine, 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 10 m*M* EDTA, 10 m*M* DTT). The purity of the sample was assessed by SDS–PAGE.

2.2. Protein refolding, purification and mass-spectrometric analysis

Refolding of the recombinant protein was performed at low temperature (277–285 K) in refolding buffer (200 m*M* Tris–HCl pH 8.0, 1 *M* L-arginine, 10 m*M* EDTA, 0.1 m*M* PMSF, 5 m*M* reduced glutathione, 0.5 m*M* oxidized glutathione). A total of 60 mg (20 mg each day for 3 d) of sample was added to cold refolding buffer (1 l) and incubated over 3 d. After refolding, the sample was concentrated in a stir cell to a volume of 30–50 ml and centrifuged at 15 000 rev min^{-1} at 277 K for 30 min. The supernatant was dialysed into gel-filtration buffer (50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl) and purified by gel-filtration chromatography using a Superdex 75 HR column (Amersham). The purified protein was desalted and its molecular weight was determined by mass spectrometry to be 12 488.0 Da (Fig. 1), which agrees well with the calculated weight of 12 488.6 Da.

Table 2
Matthews coefficient calculations.

Molecules per AU	Matthews coefficient (Å^3 Da^{-1})	Solvent content (%)
2	2.9	57.4
3	1.9	36.1

2.3. Crystallization

Crystallization trials were set up with Hampton Crystal Screens 1 and 2 at 291 K using the hanging-drop method. Drops contained equal volumes (1 µl each) of protein solution (10 mg ml^{-1}) and reservoir crystallization buffer and were placed over a well containing 200 µl reservoir solution. Crystals suitable for data collection were grown from the optimized conditions [1.5 *M* ammonium sulfate, 100 m*M* Tris–HCl pH 8.5 with 9–10%(*v*/*v*) DMSO] to full size (typical dimensions 10 × 10 × 100 µm; Fig. 2) in four weeks.

2.4. Data collection and processing

Data were collected in-house on a Rigaku MicroMax007 rotating-anode X-ray generator operating at 40 kV and 20 mA (Cu $K\alpha$; λ = 1.5418 Å) equipped with an R-AXIS VII^{++} image-plate detector. A complete set was collected to 2.8 Å (Fig. 3) at 100 K from a single crystal. The diffraction data were indexed, integrated and scaled with *DENZO* and the *HKL*2000 software package (Otwinowski & Minor, 1997). The statistics of data processing are summarized in Table 1. The space group was determined to be $P222_1$, with unit-cell parameters a = 52.6, b = 59.7, c = 95.0 Å. The Matthews coefficients (Table 2; Matthews, 1968) are consistent with either two or three molecules in the asymmetric unit.

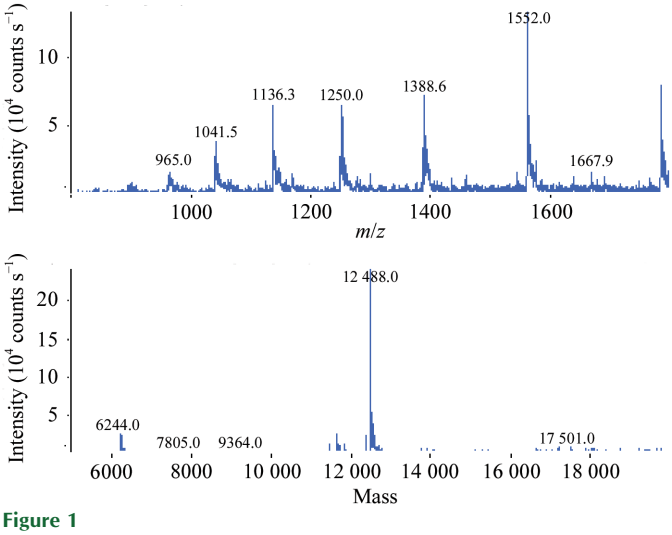

Figure 1
Mass-spectrometric results for WNV-rED3. The molecular weight of the refolded rED3 is clearly shown.

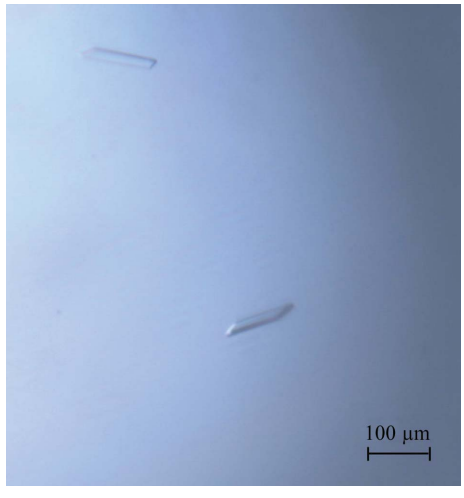

Figure 2
Typical appearance of crystals in a crystallization drop.

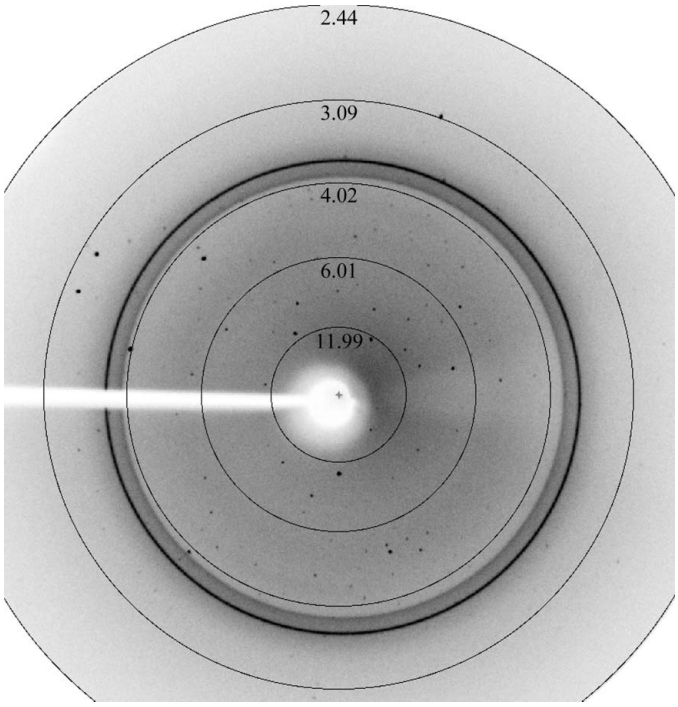

Figure 3
Typical diffraction pattern showing diffraction beyond 3.0 Å.

3. Results and discussion

The target protein is expressed at elevated levels as inclusion bodies. We demonstrate here that these insoluble materials can be successfully refolded and purified. The purified sample has a molecular weight of 12.5 kDa, as determined by mass spectrometry and SDS–PAGE, which agrees well with the monomeric molecular weight. The gel-filtration elution profile is consistent with a monomeric species in solution (data not shown). Although WNV domain III is significantly less stable than its other flaviviral homologues (Yu *et al.*, 2004), this refolded protein forms good-quality crystals that diffract to medium resolution. It is conceivable that the protein-refolding approach may be generally applicable to the purification and crystallization of other homologous viral envelope proteins. A similar refolding approach of WNV-rED3 has been reported by Chu *et al.* (2005), who showed that the preparation is biologically active. Further optimization of crystal quality and structure solution of this protein are under way.

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