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Crystallization and preliminary crystallographic analysis of the fusion core from two new zoonotic paramyxoviruses, Nipah virus and Hendra virus

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Highly conserved heptad-repeat (HR1 and HR2) regions in class I viral fusion (F) proteins, including the F protein from paramyxovirus, interact with each other post-fusion to form a six-helix bundle called a fusion core. Crystals of the fusion core of Nipah virus have been grown at 291 K using PEG 4000 as precipitant. The diffraction pattern of the crystal extends to 2.1 Å resolution at 100 K in-house. The crystals have unit-cell parameters $a = 31.664$, $b = 31.725$, $c = 51.256$ Å, $\alpha = 80.706$, $\beta = 86.343$, $\gamma = 65.812^\circ$ and belong to space group $P1$. Crystals of the fusion core of Hendra virus have also been grown at 291 K using PEG 4000 as precipitant. The diffraction pattern of the crystal extends to 2.0 Å resolution at 100 K in-house. A selenomethionine (SeMet) derivative of the HeV fusion core was overexpressed using the same *Escherichia coli* expression system and purified. The derivative crystals were obtained under similar conditions and three different wavelength data sets were collected to 2.0 Å resolution from the derivative crystal at BSRF (Beijing Synchrotron Radiation Facility). The crystals have unit-cell parameters $a = 31.997$, $b = 31.970$, $c = 53.865$ Å, $\alpha = 85.990$, $\beta = 85.842$, $\gamma = 68.245^\circ$ and belong to space group $P1$.

1. Introduction

Nipah virus (NiV) and Hendra virus (HeV) are newly emergent zoonotic members of the *Paramyxoviridae* family (Murray, Rogers *et al.*, 1995; Murray, Selleck *et al.*, 1995; Selvey *et al.*, 1995; Chua *et al.*, 1999, 2000; Harcourt *et al.*, 2000). They have several distinct characteristics when compared with other known members of the *Paramyxoviridae* and a new genus has therefore been proposed for them, the genus *Henipavirus* (Wang *et al.*, 2000, 2001; Chan *et al.*, 2001). Paramyxoviruses are a group of enveloped negative-stranded RNA viruses. Their infection is mediated by their fusion glycoproteins through membrane fusion.

The fusion glycoproteins of enveloped viruses such as the paramyxoviruses, which are typically type I integral membrane proteins, are encoded by the virus and synthesized by the infected cell (reviewed in Eckert & Kim, 2001). NiV and HeV F proteins share many common features with homologous proteins in the *Paramyxoviridae*, *Retroviridae* and *Filovirdae* families (Wang *et al.*, 2000, 2001; Chan *et al.*, 2001; Eckert & Kim, 2001). They are synthesized as an inactive precursor (F_0) and then cleaved by an intracellular proteolytic enzyme into two disulfide-bond-linked subunits. These are termed the distal subunit F_2 and the transmembrane subunit F_1, which possesses a highly hydrophobic conserved region called the fusion peptide and two heptad-repeat regions (HR1 and HR2), one (HR1 or HRA) adjacent to the fusion peptide and the other (HR2 or HRB) adjacent to the transmembrane (TM) domain of the F_1 subunit (Michalski *et al.*, 2000; Tamin *et al.*, 2002). It is believed that the two HR domains refold into a six-helix bundle during the fusion process, in which the HR1 domain forms a trimeric coiled coil surrounded by three antiparallel helices of the HR2 domain (reviewed in Eckert & Kim, 2001). Consequently, the transmembrane domain and the fusion peptide, which is known to insert into the cell membrane, are both transposed into close association. This pulls the cellular and viral membrane into proximity, facilitating membrane fusion. This six-helix bundle is called the virus fusion core (reviewed in Eckert & Kim, 2001). Our previous biochemical and biophysical analysis has demonstrated that the heptad-repeat peptides of the NiV and HeV fusion proteins both form a typical six-helix bundle (Xu *et al.*, 2004).

In this report, we describe the crystallization and preliminary X-ray diffraction studies of the fusion cores (the six-helix bundles of the HR1 and HR2 complex) of both the NiV and HeV fusion proteins.

2. Materials and methods

2.1. Preparation of the fusion cores

The fusion cores of both the NiV and HeV fusion proteins were prepared as a single chain by linking the HR1 and HR2 domains *via* an

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eight-amino-acid linker (GGSGGSGG). The constructs and the encoded proteins are also known as 2-Helix. The preparation and characterization of the 2-Helix proteins has been reported previously (Xu *et al.*, 2004), but the constructs were re-cloned to remove the original rhinovirus 3C cleavage site in order to obtain better crystals. The new constructs were cloned by PCR from the original pET-23d vector into the new pET-22b vector (Novagen). *Nde*I and *Xho*I sites were introduced by PCR as restriction sites for cloning. The 2-Helix constructs were verified by direct DNA sequencing. The primers used for making the new 2-Helix constructs were NP F, 5′-CTGA-**CATATG**GCTATGAAAAACGCTGAC-3′, and NP R, 5′-ATGA**CTCGAG**AACGGTG-TCCAGCAGACG-3′.

The protein expression and Ni-affinity purification were essentially performed as described previously (Xu *et al.*, 2004). Briefly, the plasmids were transformed into BL21 (DE3) competent cells and the cells were cultured at 310 K in 2×YT medium containing 100 µg ml^{-1} ampicillin. When the culture density (A_{600}) reached to 0.6–0.8, the culture was induced with 0.2 m*M* IPTG and grown for an additional 10 h at 289 K before the cells were harvested.

The bacterial cell pellet was resuspended in PBS and homogenized by sonication. The lysate was then centrifuged at 18 000*g* for 20 min at 277 K and the supernatant was loaded onto an Ni^{2+}–NTA column (Qiagen). The contaminated protein was washed with washing buffer (1×PBS, 60 m*M* imidazole) and the target protein was eluted with elution buffer (1×PBS, 500 m*M* imidazole). The protein purified by affinity chromatography was further purified by a gel-filtration run on a Superdex G75 column (Pharmacia) and analyzed by SDS–PAGE.

The selenomethionine derivative of the HeV 2-Helix was expressed in minimal media M9 containing 30 mg l^{-1} L-SetMet. A further six amino acids (lysine, threonine, phenylalanine, leucine, isoleucine and valine) were added to the culture for inhibition of Met biosynthesis of the BL21 (DE3) expression strain. Purification of the selenomethionene HeV 2-Helix was performed as for the native protein. The incorporation of selenium was confirmed by mass-spectrometric analysis.

2.2. Crystallization

The purified protein was dialyzed into crystallization buffer (10 m*M* Tris–HCl pH 8.0, 10 m*M* NaCl) and concentrated to 10–15 mg ml^{-1}. Protein concentrations were determined by absorbance at 280 nm, assuming an A_{280} of 0.393 for a 1.0 mg ml^{-1} solution. Initial crystallization conditions were screened using Crystal Screen (Hampton Research). The protein could be crystallized under several conditions. The conditions yielding small crystals were further optimized by changing the precipitant and protein concentration and the Buffer pH. NiV 2-Helix crystals of good quality could be obtained in 0.1 *M* Tris–HCl pH 8.5, 29%(*v*/*v*) PEG 4000; HeV 2-Helix crystals of good quality could be obtained in 0.1 *M* HEPES pH 6.5, 10%(*v*/*v*) PEG 4000.

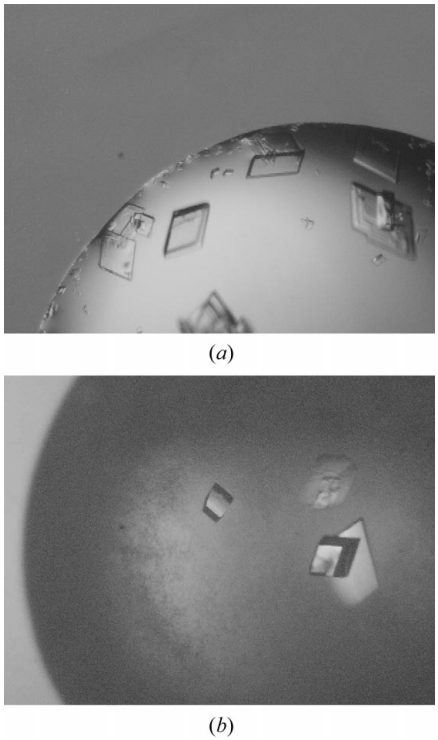

Figure 1
(*a*) Typical crystals of the NiV 2-Helix protein grown using the hanging-drop method in 29% PEG 4000, 0.1 *M* Tris pH 8.5. (*b*) Typical crystals of the HEV 2-Helix protein grown using the hanging-drop method in 10% PEG 4000, 0.1 *M* HEPES pH 6.5.

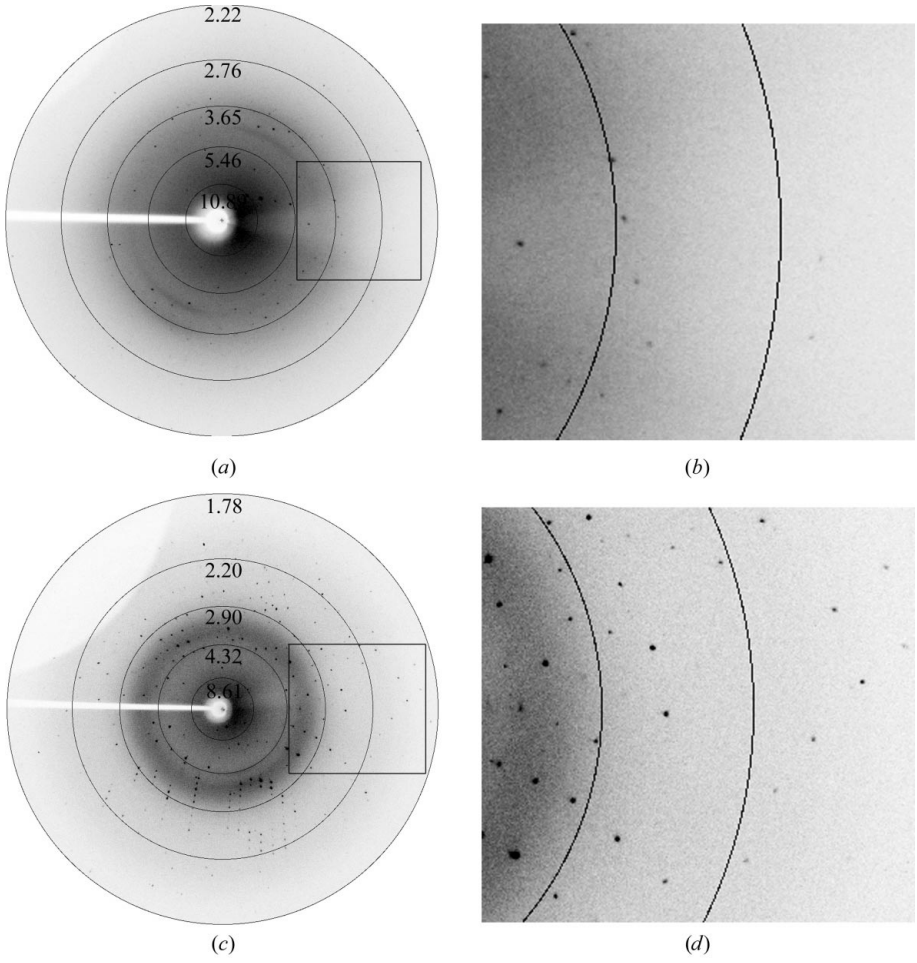

Figure 2
(*a*) A typical X-ray diffraction pattern from a crystal of NiV 2-Helix. The diffraction image was collected on a MAR Research image-plate detector. The oscillation range is 1°. (*b*) An enlarged image of the area indicated in (*a*). (*c*) A typical X-ray diffraction pattern from a crystal of HeV 2-Helix. The diffraction image was collected on a MAR Research image-plate detector. The oscillation range is 1°. (*d*) An enlarged image of the area indicated in (*c*).

Table 1
Data collection and processing statistics of the fusion cores of NiV and HeV.

	HeV			
Data	Peak	Edge	Remote	NiV
Space group	*P*1	*P*1	*P*1	*P*1
Unit-cell parameters				
a (Å)	31.938	31.997	33.003	31.664
b (Å)	31.965	31.970	33.160	31.725
c (Å)	53.321	53.865	55.618	51.256
α (°)	86.186	85.990	84.957	80.706
β (°)	85.848	85.842	86.701	86.343
γ (°)	68.127	68.245	69.053	65.812
Wavelength (Å)	0.9799	0.9801	0.9500	1.5418
Resolution range (Å)				
Overall	50–2.1	50–2.1	50–2.35	35–2.2
Lowest shell	50–4.52	50–4.52	50–5.06	35–4.74
Highest shell	2.18–2.10	2.18–2.10	2.43–2.35	2.28–2.20
Reflections observed	84348	97509	88705	27810
Unique reflections	11172	13019	12545	8630
Completeness (%)				
Overall	98.0	98.1	97.9	94.7
Lowest shell	99.4	99.4	99.0	96.8
Highest shell	96.9	96.9	97.3	91.0
$I/\sigma(I)$				
Overall	10.5	10.3	10.6	8.2
Lowest shell	15.1	15.1	13.2	11.0
Highest shell	3.3	3.5	3.2	2.8
Redundancy	7.6	7.5	7.1	3.2
$R_{\rm merge}$ (%)				
Overall	13.4	13.7	14.2	7.1
Lowest shell	8.2	8.3	8.4	4.2
Highest shell	45.5	41.3	49.3	37.4
Overall temperature factor† (Å^2)	22.9			34.8

† Estimated from the Wilson plot.

Crystallization was performed using the hanging-drop vapour-diffusion method at 291 K. 1 µl protein solution was mixed with 1 µl reservoir solution and the mixture was equilibrated against 200 µl reservoir solution at 291 K. The crystals appeared in 3–5 d.

The purified HeV 2-Helix selenomethionine derivative was concentrated to 8 mg ml^{-1}. Crystallization trials were set up based on the optimized conditions used for native protein.

2.3. Data collection and processing

Data collection of NiV 2-Helix was performed in-house on a Rigaku RU2000 rotating copper-anode X-ray generator operated at 48 kV and 98 mA (Cu $K\alpha$; λ = 1.5418 Å) with a MAR 345 image-plate detector. The crystal was mounted on a nylon loop and flash-frozen in a cold nitrogen-gas stream at 100 K using an Oxford Cryosystems cryocooler with reservoir solution as the cryoprotectant. Data were indexed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

The HeV 2-Helix selenomethionine-derivative crystal was mounted on a nylon loop and flash-frozen in a cold nitrogen-gas stream at 100 K using an Oxford Cryosystems cryocooler with 0.1 *M* HEPES pH 6.5, 25% PEG 400 as cryoprotectant. Data collection was performed by a rotation method using a MAR CCD detector with synchrotron radiation on BSRF (beamline 3W1A of Beijing Synchrotron Radiation Facility). Data were indexed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

The 2-Helix proteins of NiV and HeV can easily be crystallized under several conditions. However, good-quality crystals of NiV 2-Helix could only be obtained with 0.1 *M* Tris pH 8.5, 29%(*v*/*v*) PEG 4000 (Fig. 1*a*). For HeV 2-Helix and its selenomethionine derivative, the only conditions under which good-quality crystals could be obtained were 0.1 *M* HEPES pH 6.5, 10%(*v*/*v*) PEG 4000 (Fig. 1*b*). The NiV crystals belong to space group *P*1, with unit-cell parameters *a* = 31.664, *b* = 31.725, *c* = 51.256 Å, α = 80.706, β = 86.343, γ = 65.812° (Figs. 2*a* and 2*b*). Assuming the presence of three molecules (one stable trimer) in the asymmetric unit, the solvent content is calculated to be about 21.1% and the Matthews number ($V_{\rm M}$) is about 1.5 Å^3 Da^{-1}. The HeV selenomethionine-derivative crystals belong to space group *P*1, with unit-cell parameters *a* = 31.997, *b* = 31.970, *c* = 53.865 Å, α = 85.990, β = 85.842, γ = 68.245° (Figs. 2*c* and 2*d*). Assuming the presence of three molecules (one stable trimer) in the asymmetric unit, the solvent content is calculated to be about 25.8% and the Matthews number ($V_{\rm M}$) is about 1.7 Å^3 Da^{-1}. Selected data statistics are shown in Table 1.

Multiple-wavelength anomalous dispersion (MAD) data of HeV were collected from a single selenomethionine-derivative crystal using synchrotron radiation on BSRF (beamline 3W1A of the Beijing Synchrotron Radiation Facility) at peak (0.9799 Å), inflection (0.9801 Å) and remote (0.950 Å) wavelengths to 2.1 Å using a MAR CCD detector. The crystal-to-detector distance was 80 mm, the exposure time was approximately 60 s and the oscillation angle was 3°. Reflections with intensities of $I/\sigma(I) > 3$ were analyzed to 2.1 Å

X-ray diffraction data were collected from a single crystal of NiV using a Rigaku RU2000 rotating copper-anode X-ray generator and a MAR 345 image-plate detector. The crystal-to-detector distance was 120 mm, the exposure time was approximately 600 s, the oscillation angle was 1° and the wavelength was 1.5418 Å.

The data were indexed and reduced online using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The structures of the NiV 2-Helix and HeV 2-Helix have been solved and will be published elsewhere. Clear structural analysis of the six-helix bundles of NiV and HeV will provide a detailed picture of the viral fusion-core structure and the molecular mechanism underlying the interchangeable interaction of HR1 with both NiV and HeV HR2. This will inevitably add to the repertoire of paramyxovirus six-helix bundle fusion-core structures, only two of which are available at present (those of RSV and SV5). This will also open a new avenue towards the structure-based design of fusion-inhibitor peptides or peptide analogues, *e.g.* small molecules, for these emerging infectious diseases.

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