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Preliminary crystallographic analysis of neuraminidase N2 from a new influenza A virus

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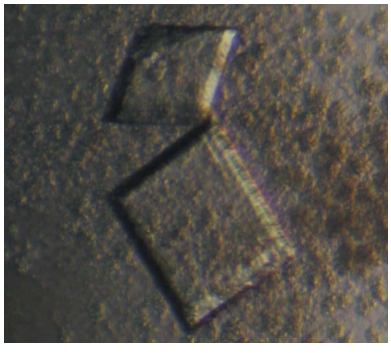
Influenza virus is a major viral respiratory pathogen that causes yearly epidemics in temperate climates. The H3N2 subtype is one of the major causative agents of severe epidemics and plays a critical role in vaccine development. The neuraminidase (NA) inhibitors oseltamivir and zanamivir are two commercially available NA-targeted competitive antiviral drugs. However, their effectiveness has been compromised by the rapid emergence of resistance. Q136K is a novel mutation in NA which confers resistance to zanamivir. In this study, a Q136K mutant N2 protein was expressed in a baculovirus system and crystals were obtained. The crystal of N2 belonged to space group $P2_12_12_1$, with unit-cell parameters a = 109.5, b = 112.8, c = 165.2 Å. Data were collected to 2.4 Å resolution. Four monomers were found in the asymmetric unit. The Matthews coefficient and solvent content were calculated to be 3.0 Å^3 Da^{-1} and 59.0%, respectively.

1. Introduction

Influenza A viral infection causes substantial annual morbidity and mortality. Approximately 20% of children and 5% of adults worldwide have symptomatic influenza infections each year. H3N2 is one of the current circulating seasonal subtypes and also affects other animals such as dogs (Lee *et al.*, 2013) and swine (Takemae *et al.*, 2013), raising concern over the potential for zoonotic transmission. Global influenza surveillance indicates that influenza viruses are isolated every month from humans, birds and other mammals somewhere in the world. The emerging novel strains may spread worldwide and impair public health, especially among the elderly, infants, young children and immunocompromised individuals.

The two surface glycoproteins of the virus, haemagglutinin (HA) and neuraminidase (NA), perform clearly defined complementary roles in virus infection. Virus HA is responsible for attachment of the virus to sialic acid-containing glycoconjugates on susceptible cells. NA is responsible for removing the terminal-linked sialic acid moieties from HA-binding receptors to facilitate the release of progeny virions from infected cells and to prevent the aggregation of virus particles (Colman, 2009). Traditional NAs of influenza A virus contain nine serotypes N1–N9, which can be classified into two groups according to their primary sequence: group 1 (N1, N4, N5 and N8) and group 2 (N2, N3, N6, N7 and N9) (Russell *et al.*, 2006). The recently identified N10 is derived from the bat H17N10 genome and has raised considerable interest in the origin and evolution of influenza viruses (Tong *et al.*, 2012). NA is a tetramer with a single membrane-spanning domain and a large ectodomain. Structural analysis shows that group-1 NAs, with the exception of 2009 pandemic H1N1 neuraminidase (09N1; Li *et al.*, 2010), have an extra cavity in the active site called the 150-cavity (formed by loop residues 147–152; N2 numbering) which provides a potential target for NA inhibitors (Russell *et al.*, 2006; Rudrawar *et al.*, 2010). Native group-2 NAs do not contain such a 150-cavity. Recent work shows that oseltamivir carboxylate can induce opening of the closed N2 150-loop, which demonstrates the inherent flexibility of the 150-loop in a typical group-2 NA (Wu *et al.*, 2013).



NA was chosen as a suitable drug target because of its essential role in influenza virus propagation and the conserved residues of the active site and framework in influenza A and B (Colman, 2009). NA inhibitors (NAIs) are the most successful designed drug based on structural studies (Varghese & Colman, 1991; Bossart-Whitaker *et al.*, 1993). Oseltamivir and zanamivir are two commercial antiviral drugs used in clinical treatment. However, drug-resistant mutants in NA limit the effectiveness of NAIs. Most of the drug-resistant mutants have been reported to be oseltamivir-resistant viruses. In influenza A (H1N1 and H5N1) viruses (Chen *et al.*, 2009), H274Y substitution is the commonest mutation (Ramirez-Gonzalez *et al.*, 2011; Le *et al.*, 2005). In N2, E119V confers oseltamivir resistance. All of these mutants are still susceptible to zanamivir. In 2010, a novel zanamivir-resistant influenza A virus was isolated from patients in Myanmar which contains a Q136K mutation in NA (Hurt *et al.*, 2009). Here, we have carried out the crystallization and preliminary X-ray crystallographic analysis of this zanamivir-resistant NA.

2. Materials and methods

2.1. Cloning and expression of influenza A virus N2

Recombinant N2 protein was prepared using a baculovirus expression system. The cDNA encoding the ectodomain (residues 83–469; N2 numbering) of influenza A/Myanmar/M187/2007 (H3N2) NA protein (GenBank ACI22545.1) was cloned into the pFastBac1 baculovirus transfer vector (Invitrogen). A GP67 signal peptide was added at the N-terminus to facilitate secretion of the recombinant protein, followed by a His tag, a tetramerizing sequence and a thrombin cleavage site (Xu *et al.*, 2008). $DH10_{Bac}$ *Escherichia coli* cells (Invitrogen) were transformed with recombinant pFastBac1 plasmid and recombinant baculovirus was prepared based on the manufacturer's protocol (Invitrogen). Hi5 cell suspension cultures were infected with high-titre recombinant baculovirus and grown at 300 K and 120 rev min^{-1} for 60 h.

2.2. Purification of N2 neuraminidase

The cell debris was removed by centrifugation (6000 rev min^{-1}, 60 min) and the supernatant was applied onto a 5 ml HisTrap HP column (GE Healthcare) for initial purification. The HisTrap HP column was eluted with 20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl containing 20, 50 and 200 m*M* imidazole. The N2 protein was identified by 12% SDS–PAGE. Fractions containing NA were dialysed against 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl for 16 h and then digested with thrombin (3 U per milligram of N2; BD Biosciences) overnight at 277 K. Thrombin cleavage leaves six additional amino acids (GSPSRS) at the N-terminus derived from the construct. The cleaved protein was loaded onto a Superdex 200 10/300 GL column (GE Healthcare) and eluted with 20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl. High-purity N2 fractions were pooled and concentrated to 10 mg ml^{-1} using 10 kDa cutoff centrifugal filter units. The protein concentration was determined by the BCA protein assay (Pierce). All purification steps were performed at 277 K.

2.3. Crystallization and X-ray data collection

Crystallization trials were set up with PEGRx 1 and 2 (Hampton Research) at 291 K using the hanging-drop vapour-diffusion method. A drop consisting of 1 µl protein solution (10 mg ml^{-1}) and 1 µl reservoir crystallization buffer was placed over a well containing 200 µl reservoir solution. N2 crystals were obtained in 3 d with 0.1 *M* HEPES pH 7.5, 12%(*w*/*v*) polyethylene glycol 3350. N2 crystals were cryoprotected in mother liquor with the addition of 20%(*v*/*v*) glycerol before being flash-cooled at 100 K. A complete data set was collected to 2.4 Å resolution on beamline BL17U at the Shanghai Synchrotron Radiation Facility. The collected data were indexed, integrated, corrected for absorption, scaled and merged using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

For N2 expression, 1 l of Hi5 cells was infected at a density of 2.0×10^6 cells ml^{-1}. The first step of the purification of the His-tagged N2 protein was affinity chromatography. The chromatographic profile showed three peaks that were eluted using increasing concentrations of imidazole in 20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl buffer. The reduced denaturing SDS–PAGE gel indicated that N2 was present in peak 3 (50 kDa; aFig. 1*a*). The partially purified protein was digested by thrombin to remove the tetramer and His tags, and was further purified by gel-filtration chromatography on a Superdex 200 10/300 GL column (Fig. 1*b*). The molecular weight of the cleaved protein was 42.5 kDa. The final yield of pure protein was 1 mg per litre of cell culture. The N2 protein was concentrated to 10 mg ml^{-1}

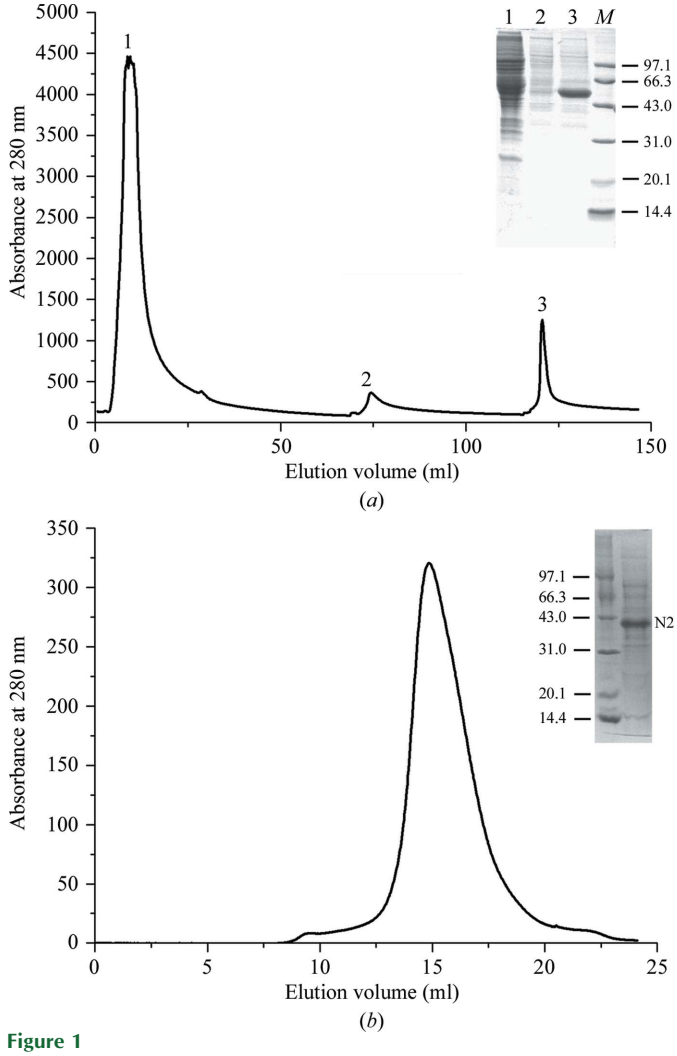
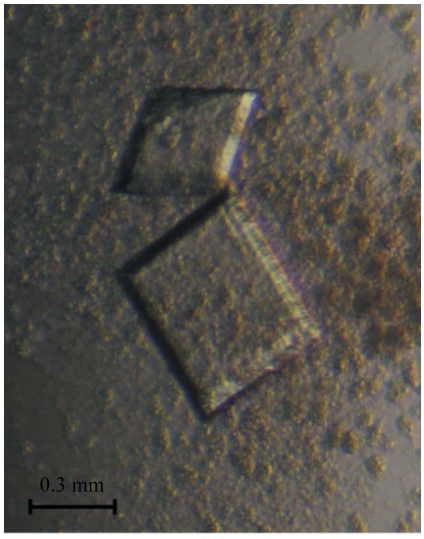
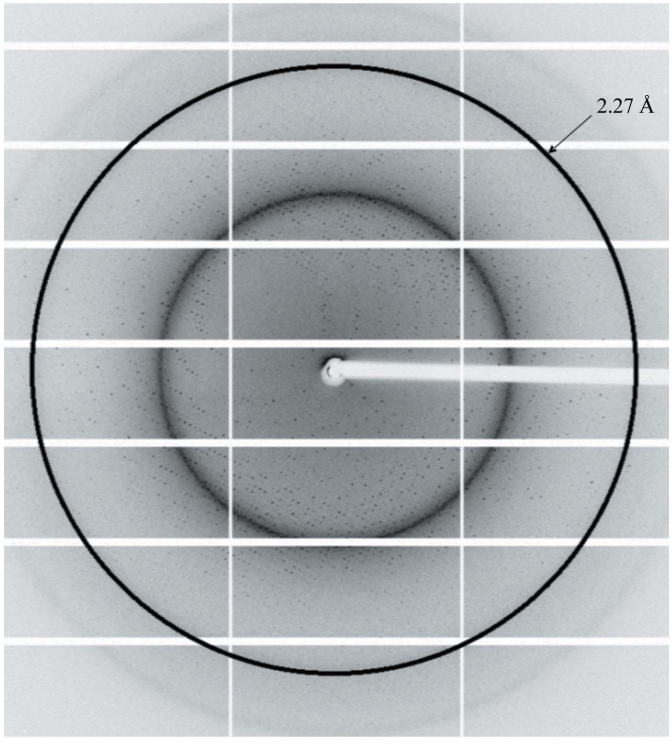


Figure 1
(*a*) Purification of the influenza A virus neuraminidase N2 using a HisTrap HP column. The concentration of imidazole during the elution of peaks 1, 2 and 3 was 20, 50 and 200 m*M*, respectively. (*b*) Gel-filtration profile of the N2 protein and the reduced denaturing SDS–PAGE gel (12%) of the purified protein.



(*a*)



(*b*)

Figure 2
(*a*) Typical crystal of influenza A virus neuraminidase N2. (*b*) X-ray diffraction image of the crystal of influenza A virus neuraminidase N2. The black ring represents 2.27 Å resolution. The oscillation angle ($\Delta\varphi$) used to collect the diffraction image was 1°.

and a crystal screen was set up. Large square crystals appeared in 3 d using 0.1 *M* HEPES pH 7.5, 12%(*w*/*v*) polyethylene glycol 3350 as a precipitant. The crystal dimensions were about 0.5 × 0.3 × 0.1 mm (Fig. 2*a*). Data were collected to 2.4 Å resolution on beamline BL17U at the Shanghai Synchrotron Radiation Facility (Fig. 2*b*). Selected data statistics are shown in Table 1. An initial model was obtained *via* molecular replacement (MR) using the program *MOLREP* (Vagin & Teplyakov, 2010). The search model was the structure of the neuraminidase from influenza virus A/Tokyo/3/67 (PDB entry 1nn2; Varghese & Colman, 1991), which shares 87% identity with the neuraminidase from influenza virus A/Myanmar/M187/2007 target protein. The MR model did not include the Asn-linked carbohydrate residues. Four molecules were found and the *R* factor reported by *MOLREP* was 62.5%. The Matthews coefficient and solvent content (Matthews, 1968) were calculated to be 3.0 Å^3 Da^{-1} and 59.0%, respectively. Refinement of the MR model with its incorrect sequence and without any noncrystallographic symmetry restraints using the program *REFMAC*5 in the *CCP*4 program suite (Murshudov *et al.*, 2011; Winn *et al.*, 2011) lowered the *R* factor to 32.1% and the $R_{\rm free}$ to 36.5%. Further refinement is under way.

Table 1
Crystal data and intensity statistics.

Values in parentheses are for the outermost resolution shell.

Wavelength (Å)	0.9793
Detector	ADSC Q315 CCD
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 109.5, b = 112.8, c = 165.2
Resolution range (Å)	50–2.4 (2.49–2.40)
No. of observed reflections	442455 (40407)
No. of unique reflections	80441 (7923)
Completeness (%)	99.4 (99.4)
$R_{\rm merge}$† (%)	19.5 (54.1)
Mean $I/\sigma(I)$	8.3 (2.7)
Multiplicity	5.5 (5.1)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the unique reflection *hkl* and $\langle I(hkl)\rangle$ is the average over symmetry-related observations of the unique reflection *hkl*.

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