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Crystallization and preliminary X-ray crystallographic studies of type A influenza virus matrix protein M1

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Abstract

The matrix protein, M1, of influenza virus strain A/PR/8/34 has been purified from virions and crystallized. The crystals consist of a stable fragment (18 kDa) of the M1 protein. X-ray diffraction studies indicated that the crystals are in space group $P3_121$ or $P3_221$, with $a = 66.17$, $c = 135.30$ Å. V_m calculations showed that there are two monomers in the asymmetric unit.

1. Introduction

Influenza virus is an enveloped virus which contains eight separate segments of negative-stranded RNA genome. There are two spike glycoproteins on the surface of the viral membrane envelope, the receptor-binding hemagglutinin (HA), and the neuraminidase (NA). The core ribonucleoprotein (RNP) encapsidated in the viral membrane envelope is composed of an RNA polymerase and RNA-binding nucleoproteins (NP) (Pons, Schulze, Hirst & Hauser, 1969). The interaction of RNP's with the membrane is mediated by the matrix protein M1 (252 amino acids, $M_r = 27$ kDa), which are tightly associated with the RNP cores while interacting with the cytoplasmic tails of the spike glycoprotein and the viral membrane (Helenius, 1992). Through the binding of hydrophobic domains to the virion lipid envelope, M1 maintains the structural integrity of the virus particle (Gregoriades & Frangione, 1981; Bucher, Kharitonenkov, Zakomirdin, Grigoriev, Klimenko & Davis, 1980). On the other hand, the interaction of M1 with RNP cores is mostly electrostatic at neutral pH (Wakefield & Brownlee, 1989). The M1 can easily be dissociated from the RNP cores by low-pH treatment (Zhirnov, 1992). The interaction of M1 with NP bound RNA templates also inhibits virus transcription and replication as a repressor (Helenius, 1992).

In addition to packaging the RNP cores during virion assembly and blocking transcription and replication, M1 also directs the transportation of RNP's into or out of the nucleus (Martin & Helenius, 1991). Upon entry of the virus into the new host cell, M1 is dissociated from RNP's as a result of reducing pH in the fusion endosome, allowing the RNP's to enter the nucleus. When progeny viral RNP's are produced, newly synthesized M1 escorts the RNP's out of nucleus and targets them to the assembly site on the cellular membrane where HA and NA are located. It is, therefore, interesting to study the crystal structure of this most abundant protein in influenza virus.

2. Experimental

2.1. *Virus preparation*

Since M1 constitutes 40% of the total protein in the influenza virus, intact virions were used as the source for purification of the M1 protein. Influenza virus strain A/PR/8/34 was inoculated in 11-day-old embryonated eggs followed by incubation at 307.5 K for 48 h. The allantoic fluid (from the embryonic diverticulum) was harvested at the end of incubation, and was centrifuged at 8000 rev min^{-1} in a Beckman JA10 rotor for 20 min at 277 K. Virus which stayed in the supernatant was concentrated by about 15-fold through an Amicon concentrator in a 277 K cold room. The virus was pelleted in a Beckman Ti 45 rotor at 20 000 rev min^{-1} for 3 h at 277 K. The virus pellet was then soaked in $Ca^{2+}Mg^{2+}$-saline (0.2 m*M* $CaCl_2$, 0.8 m*M* $MgCl_2$, 0.15 *M* NaCl) solution overnight. The softened pellet was resuspended by pipetting gently to complete suspension. The virus solution was then subjected to centrifugation on a 10–40% linear sucrose gradient in a Beckman SW28 rotor at 17 000 rev min^{-1} for 40 min at 277 K. The virus band was collected from the upper middle area, and was pelleted again in a Beckman SW28 rotor at 25 000 rev min^{-1} for 3 h at 277 K. Finally, the virus was resuspended in a $Ca^{2+}Mg^{2+}$-saline solution for storage.

2.2. *Protein extraction and purification*

In order to strip off the lipid membrane and the membrane-embedded surface proteins, 1 ml of the purified virus preparation was loaded on a three-step sucrose gradient following Zhirnov (Zhirnov, 1992). The gradient consisted of, from bottom to top, 3 ml 32% sucrose and 5 ml 17% sucrose in 0.15 *M* NaCl, 10 m*M* Hepes (pH 7.2) containing 1% of nonionic detergent NP40, and 2 ml 10% sucrose in 0.15 *M* NaCl, 10 m*M* Hepes (pH 7.2) without detergent. After centriguation in a Beckman SW41 rotor at 21 000 rev min^{-1} for 3 h at 277 K, the M1–RNP complex was pelleted to the bottom of the centrifuge tube whereas the membrane-associated proteins stayed in the gradient solution. The pellet of the M1–RNP complex was resuspended in 1 ml of 50 m*M* NaH_2PO_4 50 m*M*, 5 m*M* benzamidine and 0.02% NaN_3 (pH 4.0) to release the M1 protein from the M1–RNP complex. The RNP cores (without M1) were removed by centrifugation in a Beckman SW55 rotor at 22 000 rev min^{-1} for 1 h at 277 K. About 0.5 mg M1 protein were obtained from 1 ml virus.

The M1 protein was further purified by a Superdex 75 gel-filtration column (Pharmacia) mounted on a Pharmacia fast protein liquid chromatography (FPLC) system. The M1 protein was pooled about 62 min after sample injection at a flow rate of 1 ml min^{-1}. Compared with the molecular weights of the protein standards for gel-filtration chromatography, the apparent molecular weight of the eluted M1 protein is around

50 kDa. Since the molecular weight of the M1 monomer derived from the amino-acid sequence is 27 kDa (Winter & Fields, 1980), we inferred from this observation that the M1 protein forms a dimer at acidic pH.

However, the M1 protein was not stable when concentrated to 5 mg ml^{-1} prior to crystallization. A stable fragment of 18 kDa was identified after two week storage of the concentrated protein sample at room temperature. Dot-blotting showed that this major fragment of the M1 protein was still recognized by rabbit anti-M1 polyclonal antibodies (data not shown). Fig. 1 presents a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel of the digested M1 fragment compared with proteins from the whole virus, and the freshly purified M1 protein. The more accurate molecular weight of the fragment was determined by mass spectrometry (PE Sciex API III) as 18 230 Da. The N-terminal peptide sequencing showed the first three amino-acid residues of the fragment are: S L L. Based on the results above and the amino-acid sequence of the M1 protein, we concluded that this fragment is a polypeptide of 164 amino-acid residues corresponding to the N-terminal end of the intact M1, beginning with the second amino-acid residue. The cleavage site is unknown for any protease. When the fragment was repurified by FPLC gel-filtration chromatography (Pharmacia Superdex 75), it showed that the fragment still forms a dimer. The M1 fragment remained soluble after transferring the concentrated protein into a buffer with Hepes 10 m*M*, NaCl 0.15 *M*, pH 7.2. The soluble fragment at pH 7.2 was then incubated with insoluble M1-free RNP cores obtained during M1 purification. The mixture were pelleted and analyzed by an SDS–PAGE gel (Fig. 2). As shown by the gel, the M1 fragment is still able to bind RNP cores at neutral pH as the native M1 protein does.

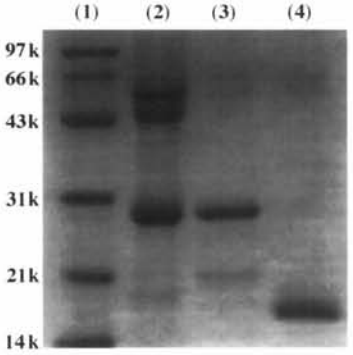

Fig. 1. 12.5% SDS–PAGE gel showing (1) protein standards with molecular weight 97, 66, 45, 31, 21 and 14 kDa; (2) influenza virus A/PR/8; (3) fresh M1 protein; (4) digested M1 protein fragment. The gel was stained by Coomassie brilliant blue.

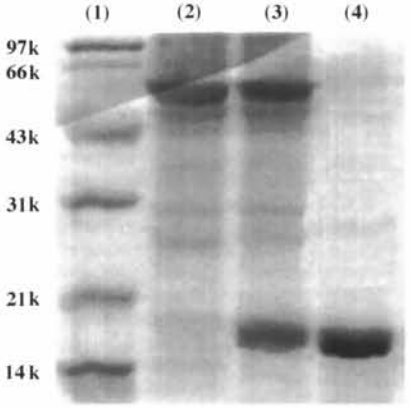

Fig. 2. 12.5% SDS–PAGE gel showing (1) protein standards with molecular weight 97, 66, 45, 31, 21 and 14 kDa; (2) M1-free RNP cores from influenza virus A/PR/8/34; (3) pellet of the mixture of M1 fragment and M1-free RNP cores in neutral pH; (4) the stable M1 fragment from influenza virus A/PR/8/34.

2.3. *Crystallization, data collection and processing*

Crystallization trials were carried out in hanging drops by the vapor-diffusion method. The protein concentration was about 5 mg ml^{-1}. The well solution was 50 m*M* NaH_2PO_4, 5 m*M* benzamidine, 0.02% NaN_3 and 20% PEG 3350 with pH 4.0. The hanging drops have a 1:1 ratio of protein to well solution. Large crystals (0.05 × 0.05 × 0.3 mm) of the 18 kDa M1 protein fragment could be grown at 293 K over two months (Fig. 3). X-ray diffraction data were collected on a MAR Research image plate at the Brookhaven National Laboratory on beamline X-12B using a 1.5° oscillation. All crystals were frozen at 100 K in a nitrogen gas stream with 20% glycerol in the mother liquid serving as cryoprotectent. The data were processed using the *HKL* package (Otwinowski, 1993; Minor, 1993). The crystals belong to space group $P3_121$ or $P3_221$ with $a=66.17$, $c=135.30$ Å. The crystals diffracted to 2.08 Å. The data were 94.7% complete and the R_{symm} was 0.048. The V_m value is 2.50 $Å^3$ Da^{-1} for two monomers per asymmetric unit, which is within the range of normal protein crystals (Matthews, 1968). The crystal structure is to be solved by MIR method because no similar structures are available. Two useful heavy-atom derivatives were obtained by soaking the crystals in the well solution with K_2PtCl_4 1 m*M* or K_2OsCl_6 5 m*M* for 12 h, respectively. The structure determination is under way.

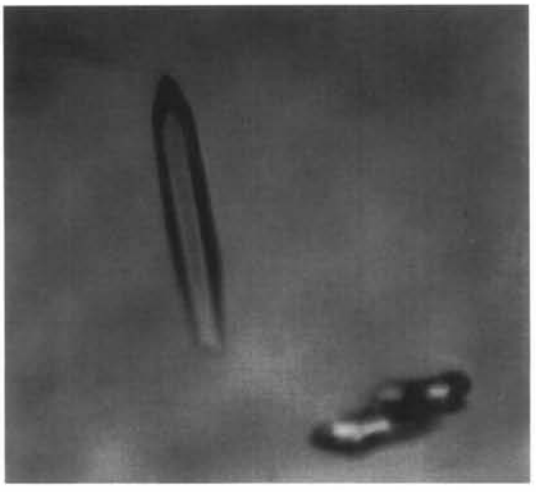

Fig. 3. Crystals of M1 protein fragment. Crystals were grown by vapor diffusion in hanging drops at 293 K over 20% PEG 3350 for two months (0.05 × 0.05 × 0.3 mm).

This stable N-terminal fragment is a multi-functional protein. It can still bind RNP cores. This is consistent with previous studies. It is shown that the RNA binding and anti-transcription domain of the influenza virus matrix protein M1 is located within the amino-acid sequence at positions 80–111 by monoclonal antibody mapping and by deletions in *E. coli* expressed M1 (Ye, Baylor & Wagner, 1989; Watanabe, Handa, Mizumoto & Nagata, 1996). On the other hand, it is also suggested that the N-terminal part of the matrix protein can bind membrane by hydrophobic interactions (Ye, Pal, Fox & Wagner, 1987). Though this protein is an 18 kDa fragment of intact protein, it may still have the ability to bind viral membrane and RNP cores. Both intact matrix protein and the fragment form a dimer, which may be necessary for their biological function. There are two monomers per asymmetric unit. Self-rotation function calculations showed that the two monomers are related by a non-crystallographic symmetry (NCS) twofold axis, which suggested that the two monomers may form a dimer by the NCS twofold axis in the crystal structure. However, the detailed mechanism of M1 interactions with RNP cores and the membrane-glycoprotein tails is unknown. The crystallization of the M1 protein may lead to the solution of the three-dimensional structure which will help in understanding the function of the influenza virus matrix protein M1.

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