Crystallization and Preliminary X-ray Analyses of Two Neuraminidases from Influenza B Virus Strains B/Hong Kong/8/73 and B/Lee/40*

(Received for publication, October 19, 1987)

Patricia J. Bossart‡, Y. Sudhakar Babu§, William J. Cook§‖, Gillian M. Air§†, and W. Graeme Laver**

From the †Departments of Microbiology, ‡Center for Macromolecular Crystallography, §Comprehensive Cancer Center, and ‖Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294 and the **John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory 2601, Australia

Crystallization of neuraminidase heads from different influenza B virus strains have been grown. Neuraminidase crystals of influenza B/Hong Kong/8/73 were grown from solutions of potassium phosphate. The crystals are tetragonal prisms, space group I222; the axes are a = 123 Å and c = 165 Å. Influenza B/Lee/40 neuraminidase crystals were grown from solutions of polyethylene glycol 4000. The crystals are tetragonal pyramids, space group P42,2 or its enantiomorph P4,2,2; the axes are a = 125 Å and c = 282 Å.

Neuraminidases comprise 5-10% of total influenza virus protein and are one of two glycosylated surface antigens. The primary antigen is the hemagglutinin which is responsible for the initial attachment of the virion to cell surface receptors which contain sialic acid residues (1). The neuraminidase (acyl neuraminyl hydrolase, EC 3.2.1.18) catalyzes the cleavage of b-ketosidic linkages between those terminal sialic acid residues and the adjacent sugar residues of cell surface receptors. This cleavage of sialic acids from mucin appears to facilitate viral mobility to and from the site of infection (2, 3). Neuraminidase also acts by removing sialic acid residues from the cell surface and from carbohydrate chains of newly synthesized hemagglutinin molecules to prevent self-aggregation of the virions upon budding and to allow subsequent release of matured virions from the host cell membrane (4).

Influenza B neuraminidase molecules are translated from a messenger RNA which encodes two polypeptides, neuraminidase and NB protein, using overlapping reading frames. There are four potential glycosylation sites contained within the 466-amino acid sequence of B/Lee/40 neuraminidase (5). Newly translated molecules are firmly anchored into the plasma membrane by a stretch of hydrophobic amino acids near the amino terminus. Neuraminidase molecules have an average monomer molecular weight of 58,000-68,000 (5) and exist as tetramers on the virus surface stabilized by disulfide bridges. The tetrameric neuraminidase "heads" are positioned on the top of a stalk which rises above the virus membrane (6). Biologically active heads can be released from the stalk by trypsin which cleaves the molecule near the top of the stalk.

Because influenza viruses typically exhibit a high degree of antigenic variation in their surface antigens, strains isolated in a given year may differ greatly from one another and from strains of previous years (7). The result is that current vaccines are unavailing from one year to the next and any vaccine's effectiveness for controlling future influenza eruptions is debatable. However, the activity is conserved, and for this reason, neuraminidase has been selected as one target for the design of specific inhibitors to curtail both the incidence and severity of influenza outbreaks.

Several influenza A neuraminidases have been crystallized (6-12), and the structures of two subtype neuraminidases (N2 and N9) have been determined (10, 11). The N2 neuraminidase head resolved to 2.9 Å resolution is a tetramer consisting of four identical subunits (monomer M, 50,000) arranged with circular 4-fold symmetry. Each monomer is composed of six topographically identical b-sheets arranged in a propeller formation (10). The catalytic sites are located in deep pockets which occur on the upper corners of the box-shaped tetramer. The N9 neuraminidase head shares about 45% amino acid sequence homology with that of N2 neuraminidase and at 3 Å resolution (11) is seen to be folded similarly to N2 neuraminidase. There are some differences in the way in which the subunits are organized around the molecular 4-fold axis, and insertions and deletions with respect to N2 neuraminidase occur in four regions.

Influenza B/Lee/40 neuraminidase has less than 25% sequence identity compared with either N2 or N9 neuraminidase in the head region. Conservation of several of the cysteine residues which form disulfide bonds in N2 and N9 neuraminidases and of several amino acid side-chains which line the active site pocket suggest that the polypeptides may be similarly folded, but this can only be confirmed by a full structure determination. This is the first report of influenza type B neuraminidase crystals grown reproducibly for single crystal x-ray diffraction analysis.

EXPERIMENTAL PROCEDURES

Viruses were propagated in 11-day-old embryonated chicken eggs and purified from allantoic fluid (13). B/Hong Kong neuraminidase was prepared from the viral strain B/Hong Kong/8/73. The B/Lee neuraminidase was prepared from the high growth reassortant virus, B/Hong Kong/8/73 (HC), which possesses the hemagglutinin of B/Hong Kong/8/73 and the neuraminidase of B/Lee/40 (14). Purified virus was treated with L-1-tosyl-amido-2-phenylethyl chloromethyl ketone-trypsin (1.0 mg/ml, Worthington Diagnostic Systems, Inc.) for 2 h at 37°C (15). Residual viral cores that contained the membrane-embedded amino-terminal portion of the neuraminidase as well as intact hemagglutinin were pelleted in a Beckman Ti-60 rotor at 40,000 rpm for 30 min. The supernatant which contained 100% of the neuraminidase activity was centrifuged for 18 h at 5°C at 55,000 rpm for 30 min.
The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
shows 94% amino acid sequence homology over the whole molecule. Much of this variation is in the stalk, and in the trypsin-released heads used for the crystallographic studies, the homology is 95%.

The B/Lee neuraminidase crystals exhibit either tetragonal, pyramidal, or rectangular morphology. Most of the crystals are tetragonal pyramids, but sometimes the rectangular plates grow in the same drop (Fig. 1A). Crystals grow in the pH range 6.2-8.2, but the largest grow around pH 7.4 to maximum dimensions of 0.6 × 0.22 × 0.06 mm. X-ray precession photographs taken on a Rigaku RU-200 rotating anode indicate that the crystals are tetragonal and belong to the Laue group 4/mmm. Fig. 2A shows a zero level precession photograph taken with the x-ray beam parallel to the 4-fold axis. The space group P4₂₁₂ or its enantiomorph is specified by the systematic absence of reflections 00l with l ≠ 4n and h00 with h ≠ 2n. Unit cell parameters are a = 125 Å and c = 282 Å. The crystals are stable to x-rays at room temperature for 5 days and diffract to 3.0-Å resolution.

Using an average subunit molecular weight of 50,000, calculated values of Vₘ (17) for one, two, or four subunits/crystallographic asymmetric unit are 10.94, 5.47, and 2.74 Å³/dalton; the corresponding solvent volume fractions are 89, 78, and 55%, respectively. Either of the latter two values are in the range found for other proteins, although the Vₘ for two subunits would indicate a higher solvent content than is usually seen for protein crystals grown from PEG-4000. It appears that either a complete tetramer or half of a tetramer in the crystallographic asymmetric unit is present.

In contrast, B/Hong Kong neuraminidase crystals grow as thick rectangles or as wedges with both morphologies appearing in the same drop, although the wedge morphology dominates the population. These crystals grow with average dimensions of 0.3 × 0.22 × 0.08 mm and were the crystals used for the preliminary space group determination (Fig. 1B). Left undisturbed for 6 weeks, beautiful tetragonal prisms are grown to maximum dimensions of 0.6 × 0.6 × 0.2 mm at room temperature.

The B/Hong Kong neuraminidase crystals diffract to 3.0-Å resolution, but are relatively unstable. The average crystal lifetime in the x-ray beam on a Rigaku rotating anode generator operated at 40 kV × 50 mA is about 12 h. X-ray precession photographs taken on a rotating anode indicate that the B/Hong Kong neuraminidase crystals are also tetragonal and belong to the Laue group 4/mmm. Fig. 2B shows a zero level precession photograph taken with the x-ray beam parallel to the 4-fold axis. The space group I4₂₂ is specified by the systematic absence of reflections hkl with h + k + l ≠ 2n. The unit cell dimensions are a = 123 Å and c = 165 Å. Calculated Vₘ values for one or two subunits/asymmetric unit are 3.12 and 1.56 Å³/dalton. The corresponding solvent volumes are 61% and 21%, respectively. The value of 3.12 Å³/dalton lies within the range consistent for that of most other proteins. Thus, there is one subunit in the asymmetric unit.

Presently the B/Hong Kong neuraminidase crystals are more likely to furnish a complete native data set required for a full influenza B neuraminidase structure determination, and efforts are currently underway to obtain high-resolution structural information from these crystals.

Acknowledgments—We especially thank Dr. Charles E. Bugg for the use of laboratory equipment. We also thank Gayla Legrone and Chunling Ma for expert technical assistance. This collaborative project was greatly helped by international telephone facilities donated by the Australian Overseas Telecommunications Commission.

REFERENCES

1. Lazaro-witz, S. G., and Choppin, P. W. (1975) Virology 68, 440-454
2. Burnet, F. M., and Stone, J. D. (1947) Aust. J. Exp. Biol. Med. Sci. 25, 227-233
3. Gottschalk, A. (1966) The Glycoproteins. Their Composition, Structure and Function, Elsevier, Amsterdam
4. Palese, P., Tobita, K., Ueda, M., and Compans, R. W. (1974) Virology 61, 397-410
5. Shaw, M. W., Lamb, R. A., Erickson, B. W., Breidis, D. J., and Choppin, P. W. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6817-6821
6. Webster, R. G., Laver, W. G., and Air, G. M. (1983) Genetics of Influenza Viruses, pp. 127-168, Springer-Verlag, Berlin and New York
7. Air, G. M., and Laver, W. G. (1986) Adv. Virus Res. 31, 53-102
8. Wright, C. E., and Laver, W. G. (1978) J. Mol. Biol. 120, 133-136
9. Laver, W. G. (1978) Virology 68, 78-87
10. Varghese, J. N., Laver, W. G., and Colman, P. M. (1983) Nature 303, 35-40
11. Baker, A. T., Varghese, J. N., Laver, W. G., Air, G. M., and Colman, P. M. (1987) Proteins 2, 111-117
12. Laver, W. G., Colman, P. M., Webster, R. G., Hillshaw, V. S., and Air, G. M. (1984) Virology 137, 314-323
13. Laver, W. G. (1969) Fundamental Techniques in Virology, pp. 82-86, Academic Press, New York
14. Wei, X., Els, M. C., Webster, R. G., and Air, G. M. (1987) Virology 156, 253-258
15. Noll, H., Aoyagi, T., and Orlando, J. (1962) Virology 18, 154-157
16. Wrigley, N. G., Skehel, J. J., Charlwood, P. A., and Brand, C. M. (1973) Virology 51, 525-529
17. Matthews, B. W. (1968) J. Mol. Biol. 33, 491-497