CRYSTALLIZED N-TERMINAL DOMAIN OF INFLUENZA VIRUS MATRIX PROTEIN M1 AND METHOD OF DETERMINING AND USING SAME

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Related U.S. Application Data

Provisional application No. 60/023,564, Aug. 7, 1996.

References Cited

PUBLICATIONS


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 Kd) of the M1 protein. X-ray diffraction studies indicated that the crystals have a space group of P321 or P3121. Vm calculations showed that there are two monomers in an asymmetric unit. A crystallized N-terminal domain of M1, wherein the N-terminal domain of M1 is crystallized such that the three dimensional structure of the crystallized N-terminal domain of M1 can be determined to a resolution of about 2.1 Å or better, and wherein the three dimensional structure of the uncrystallized N-terminal domain of M1 cannot be determined to a resolution of about 2.1 Å or better. A method of purifying M1 and a method of crystallizing M1. A method of using the three-dimensional crystal structure of M1 to screen for antiviral, influenza virus treating or preventing compounds. A method of using the three-dimensional crystal structure of M1 to screen for improved binding to or inhibition of influenza virus M1. The use of the three-dimensional crystal structure of the M1 protein of influenza virus in the manufacture of an inhibitor of influenza virus M1. The use of the three-dimensional crystal structure of the M1 protein of influenza virus in the screening of candidates for inhibition of influenza virus M1.

18 Claims, 8 Drawing Sheets
FIG. 1b
Xmerge Merging R for tmp.df

Resolution (\sin(\theta/\lambda))

all 4715 reflections
3866 acentric reflections
849 centric reflections

FIG. 1c
FIG. 2a

$X_{\text{merge}} |(f_1 - f_2)|$ for tmp.df

<table>
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<tr>
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</table>

Resolution ($\sin(\theta)/\lambda$)

- all 3671 reflections
- 2929 acentric reflections
- 742 centric reflections

FIG. 2a
Xmerge - Merging R for tmp.df

<table>
<thead>
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<td>0.0588</td>
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- all 3671 reflections
- 2929 acentric reflections
- 742 centric reflections

FIG. 2b
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<tr>
<th>Res. limits (Å)</th>
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<th>Rsymm %</th>
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<td>4.6</td>
<td>94.7</td>
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Space group: P3_121, a=b=67.17Å, c-135.30Å

FIG. 3
CRYSTALLIZED N-TERMINAL DOMAIN OF INFECTIOUS VIRUS MATRIX PROTEIN M1 AND METHOD OF DETERMINING AND USING SAME

BACKGROUND OF THE INVENTION

This application claims benefit, pursuant to 35 U.S.C. § 119, of applicants’ provisional U.S. Ser. No. 60/023,564, filed Aug. 7, 1996, the contents of which are hereby incorporated by reference.

This application was supported, in part, by a grant from NASA (NAGW-819).

FIELD OF THE INVENTION

The present invention relates to the field of crystallography and, particularly, to the characterization of the structure of matrix protein M1 of influenza virus neuraminidase and, specifically, to the determination of the crystal structure of the N-terminal domain of M1, the domain itself, the crystallized domain, methods of purifying and crystallographically determining the three-dimensional structure of that domain, and methods of using the crystal structure of N-terminal domain of M1 to design pharmaceuticals.

BACKGROUND OF THE INVENTION

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) encapsulated in the viral membrane envelope is composed of an RNA polymerase and RNA-binding nucleoproteins (NP) (1). The interaction of RNPs with the membrane is mediated by the matrix protein M1 (252 amino acids, Mr=27 kd), which are tightly associated with the RNP cores while interacting with the cytoplasmic tails of the spike glycoprotein and the viral membrane (2). Through the binding of hydrophobic domains to the virion lipid envelope, M1 maintains the structural integrity of the virus particle (3,4). On the other hand, the interaction of M1 with RNP cores is mostly electrostatic at neutral pH (5,6). The M1 can easily be dissociated from the RNP cores by low pH treatment (7,8).

In addition to packaging the RNP cores during virion assembly, M1 also directs the transportation of RNPs into or out of the nucleus (9,10). Upon entry of the virus into the new host cell, M1 is dissociated from RNPs as the result of reducing pH in the fusion endosome, allowing the RNPs to enter the nucleus. When progeny viral RNPs are produced, newly synthesized M1 escort the RNPs out of nucleus and target them to the assembly site on the cellular membrane where HA and NA are located. It is therefore highly desirable to be able to provide a method of deducing the crystal structure of M1 and of providing a method of using this structure provide antiviral candidates and M1 inhibitors.

SUMMARY OF THE INVENTION

The present invention provides a crystallized N-terminal domain of M1, wherein the N-terminal domain of M1 is crystallized such that the three dimensional structure of the crystallized N-terminal domain of M1 can be determined to a resolution of 2.1 Å or better, and wherein the three-dimensional structure of the uncrystallized N-terminal domain of M1 cannot be determined to a resolution of 2.1 Å or better.

In an alternate embodiment, the present invention provides a method of extracting the M1 protein of influenza virus comprising stripping the membrane proteins from influenza virus, removing the stripped membrane proteins, thereby leaving the M1-RNP complex, and releasing M1 from the M1-RNP complex by suspending the complex in a solution comprising NaH₂PO₄, Benzamidine, and NaN₃, at a pH of from 3 to 5, preferably about 4.0. In a further embodiment, the released M1 is purified.

In yet another embodiment, the present invention provides a method of extracting the N-terminal domain of the M1 protein of influenza virus comprising stripping the membrane proteins from influenza virus, removing the stripped membrane proteins, thereby leaving the M1-RNP complex, releasing M1 from the M1-RNP complex by suspending the complex in a solution comprising NaH₂PO₄, Benzamidine, and NaN₃, at a pH of from 3 to 5, preferably about 4.0, purifying the released M1, concentrating the purified M1 to a concentration of from about 3 to about 20 mg/ml, more preferably from about 4 to about 10 mg/ml, and most preferably about 5 mg/ml and allowing a period of time sufficient for the formation of an 18 kd protein fragment corresponding to the N-terminal domain of M1, collecting the 18 kd polypeptide corresponding to the N-terminal domain of M1 in a further embodiment, the method also includes crystallizing the 18 kd polypeptide corresponding to the N-terminal domain of M1 in hanging drops (which is one crystal formation method, see elsewhere herein for others) using the vapor diffusion method to a resolution of less than about 2.1 Å, wherein the N-terminal domain of M1 is present at a concentration of from 3 to 20 mg/ml, preferably about 5 mg/ml, and the crystallization takes place at 4 to 10° C. over 20% PEG 3350, to thereby obtain crystals of space group P3₂1 or P3₁₂₁ with approximate a=68.0 Å and approximate c=136.57 Å.

In yet another embodiment, the present invention provides a method for determining the three dimensional structure of the crystallized N-terminal domain of the M1 protein of influenza virus to a resolution of 2.1 Å or better comprising the steps of crystallizing the N-terminal domain of M1 in hanging drops using the vapor diffusion method to a resolution of less than about 2.1 Å, wherein the N-terminal domain of M1 is present at a concentration of from 3 to 20 mg/ml, preferably about 5 mg/ml, and allowing a period of time sufficient for the formation of an 18 kd protein fragment corresponding to the N-terminal domain of M1, collecting the 18 kd polypeptide corresponding to the N-terminal domain of M1 in hanging drops (which is one crystal formation method, see elsewhere herein for others) using the vapor diffusion method to a resolution of less than about 2.1 Å, wherein the N-terminal domain of M1 is present at a concentration of from about 3 to about 20 mg/ml, preferably about 5 mg/ml, and the crystallization takes place at 4 to 10° C. over 20% PEG 3350, to thereby obtain crystals of space group P3₂1 or P3₁₂₁ with approximate a=68.0 Å and approximate c=136.57 Å, and then analyzing the N-terminal domain of M1 to determine the three-dimensional structure of the crystallized N-terminal domain of M1. In a further embodiment, the invention provides the crystallized N-terminal domain of M1 produced by this process.

In yet another embodiment, the present invention provides a method for designing an antiviral compound for the prevention or treatment of influenza virus infection, comprising evaluating the three dimensional structure of the crystallized N-terminal domain of M1 produced by crystallizing a purified N-terminal domain of M1 in hanging drops using the vapor diffusion method to a resolution of less than about 2.1 Å, wherein the N-terminal domain of M1 is present at a concentration of about 3 to about 20 mg/ml and the crystallization takes place at from about 4 to about 10° C. over 20% PEG 3350, to thereby obtain crystals of space group P3₂1 or P3₁₂₁ with approximate a=68.0 Å and approximate c=136.57 Å, and synthesizing an antiviral compound based on the three-dimensional crystal structure of the crystallized N-terminal domain of M1, wherein the...
antiviral compound can be screened for having improved binding to M1.

In yet another embodiment, the present invention provides a purified N-terminal domain of M1. In a further embodiment, the present invention provides this purified N-terminal domain of M1, comprising the amino acid sequence of SEQ. ID. NO. 1 (see below). In one embodiment, “N-terminal domain” means the amino acid sequence of SEQ. ID. NO. 1. One of skill in the art would recognize that various amino acid substitutions could be made to this polypeptide. Such modifications, so long as the basic and novel utility of the present invention is not disturbed, are understood to be within the scope of the present invention. For instance, a polypeptide could be constructed containing the first 150 amino acids, from position 1 (perhaps by cleavage of some of the amino acids and addition at the N-terminal end). Such a polypeptide is intended to be within the scope of the present invention. To the extent that the crystal structures of such analogous polypeptides are similar to the structure set forth herein, such structures fall within the scope of the present invention.

In yet another embodiment, the present invention provides a method for designing a candidate compound for screening for improved binding to or inhibition of influenza virus M1, comprising evaluating the three dimensional structure of the crystallized N-terminal domain of M1, and synthesizing a candidate binding compound based on the three-dimensional crystal structure of the crystallized N-terminal domain of M1 for improved binding to M1.

In a further embodiment, the present invention provides the three-dimensional crystal structure of influenza virus protein M1 as set forth elsewhere herein. In a further embodiment, the present invention provides a purified N-terminal domain of M1 and synthesizing a polypeptide having that three-dimensional crystal structure.

In yet another embodiment, the invention provides a method for designing a candidate compound for screening for improved binding to or inhibition of influenza virus M1, comprising evaluating the three dimensional structure of the crystallized N-terminal domain of M1, and synthesizing a candidate binding compound based on the three-dimensional crystal structure of the crystallized N-terminal domain of M1.

In a further embodiment, the present invention provides a method of extracting the N-terminal domain of M1 from a solution containing influenza virus M1, comprising stripping the membrane proteins from influenza virus, removing the stripped membrane proteins, thereby leaving the M1-RNP complex, and releasing M1 from the M1-RNP complex by suspending the complex in a solution comprising NaH₂PO₄, Benzamidine, and NaN₃, purifying the released M1, constructing containing the first 150 amino acids, from position 1 (perhaps by cleavage of some of the amino acids and addition at the N-terminal end). Such a polypeptide is intended to be within the scope of the present invention. To the extent that the crystal structures of such analogous polypeptides are similar to the structure set forth herein, such structures fall within the scope of the present invention.

In a further embodiment, the present invention provides a method for designing a candidate compound for screening for improved binding to or inhibition of influenza virus M1, comprising evaluating the three dimensional structure of the crystallized N-terminal domain of M1, and synthesizing a candidate binding compound based on the three-dimensional crystal structure of the crystallized N-terminal domain of M1 for improved binding to M1.

The present invention therefore provides a crystallized N-terminal domain of M1, wherein the N-terminal domain of M1 is crystallized such that the three dimensional structure of the crystallized N-terminal domain of M1 can be determined to a resolution of 2.1 Å or better, and wherein the three dimensional structure of the uncrystallized N-terminal domain of M1 cannot be determined to a resolution of 2.1 Å or better.

In yet another embodiment, the present invention provides a method of extracting the M1 protein of influenza virus comprising stripping the membrane proteins from influenza virus, removing the stripped membrane proteins, thereby leaving the M1-RNP complex, and releasing M1 from the M1-RNP complex by suspending the complex in a solution comprising NaH₂PO₄, Benzamidine, and NaN₃. In a preferred embodiment, the solution has a pH of from about 3 to about 5, more preferably about 4.0. In a further embodiment, this method is extended to include purifying the released M1.

In yet another embodiment, the present invention provides a method of extracting the N-terminal domain of the M1 protein of influenza virus comprising stripping the membrane proteins from influenza virus, removing the stripped membrane proteins, thereby leaving the M1-RNP complex, and releasing M1 from the M1-RNP complex by suspending the complex in a solution comprising NaH₂PO₄, Benzamidine, and NaN₃, purifying the released M1, constructing containing the first 150 amino acids, from position 1 (perhaps by cleavage of some of the amino acids and addition at the N-terminal end). Such a polypeptide is intended to be within the scope of the present invention. To the extent that the crystal structures of such analogous polypeptides are similar to the structure set forth herein, such structures fall within the scope of the present invention.

In a further embodiment, the present invention provides a method for designing a candidate compound for screening for improved binding to or inhibition of influenza virus M1, comprising evaluating the three dimensional structure of the crystallized N-terminal domain of M1, and synthesizing a candidate binding compound based on the three-dimensional crystal structure of the crystallized N-terminal domain of M1 for improved binding to M1.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1a shows the Harker section (Z=1/3) of the difference Pattern of Os derivative of M1 crystals.

FIG. 1b shows the structure factor amplitude difference between native and Os derivative data sets versus resolution. From top to bottom, the curves are for all reflections, acentric reflections and centric reflections, respectively.

FIG. 1c shows the merging R-factor between native and Os derivative data sets versus resolution. From top to bottom, the curves are for all reflections, acentric reflections and centric reflections, respectively.

FIG. 1d shows the structure factor amplitude difference between native and Pt derivative data sets versus resolution. From top to bottom, the curves are for all reflections, acentric reflections and centric reflections, respectively.

FIG. 2a shows a section of solvent flattened map (FOM=0.89) at 5 Å resolution.

FIG. 2b shows the merging R-factor between native and Pt derivative data sets versus resolution. From top to bottom, the curves are for all reflections, acentric reflections and centric reflections, respectively.

FIG. 2c shows a slice of electron density map at 3.5 Å resolution corresponding to an alpha-helix.

FIG. 2d shows a section of solvent flattened map (FOM=0.89) at 5 Å resolution.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Figures.

Before the present methods and structures are disclosed and described, it is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. It must be noted that, as used in the specification and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise.

Throughout this application, where publications are referenced, the disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains. Publications have been referenced herein by placing the number in parentheses. These publications are listed according to their respective numbers in the “Reference” section hereinbelow.

The present invention therefore provides a crystallized N-terminal domain of M1, wherein the N-terminal domain of M1 is crystallized such that the three dimensional structure of the crystallized N-terminal domain of M1 can be determined to a resolution of 2.1 Å or better, and wherein the three dimensional structure of the uncrystallized N-terminal domain of M1 cannot be determined to a resolution of 2.1 Å or better.

In a further embodiment, the present invention provides a method of extracting the M1 protein of influenza virus comprising stripping the membrane proteins from influenza virus, removing the stripped membrane proteins, thereby leaving the M1-RNP complex, and releasing M1 from the M1-RNP complex by suspending the complex in a solution comprising NaH₂PO₄, Benzamidine, and NaN₃. In a preferred embodiment, the solution has a pH of from about 3 to about 5, more preferably about 4.0. In a further embodiment, this method is extended to include purifying the released M1.

In yet another embodiment, the present invention provides a method of extracting the N-terminal domain of the M1 protein of influenza virus comprising stripping the membrane proteins from influenza virus, removing the stripped membrane proteins, thereby leaving the M1-RNP complex, and releasing M1 from the M1-RNP complex by suspending the complex in a solution comprising NaH₂PO₄, Benzamidine, and NaN₃, purifying the released M1, constructing containing the first 150 amino acids, from position 1 (perhaps by cleavage of some of the amino acids and addition at the N-terminal end). Such a polypeptide is intended to be within the scope of the present invention. To the extent that the crystal structures of such analogous polypeptides are similar to the structure set forth herein, such structures fall within the scope of the present invention.

In a further embodiment, the present invention provides a method for designing a candidate compound for screening for improved binding to or inhibition of influenza virus M1, comprising evaluating the three dimensional structure of the crystallized N-terminal domain of M1, and synthesizing a candidate binding compound based on the three-dimensional crystal structure of the crystallized N-terminal domain of M1 for improved binding to M1.
of M1 to a resolution of less than about 2.1 Å, wherein the N-terminal domain of M1 is present at a concentration of about 3 to about 20 mg/mL, more preferably from about 4 to about 10 mg/mL and most preferably about 5 mg/mL, and the crystallization takes place at from 4 to 32°C, more preferably from 10 to 26°C, even more preferably at about 16 to about 24°C, and even more preferably about 20°C, to thereby obtain crystals of space group P321 or P322. The crystals may have an approximate a = 68.0 Å and approximate c = 136.57 Å. The crystallization, in one embodiment, may occur using hanging drops and the vapor diffusion method. Alternatively, other crystallization methods may be used. For instance, a temperature variation may be used to produce crystals, or crystallization in space may be used to improve resolution. The crystallization, in another embodiment, may occur over about 20% PEG 3350. In addition, other chemicals may be used in the place of MPD to PEG 2000. For instance, organic chemicals (e.g., isopropanol), inorganic chemicals (e.g., (NH₄)₂SO₄, NaH₂PO₄), and other molecular weight PEG (from MPD to PEG 2000) may be used.

In yet another embodiment, the present invention provides a method for determining the three-dimensional structure of the crystallized N-terminal domain of M1 of the protein of influenza virus to a resolution of more preferably from 10 to 26°C, even more preferably at about 16 to about 24°C, and even more preferably about 20°C, to thereby obtain crystals of space group P321 or P322, and then analyzing the N-terminal domain of M1 to determine the three-dimensional structure of the crystallized N-terminal domain of M1. In a preferred embodiment, the analysis is by x-ray diffraction. The crystallization, in one embodiment, may occur over about 20% PEG 3350. The crystallization, in one embodiment, may occur using hanging drops and the vapor diffusion method. Alternatively, other crystallization methods may be used. For instance, a temperature variation method may be used to produce crystals, or crystallization in space may be used to improve resolution.

In yet another embodiment, the present invention provides a method for designing an antiviral compound for the prevention or treatment of influenza virus infection, comprising evaluating the three-dimensional structure of the crystallized N-terminal domain of M1 produced by crystallizing a purified N-terminal domain of M1 to a resolution of less than about 2.1 Å, wherein the N-terminal domain of M1 is present at a concentration of about 3 to about 20 mg/mL, more preferably from about 4 to about 10 mg/mL, even more preferably about 5 mg/mL, and even more preferably about 5 mg/mL, and the crystallization takes place at from about 4 to about 32°C, more preferably from about 10 to about 26°C, even more preferably at about 16 to about 32°C, and even more preferably about 20°C, to thereby obtain crystals of space group P321 or P322, and then synthesizing an antiviral compound based on the three-dimensional crystal structure of the crystallized N-terminal domain of M1, wherein the antiviral compound can be screened for having improved binding to M1. In a further embodiment, the present invention provides a purified N-terminal domain of M1. In a further embodiment, the invention provides that purified N-terminal domain of M1, comprising the amino acid sequence of SEQ. ID. NO. 1.

In yet another embodiment, the present invention provides a method for designing a candidate compound for screening for improved binding to or inhibition of influenza virus M1, comprising evaluating the three-dimensional structure of the crystallized N-terminal domain of M1, and synthesizing a candidate binding compound based on the three-dimensional crystal structure of the crystallized N-terminal domain of M1 for improved binding to M1. In a further embodiment, the candidate compound is a peptide or polypeptide. The crystallization, in one embodiment, may occur using hanging drops and the vapor diffusion method. Alternatively, other crystallization methods may be used. For instance, a temperature variation method may be used to produce crystals, or crystallization in space may be used to improve resolution.

In yet another embodiment, the present invention provides a method for improving binding to M1. In one embodiment, the method comprises using a purified N-terminal domain of M1, comprising the amino acid sequence of SEQ. ID. NO. 1.

In yet another embodiment, the present invention provides a crystallized N-terminal domain of M1 produced by the process described above.

In yet another embodiment, the present invention provides a method for designing an antiviral compound for the prevention or treatment of influenza virus infection, comprising evaluating the three-dimensional structure of the crystallized N-terminal domain of M1 produced by crystallizing a purified N-terminal domain of M1 to a resolution of less than about 2.1 Å, wherein the N-terminal domain of M1 is present at a concentration of about 3 to about 20 mg/mL, more preferably about 4 to about 10 mg/mL, even more preferably about 5 mg/mL, and even more preferably about 5 mg/mL, and the crystallization takes place at from about 4 to about 32°C, more preferably from about 10 to about 26°C, even more preferably at about 16 to about 32°C, and even more preferably about 20°C, to thereby obtain crystals of space group P321 or P322, and then synthesizing an antiviral compound based on the three-dimensional crystal structure of the crystallized N-terminal domain of M1, wherein the antiviral compound can be screened for having improved binding to M1. In a further embodiment, the antiviral compound is a peptide or polypeptide. The crystallization, in one embodiment, may occur using hanging drops and the vapor diffusion method. Alternatively, other crystallization methods may be used. For instance, a temperature variation method may be used to produce crystals, or crystallization in space may be used to improve resolution.

In yet another embodiment, the present invention provides a purified N-terminal domain of M1. In a further embodiment, the invention provides that purified N-terminal domain of M1, comprising the amino acid sequence of SEQ. ID. NO. 1.
protein under study is authentic. Attempts to express the peptide. In yet another embodiment, the present invention was concentrated by about 15 fold through an Amicon concentrator in a 4°C cold room. The virus was pelleted in a Beckman SW28 rotor at 21 K rpm for three hours at 4°C, 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 M1 of 50 mM Na2HPO4, 50 mM, 5 mM Benzamidine and 0.02% NaN3 (pH of from 3.0 to 5.0, preferably 4.0) to release the M1 protein from the M1-RNP complex (8). The RNP cores (lacking M1) were removed by centrifugation in a Beckman SW55 rotor at 22K rpm for one hour at 4°C. The M1 protein was further purified by gel filtration in a column of Superdex 75 (Pharmacia) mounted on a Pharmacia FPLC system. The M1 protein was pooled about 62 minutes after sample injection at a flow rate of 1 M1/min. Compared with the molecular weights of the protein standards, the apparent molecular weight of the eluted M1 protein was about 50 kd. Because the molecular weight of M1 monomer derived from its amino acid sequence was expected to be 27 kd (11), the M1 protein appears to form a dimer in solution at acidic pH.

However, the M1 protein was not stable when concentrated to 5 mg/ml prior to crystallization. A stable fragment of 18 Kd 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. The molecular weight of the fragment was determined by Mass Spectrum (PE Sciex API III) to be 18,230 dalton. The soluble fragment, at pH 7.2, was then incubated with M1-free RNP cores obtained during M1 purification. The mixture was pelleted and analyzed by SDS-PAGE gel. The gel showed that, similar to the native M1 protein, the M1 fragment was still able to bind RNP cores at neutral pH.

Since this fragment can still bind to RNP cores, M1 can be conceptualized as a two-domain protein. The C-terminal domain binds the RNP cores through hydrophilic interactions which can be interrupted by reducing the pH. The N-terminal domain binds the membrane. Without wishing to be bound by theory, the N-terminal domain probably has hydrophobic properties because the intact M1 protein aggregates at neutral pH while the C-terminal domain binds the RNP cores through hydrophilic interactions which can be interrupted by reducing the pH. The N-terminal end thus binds the membrane. The concept of a two domain protein is consistent with the function of M1. During virus production, M1 is synthesized and transported to the nucleus as a dimer where it binds to RNP cores by the C-terminal domain. This is consistent with the RNA binding and anti-transcriptase activity data which were mapped by
monoclonal antibodies to be within the amino acid sequence at position 90 to position 164. The association of M1 with RNP cores signals, perhaps through a conformational change of M1, the transportation of the M1-RNP complex to the assembly site on the cellular membrane. Only the RNP-associated M1 N-terminal domain can bind to the membrane and the C-terminal tails of the spike glycoproteins, HA and NAA. This completes the function of M1 during virion assembly. During virion entry, the C-terminal domain of M1 is dissociated from the RNP cores due to the low pH of the fusion endosome while the N-terminal domain remains associated with the membrane and the spike glycoprotein tails by hydrophobic interactions. The dissociation of M1 releases naked RNP cores and uncovers the signal for nucleus targeting carried by RNP cores. The virion M1 could not block the transportation of entering RNP cores into the nucleus because of conformational changes induced by low pH. Unlike the matrix protein of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), which are single domain proteins forming a trimer, the M1 protein is a two domain protein functioning as a dimer. The membrane anchoring of M1 occurs through an entirely separate N-terminal protein domain, rather than a covalent myristoyl modification, as found in HIV and SIV matrix proteins.

EXAMPLE III

Crystalization, Data Collection and Processing

Crystalization was carried out in hanging drops by the vapor diffusion method. However, one skill in the art would recognize that other crystalization methods may be used. For instance, a temperature variation method may be applied. For instance, a temperature variation method may be used to improve resolution. Nonetheless, for the hanging drop method, the protein concentration was about 5 mg/ml. Large crystals (0.05 mmx0.05 mmx0.3 mm) of the 18 kD M1 protein fragment could be grown at 20°C. over 20% PEG 3350 in two months. X-ray diffraction data were collected at the Brookhaven National Laboratory on beamline X-12C. 60 frames from two crystals were collected and the data were processed by the HKL package. The crystals were classified as belonging to space group P3,21 or P3,21 with a = 68.74 Å, c = 136.57 Å. The data were about 52% complete at 2.6 Å resolution and the Rsym was 0.11

**TABLE 1**

<table>
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<tr>
<th>Resolution (Å)</th>
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<td>All</td>
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<td></td>
</tr>
</tbody>
</table>

Rsym 66.17 Å, 2β=135.30 Å

Because there are no reported homologous structures to M1, the structural solution relies on the conventional multiple isomorphous replacement method. Three data sets were collected under identical conditions from a SIEMENS Highstar multwire detector, mounted on a Rigaku rotating anode source with the Oxford cryo system. These three data sets are a new native and two derivative data sets. The statistics are shown in Table 2.

**TABLE 2**

<table>
<thead>
<tr>
<th>Res. limits (Å)</th>
<th>Native</th>
<th>K,PO4</th>
<th>K2SO4FO6</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.00</td>
<td>3.78</td>
<td>44.1</td>
<td></td>
</tr>
<tr>
<td>3.78</td>
<td>3.00</td>
<td>42.3</td>
<td></td>
</tr>
<tr>
<td>3.00</td>
<td>2.62</td>
<td>33.0</td>
<td></td>
</tr>
<tr>
<td>2.62</td>
<td>2.38</td>
<td>24.6</td>
<td></td>
</tr>
<tr>
<td>2.38</td>
<td>2.21</td>
<td>18.5</td>
<td></td>
</tr>
<tr>
<td>2.21</td>
<td>2.08</td>
<td>11.9</td>
<td></td>
</tr>
</tbody>
</table>

**REFERENCES**

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 1
<210> SEQ ID NO 1
<211> LENGTH: 163
<212> TYPE: PRT
<213> ORGANISM: Influenza Virus

<400> SEQUENCE: 1
Ser Leu Leu Thr Glu Val Glu Thr Tyr Val Leu Ser Ile Ile Pro Ser
1 5 10 15
Gly Pro Leu Lys Ala Glu Ile Ala Gln Arg Leu Glu Val Phe Ala
20 25 30
Gly Lys Asn Thr Asp Leu Glu Val Leu Met Glu Trp Leu Lys Thr Arg
35 40 45
Pro Ile Leu Ser Pro Leu Thr Lys Gly Ile Leu Gly Phe Val Phe Thr
50 55 60
Leu Thr Val Pro Ser Glu Arg Gly Leu Gln Arg Arg Phe Val Gln
65 70 75 80
Aaa Ala Leu Aaa Gly Aaa Gly Asp Pro Aaa Aaa Met Aaa Ala Val
85 90 90
Lys Leu Tyr Arg Lys Leu Lys Arg Glu Ile Thr Phe His Gly Ala Lys
100 105 110
Glu Ile Ser Leu Ser Tyr Ser Ala Glu Ala Ala Ala Cys Met Gly
115 120 125
Leu Ile Tyr Arg Ser Met Gly Ala Val Thr Glu Val Ala Phe Gly
130 135 140
Leu Val Cys Ala Thr Cys Gly Gln Ile Ala Aaa Ser Gln His Arg Ser
145 150 155 160
His Arg Glu

15. Hankins, R. W., Nagata, K., Bucher, D. J., Popples, S. and Ishihama, A., Monoclonal antibody analysis of influ-

What is claimed is:

1. A crystallized N-terminal domain of the M1 protein of influenza virus comprising the amino acid sequence set forth
in the Sequence Listing as SEQ ID NO:1, wherein the N-terminal domain of M1 is crystallized to obtain crystals of
space group P3_212 with approximate a=68.0 Å and approximate c=136.57 Å such that the three dimensional
structure of the crystallized N-terminal domain of M1 can be
determined to a resolution of about 2.1 Å or better.

SEQ. ID. NO. 1 represents amino acids 2-164 of the N-terminal domain of influenza virus matrix protein M1:

SEQ. ID. NO.1: SLLTEVTEVLSHPS- GPLKAEGAKLREDVFAGKNTDLEVMEWLKTR- PILSPLFKGILGEFTLVSEIGLQRQRFQVQNALNGNGDPPNMKAKVLKLYKLKREIFTIF
HGAEKISLSYAGALASCMLIYNRM-GAVTTEVFGLVCATCEIQDOSHRSHQ

It will be apparent to those skilled in the art that various modifications and variations can be made in the present
invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be
apparent to those skilled in the art from consideration of the

specification and practice of the invention disclosed herein. It is intended that the specification and examples be con-
sidered as exemplary only, with the true scope and spirit of the invention being indicated by the following claims.
6,090,609

a) stripping the membrane proteins from influenza virus;  
b) removing the stripped membrane proteins, thereby  
leaving the M1-RNP complex;  
c) releasing M1 from the M1-RNP complex by suspend-
    ing the complex in a solution comprising NaH2PO4,  
    Benzamidine, and NaN3;  
d) purifying the released M1;  
e) concentrating the purified M1 to a concentration of  
    from 3 mg/ml to 20 mg/ml; and  
f) after a period of time sufficient for the formation of an  
    18 kD protein fragment corresponding to the N-terminal  
    domain of M1, collecting the 18 kD polypeptide cor-
    responding to the N-terminal domain of M1.

4. The method of claim 3, wherein the time period is from  
7 to 21 days.

5. The method of claim 3, further comprising crystallizing  
the 18 kD polypeptide corresponding to the N-terminal  
domain of M1 to a resolution of less than about 2.1 Å,  
wherein the N-terminal domain of M1 is present at a  
concentration of from 3 to 20 mg/ml and the crystallization  
takes place at from 4 to 32°C to thereby obtain crystals of  
space group P321 or P3,21.

6. The method of claim 3, wherein the pH of the solution  
of step e) is from 3.0 to 5.0.

7. The method of claim 5, wherein the crystallization  
occur in hanging drops using the vapor diffusion method.

8. A method for determining the three dimensional struc-
ture of the crystallized N-terminal domain of the M1 protein  
of influenza virus comprising the amino acid sequence set  
forth in the Sequence Listing as SEQ ID NO:1 to a resolu-
tion of about 2.1 Å or better comprising the steps of  
crystallizing the N-terminal domain of M1 to a resolution of  
about 2.1 Å, wherein the N-terminal domain of M1 is present at a  
concentration of from 3 to 20 mg/ml and the crystallization  
takes place at from 4 to 32°C, to thereby obtain crystals of  
space group P3,21 or P3,21.

9. The method of claim 8, wherein the analyzing is by  
x-ray diffraction.

10. The method of claim 8, wherein the crystallization  
occur in hanging drops using the vapor diffusion method.

11. The crystallized N-terminal domain of M1 produced  
by the process of claim 5.

12. A method for designing a potential antiviral com-
 pound for the prevention or treatment of influenza virus  
infection, comprising:
   a) obtaining crystals of the N-terminal domain of M1,  
      wherein the crystals have the space group P3,21 or  
      P3,21 with approximate a=68.0 Å and approximate  
c=136.57 Å such that the three dimensional structure of  
the crystallized N-terminal domain of M1 can be deter-
mined to a resolution of about 2.1 Å or better,  
b) evaluating the three dimensional structure of the crys-
tallized N-terminal domain of M1;  
c) synthesizing the potential antiviral compound based on  
the three-dimensional crystal structure of the crys-
tallized N-terminal domain of M1;  
d) contacting an influenza virus or an influenza viral  
protein with the potential antiviral compound; and  
e) assaying the influenza virus for infectivity or monitor-
ing the influenza viral protein for activity, or both,  
whereby a decrease in the infectivity of the influenza  
virus or a change in the activity of the influenza virus  
protein indicates the compound may be used for the  
prevention or treatment of influenza virus infection.

13. The method of claim 12, wherein the antiviral com-
 pound is a peptide or polypeptide.

14. The method of claim 12, wherein the crystallization  
occur in hanging drops using the vapor diffusion method.

15. The method of claim 12, wherein the crystal has the  
three dimensional crystal structure of influenza virus protein  
M1 as set forth in FIG. 3.

16. The method of claim 12, wherein the influenza viral  
protein comprises M1.

17. A method for designing a candidate compound for  
screening for binding to or inhibition of influenza virus M1,  
comprising:
   a) utilizing the three dimensional structure of a crystal-
lized N-terminal domain of M1 which is defined by the atomic  
coordinates in FIG. 3; and  
b) designing a candidate binding compound based on the  
three-dimensional crystal structure of the crystallized  
N-terminal domain of M1 for binding to M1.

18. The method of claim 17, wherein the candidate  
compound is a peptide or polypeptide.

* * * * *