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


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 P321. Vm calculations showed that there are two monomers in an asymmetric unit. A crystalized N-terminal domain of M1, 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, 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 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.
FIG. 1b
Xmerge Merging R for tmp.df

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

FIG. 1c
FIG. 2a

\[
\text{Xmerge } |(f_1 - f_2)| \text{ for tmp.df}
\]

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

|\Delta| vs Resolution (\sin(\theta/\lambda))

- 96.7
- 77.4
- 58.0
- 38.7
- 19.3
- 0

Resolutions:
- 0.0141
- 0.0382
- 0.0623
- 0.0864
- 0.111
- 0.135
Xmerge – Merging R for tmp.df

FIG. 2b
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Space group: P3_121, a=b=67.17Å, c=135.30Å

FIG. 3
1 CRYSTALLIZED N-TERMINAL DOMAIN OF INFLUENZA 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, 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 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 by low pH.

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.

In yet another embodiment, the present invention provides a method of extracting 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 after a period of time sufficient for the formation of an 18 kd polypeptide 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 5 mg/ml, and the crystallization takes place at 4 to 32° C. over 20%PEG 3350, to thereby obtain crystals of space group P321 or P3,21 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 5 mg/ml, and the crystallization takes place at 4 to 32° C. over 20%PEG 3350, to thereby obtain crystals of space group P3,21 or P3,21 with approximate a=68.0 Å and approximate c=136.57 Å, and 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 32° C. over 20% PEG 3350, to thereby obtain crystals of space group P3,21 or P3,21 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 crystallized polypeptide having that three-dimensional crystal structure.

In yet another embodiment, the invention provides a method for designing a candidate compound for screening as an antiviral for the prevention or treatment of influenza virus infection, comprising evaluating the three-dimensional crystal structure set forth herein, and synthesizing a candidate compound based on the three-dimensional crystal structure. Moreover, the present invention provides for the use of the three-dimensional crystal structure as set forth herein for screening candidate compounds for inhibition of influenza virus M1.

In a further embodiment, the present invention also provides for the use of the N-terminal domain of M1 for screening candidate compounds for inhibition of influenza virus M1.

Finally, the present invention provides a crystallized N-terminal domain of 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 Pt derivative data sets versus resolution. From top to bottom, the curves are for all reflections, acentric reflections and centric reflections, respectively.

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 section of solvent flattened map (FOM=0.89) at 5 Å resolution.

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

FIG. 3 shows atomic coordinates of the three dimensional crystal structure of influenza virus protein M1.

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, Benzaflamide, 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, Benzaflamide, and NaN₃.

In a further embodiment, the present invention provides a method of designing a candidate compound for screening such structures fall within the scope of the present invention.
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 20°C, to thereby obtain crystals of space group P321 or P32.21. The crystals may have an approximate a=68.0 Å and approximate c=136.5 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 method may be used to produce crystals of space group P321 or P32.21, and synthesizing a candidate binding compound based on the three-dimensional crystal structure of the crystallized N-terminal domain of M1, 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 the crystallization takes place at from about 4 to about 32°C, more preferably from about 10 to 26°C, even more preferably at about 16 to about 24°C, and even more preferably 20°C, to thereby obtain crystals of space group P321 or P32.21, 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 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 outer 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.

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 the crystallization takes place at from about 4 to about 32°C, more preferably from about 10 to 26°C, even more preferably at about 16 to about 24°C, and even more preferably 20°C, to thereby obtain crystals of space group P321 or P32.21, 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 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 outer 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.
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<td>54.40</td>
<td></td>
</tr>
<tr>
<td>229</td>
<td>OD2</td>
<td>ASP</td>
<td>39.942</td>
<td>43.872</td>
<td>44.790</td>
<td>1.00</td>
<td>56.56</td>
<td></td>
</tr>
</tbody>
</table>

[Continued]
In yet a further embodiment, the present invention provides a crystallized polypeptide having the three-dimensional crystal structure set forth in the previous table. The present invention also provides a method for designing candidate compounds for screening as an antiviral for the prevention or treatment of influenza virus infection, comprising evaluating the three-dimensional crystal structure set forth in the previous table and synthesizing a candidate compound based on the three-dimensional crystal structure. In a further embodiment, the candidate compound is a peptide. In yet another embodiment, the present invention provides for the use of the three-dimensional crystal structure as set forth in the previous table for screening candidate compounds for inhibition of influenza virus M1.

In a further embodiment, the present invention provides for the use of the N-terminal domain of M1 as described herein for screening candidate compounds for inhibition of influenza virus M1.

The present invention also provides a crystallized N-terminal domain of M1.

The use of the crystal structure to design candidate antivirals may be accomplished in the following fashion. Once the crystal structure of the target (e.g., the N-terminal domain of M1) is determined, computer modeling is conducted (using such as programs DOCK or Multiple Copy Simultaneous Search (MCSS)) to construct candidate inhibitor compounds based on the crystal structure. These compounds are chemically synthesized and their biological activity is assayed. For those compounds which show activity, they are associated or complexed with the crystal for further X-ray diffraction analysis to map the interactions of the compound with the crystal structure.

From the resulting inhibitor-target crystal structure, one of ordinary skill in the art could construct further improved candidate compounds. The steps set forth in the preceding paragraph are repeated and refined as desired. With this in mind, examples using the preferred embodiments of the above-described methods and structures are set forth hereinbelow. Other features of the invention will become apparent from the following examples, which are for illustrative purposes only and are not intended as a limitation upon the present invention.

**EXAMPLE I**

**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. This also ensures that the M1 protein under study is authentic. Attempts to express the whole protein or M1 fragments in E. coli failed to produce any useful protein. Influenza virus strain A/PR/8/34 was inoculated in 11-day-old embryonated eggs (Hyvac Lab, Iowa) followed by incubation at 34.5°C for 48 hours. The allantoic fluid was harvested at the end of incubation, and was centrifuged at 8,000 rpm in a Beckman JA10 rotor for 20 minutes at 4°C. Virus which stayed in the supernatant was concentrated by about 15 fold through an Amicon filter tube whereas the membrane associated proteins stayed in the gradient solution. The pellet of the M1-RNP complex was pelleted to the bottom of the centrifuge tube whereas the membrane associated proteins stayed in the gradient solution.

### Protein Extraction and Purification

In order to strip off the lipid membrane and the membrane embedded surface proteins, 1 M1 of the purified virus preparation was loaded on a three-step sucrose gradient (8). The gradient consisted of, from bottom to top, 3 M1 32% sucrose and 5 M1 17% sucrose in 0.15 M NaCl, 10 mM HEPES (pH 7.2) containing 1% of nonionic detergent NP40, and 2 M1 10% sucrose in 0.15 M NaCl, 10 mM HEPES (pH 7.2) without detergent. After centrifugation in a Beckman SW41 rotor at 21 K rpm for three hours at 4°C, the M1-RNP complex was pelleted to the bottom of the centrifuge tube. To release the M1 protein from the M1-RNP complex, a stable fragment was resuspended in 1 M1 of 50 mM NaH2PO4, 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 22 K 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 (II), 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 38 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 Sciei API III) to be 18,230 dalton. The M1 fragment remained soluble after transfer of the concentrated protein into a buffer with pH 7.2. 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 hydophobic 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 domain 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

**EXAMPLE II**

In this embodiment, the present invention provides a crystallized polypeptide having the three-dimensional crystal structure set forth in the previous table. The present invention provides a method for designing candidate compounds based on the crystal structure. These compounds are chemically synthesized and their biological activity is assayed. For those compounds which show activity, they are associated or complexed with the crystal for further X-ray diffraction analysis to map the interactions of the compound with the crystal structure.

From the resulting inhibitor-target crystal structure, one of ordinary skill in the art could construct further improved candidate compounds. The steps set forth in the preceding paragraph are repeated and refined as desired. With this in mind, examples using the preferred embodiments of the above-described methods and structures are set forth hereinbelow. Other features of the invention will become apparent from the following examples, which are for illustrative purposes only and are not intended as a limitation upon the present invention.

**EXAMPLE III**

**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. This also ensures that the M1 protein under study is authentic. Attempts to express the whole protein or M1 fragments in E. coli failed to produce any useful protein. Influenza virus strain A/PR/8/34 was inoculated in 11-day-old embryonated eggs (Hyvac Lab, Iowa) followed by incubation at 34.5°C for 48 hours. The allantoic fluid was harvested at the end of incubation, and was centrifuged at 8,000 rpm in a Beckman JA10 rotor for 20 minutes at 4°C. Virus which stayed in the supernatant was concentrated by about 15 fold through an Amicon filter tube whereas the membrane associated proteins stayed in the gradient solution. The pellet of the M1-RNP complex was pelleted to the bottom of the centrifuge tube whereas the membrane associated proteins stayed in the gradient solution.

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 (II), 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 38 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 Sciei API III) to be 18,230 dalton. The M1 fragment remained soluble after transfer of the concentrated protein into a buffer with pH 7.2. 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 hydophobic 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 domain 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

Crystallization, Data Collection and Processing

Crystallization was carried out in hanging drops by the vapor diffusion method. However, one of skill in the art would recognize that other crystallization methods may be used. For instance, a temperature variation method may be recognized that other crystallization methods may be used. For instance, a temperature variation method may be recognized that other crystallization methods may be used. For instance, a temperature variation method may be used to improve resolution. Nonetheless, for the Harker section of the difference patterns of the Os and Pt derivatives are shown in FIGS. 1a, 1b, 1c, 1d, 2a, 2b, 2c, and 2d. The amplitude difference and merging R factors versus resolution for the two derivatives are shown also in FIGS. 1a, 1b, 1c, 1d, 2a, 2b, 2c, and 2d. Finally, a section of solvent flattened map (FOM=0.89) at 5 Å resolution is shown in FIG. 2c, and a region corresponding to an α-helix at 3.5 Å is shown in FIG. 2d.

REFERENCES

The following references are incorporated in their entirety herein by this reference:

14. Ye, Z., Baylor, N. W. and Wagner, R. R., *Transcription-inhibition and RNA-binding domains of influenza A virus* specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

**SEQUENCE LISTING**

163 NUMBER OF SEQ ID NOS: 1
163 SEQ ID NO 1
163 LENGTH: 163
163 TYPE: PRT
163 ORGANISM: Influenza Virus

SEQUENCE:

```
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 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 Arg Phe Val Gln
65 70 75 80
Aan Ala Leu Aan Gly Aan Gly Asp Pro Aan Aan Met Aap Lys Ala Val
85 90 95
Lys Leu Tyr Arg Lys Leu Arg Glu Ile Thr Phe His Gly Ala Lys
100 105 110
Glue Ser Leu Ser Tyr Ser Ala Gly Leu Ala Ala Ser Cys Met G1y
115 120 125
Leu Ile Tyr Asn Arg Met Gly Ala Val Thr Glu Val Ala Phe Gly
130 135 140
Leu Val Cys Ala Thr Cys Glu Gln Ile Ala Asp Ser Gln His Arg Ser
145 150 155 160
His Arg Gln
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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 P321 or P3121 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.

Sequence Listing

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

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SLLTEVETYVLSIIPS- GPLKAEC-
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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
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 suspending 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 corresponding 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 c) is from 3.0 to 5.0.

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

8. A method for determining the three dimensional structure of influenza virus comprising the amino acid sequence set forth in the Sequence Listing as SEQ ID NO:1 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 P3₂1 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 occurs 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 compound 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 P3₂1 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 determined to a resolution of about 2.1 Å or better,
b) evaluating the three dimensional structure of the crystallized N-terminal domain of M1;
c) synthesizing the potential antiviral compound based on the three-dimensional crystal structure of the crystallized 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 monitoring 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 viral 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 compound is a peptide or polypeptide.

14. The method of claim 12, wherein the crystallization occurs 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 crystallized 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.