Influence of the water molecules near surface of viral protein on virus activation process

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Abstract. The infection of a cell with influenza virus comprises the stages of receptor binding to the cell membrane, endocytosis of virus particle, and fusion of the virus envelope and cell endosome membrane, which is determined by the conformational changes in hemagglutinin, a virus envelope protein, caused by pH decrease within the endosome. The pH value that induces conformation rearrangements of hemagglutinin molecule considerably varies for different influenza virus strains, first and foremost, due to the differences in amino acid structure of the corresponding proteins. The main goal of this study was to construct a model making it possible to assess the critical pH value characterizing the fusogenic activity of influenza virus hemagglutinin from the data on hemagglutinin structure and experimental verification of this model. Under this model, we assume that when the electrostatic force between interacting hemagglutinin molecules in the virus envelope exceeds a certain value, the hemagglutinin HA1 subunits are arranged so that they form a cavity sufficient for penetration of water molecules. This event leads to an irreversible hydration of the inner fragments of hemagglutinin molecule in a trimer and to the completion of conformational changes. The geometry of electrostatic field in hemagglutinin trimer was calculated taking into account the polarization effects near the interface of two dielectrics, aqueous medium and protein macromolecule. The critical pH values for the conformational changes in hemagglutinin were measured by the erythrocyte hemolysis induced by influenza virus particles when decreasing pH. The critical pH value conditionally separating the pH range into the regions with and without the conformational changes was calculated for several influenza virus H1N1 and H3N2 strains based on the data on the amino acid structure of the corresponding hemagglutinin molecules. Comparison of the theoretical and experimental values of critical pH values for influenza virus strains suggests that the proposed model of the interaction between water molecules and influenza virus envelope proteins has a high prediction efficiency.

Introduction

The life cycle of the majority of viruses is a series of successive stages of virion interaction with the sensitive cell, namely, attachment of the virus particle to the cell surface; penetration into the cell; release of the virus genome; transcription, translation, and processing of the virus proteins; assembly of daughter virions; and release of virus from the infected cell. The infection of a cell with influenza
virus commences from the interaction between virus glycoprotein, hemagglutinin, and the terminal sialooligosaccharides of cell membrane proteins. Such receptor-type binding to the membrane induces the endocytosis of the virus particle by the host cell, which is accompanied by the inflow of hydrolases to the endosome. Activation of hydrolases by acidification of the endosome medium leads to degradation of the virus particle. In turn, a decrease in pH also activates virus envelope proteins inducing conformational molecular rearrangement in their structure, which leads to juxtaposition of the virus and cell endosome membranes and their fusion. Consequently, a pore is formed between the virus and the cytoplasm, through which the virus genome enters the cell [1] [2]. Thus, the biological activity of a virus is determined by the ability of virus hemagglutinin to rearrange the conformation of its molecule in a pH-dependent manner and to induce the fusion of virus and endosome membrane before the virus proteins are degraded by hydrolases upon pH decrease in the endosome.

The monomer of hemagglutinin is a prolate globule located on the envelope perpendicular to the virion surface. The monomer consists of two domains, HA1 and HA2, connected with a disulfide bond located at a distance of 115 Å from the molecule’s top and of 20 Å from the lipid bilayer envelope of the virus [3]. Hemagglutinin forms a quaternary structure comprising three monomers with their HA1 domains turned outside and HA2 domains concealed inside.

![Figure 1. A scheme of the monomer of hemagglutinin in a native state.](image)

The secondary structure of HA1 domain is mainly formed by β-sheets. Its surface contains the binding site for sialic acids, located on the membrane of target cell [4]. The secondary structure of HA2 domain is mainly formed by α-helices; three successive regions in HA2 polypeptide chain are able to form stable secondary structure as α-helices, yet only the outermost parts form α-helices in a native state [5]. The middle region in a native state fails to form this structure and is separated by HA1 subunits from water molecules, which are able to induce the formation of helices in this region. Thus, the structure of this molecule possesses an additional Gibbs free energy, which is likely to provide for the intramolecular rearrangement [6]. The contact of HA domain with water molecules induces formation of a long α-helix of all the three regions; this α-helix is perpendicular to the virus envelope and protrude from the previous protein boundary towards the possible contact with the cell membrane. The protruding part of this helix ends with the polypeptide region formed mainly of hydrophobic amino acid residues, which allow it to penetrate inside the cell membrane. This region got the name “fusion peptide” [3]. The fusion peptide is marked with the representation of atoms by spheres.
When pH is decreased or temperature is elevated, hemagglutinin changes its conformation. It is assumed that this conformational changes proceeds as follows [7] [8]. At neutral pH, the attractive forces of HA1 subunits in the hemagglutinin trimer compensate the repulsive forces and stabilize the trimer. A decrease in pH leads to an increase in the protonation of subunits, positive shift of their charges, and increase in the repulsive forces. [6] In this process, the subunits of trimer become more distant, thereby forming a cavity sufficient for water molecules to penetrate and hydrate the above mentioned regions in the polypeptide chains of three HA2 subunits, causing an irreversible formation of three $\alpha$-helices coiled in parallel to the trimer axis [8]. If the conformational changes take place inside the endosome in the glycoprotein bound in a receptor manner to the cell membrane, these helices penetrate with their fusion peptides into the cell membrane. It is assumed that the state of the trimer having concurrently penetrated into the cell membrane and virus envelope is unstable and induces the changes leading eventually to the fusion of virus envelope and cell endosome membrane followed by formation of the pore connecting the virion with the cell cytoplasm [9]. Thus, it is likely that the most critical and the only reversible stage in the overall process of pH-dependent conformational changes of hemagglutinin molecules in the influenza virus envelope is the initiation, when the possibility is created for water molecules to penetrate into the cavity of hemagglutinin trimer between its subunits.

In this work, we focused on description and study of this stage, which is the consequence of disbalance between the forces stabilizing the structure of hemagglutinin trimer.

As the disulfide bond in the part of the molecule closest to the virus envelope is the only covalent bond between HA1 and HA2 domains and it does not prevent the deviation of HA1 subunit from the trimer axis [3], then the dense folding of hemagglutinin molecule in the trimer, existing under normal conditions, must be determined by van der Waals and electrostatic forces and the interactions of amino acid residues with the solvent. HA1 and HA2 subunits contain a large number of amino acid residues accessible to water and capable of being charged. Therefore, the changes in electrostatic forces caused by a decrease in pH must considerably contribute to the change in the force balance within the trimer [6]. As the hydration of previously inaccessible protein parts completes the initiation stage, we can consider that the area of water contact at this stage is constant, allowing us to neglect the contribution
of solvation to the change in the force balance within the trimer. We can also neglect the contribution of van der Waals interaction, as no considerable molecular movements take place at the initiation stage. Thus, we can infer that the electrostatic force is the main contributor to the disbalance that initiates hemagglutinin conformational changes. The value of this force is determined by the equilibrium constants of water dissociation, which, in turn, depends on the reaction temperature.

The main goal of our study was to construct a model making it possible to assess the critical pH value characterizing the fusogenic activity of influenza virus hemagglutinin from the data on hemagglutinin structure and experimental verification of this model. Under this model, we assume that when the electrostatic force between interacting hemagglutinin molecules in the virus envelope exceeds a certain value, hemagglutinin HA1 subunits are arranged so that they form a cavity sufficient for penetration of water molecules. This event leads to an irreversible hydration of the inner fragments of hemagglutinin molecule in the trimer and to completion of conformational changes.

Materials and methods

Computer modeling of the process. A popular model describing the protein behavior in aqueous solutions forms the background for the modeling procedure and calculation of the protein interactions, accomplished as original software; in this model, it is assumed that the molecules in question carry electrostatic charges acquired due to redox reactions [10] [11] [12]. In the developed algorithm, the charged amino acid residues were modeled as point charges placed at the center of the most electronegative atom involved in water dissociation. The geometry of electrostatic field strength for the protein molecule is determined by the geometry of its surface and pH of solution. The geometry of molecule surface is the interface between two dielectrics, protein globule and water, which influences the electrostatic field due to polarization effects [12]. These effects can considerably contribute to the interactions of globular proteins displaying a domain structure. The pH of solution encompassing protein molecule also influences the geometry of electrostatic field, as the values for isoelectric points for various amino acid residues differ considerably. The electrostatic charge of amino acid residues within the protein molecule was calculated assuming that an atom could be either dissociated in the presence of water or not. Here we used the values of isoelectric points of amino acid residues [13], also taking into account the dipole amino acid pairs formed during protein folding when the structure is stabilized by the bonds between oppositely charged amino acid residues. To find the critical pH values, we used the introduced concept of the threshold force as a minimal electrostatic repulsive force of hemagglutinin subunits necessary for formation of the cavity with size sufficient for water molecule penetration into the hemagglutinin trimer on the influenza virus envelope.

Study of hemolysis kinetics. The hemolysis of erythrocytes in the presence of influenza virus particles [14] was used for the experimental study of the kinetics of pH-dependent hemagglutinin conformational changes. In this approach, the endosome membrane is simulated by the erythrocyte membrane, covered with sialic acids, which provide the absorption of influenza virus virions.

Figure 3. A scheme of the hemolysis of erythrocytes in the presence of influenza virus particles. At the first stage of this reaction, erythrocytes are hemagglutinated due to the sorbed virus particles. At the second stage, pH of the solution with agglutinated erythrocytes is artificially decreased, which changes the conformation of hemagglutinin molecules on the virus envelope and leads to lysis of erythrocytes.
At the first stage of this reaction, erythrocytes are hemagglutinated due to the sorbed virus particles [15] [16]. At the second stage, pH of the solution with agglutinated erythrocytes is artificially decreased, which changes the conformation of hemagglutinin molecules on the virus envelope and leads to lysis of erythrocytes. The degree of lysis is determined spectrophotometrically according to the measured concentration of hemoglobin released into solution depending on the reaction duration. It is very important to control the temperature of hemagglutination and hemolysis of erythrocytes. When calculating the rate of erythrocyte hemolysis, we assumed that (1) all virus particles were absorbed on erythrocytes; (2) the conformational changes of influenza virus hemagglutinin were described by the equation for a simple irreversible chemical reaction where the rate constant is a single-valued function of temperature and pH; and (3) completion of the conformational changes obligatory causes erythrocyte hemolysis. The concentrations of erythrocytes and virus particles were selected so that each erythrocyte was bound to no more than one virion, as this provided that the amount of hemoglobin released from erythrocytes was proportional to the amount of hemagglutinin trimers involved in the reaction. The kinetic constant of hemolysis was calculated according to the following equation:

\[
C = C_0 \cdot \left(1 - e^{-kt}\right)
\]

where \(k\), reaction kinetic constant; \(t\), reaction time; \(C_0\), concentration of all virus receptors on erythrocyte membranes; and \(C\), concentration of the reacted virus receptors on erythrocyte membranes.

The following influenza virus strains were used in the work: A/Aichi/2/86 (H3N2), obtained from the collection of microorganisms with the FSRI SRC VB VECTOR, and A/NIB/23/89 M and A/NIB/23/89 MA, kindly provided by Dr. A.S. Gambaryan.

**Results and discussion**

Computer modeling of the interaction between hemagglutinin molecules within the trimer makes it possible to calculate the dependence of the force and the moments of force acting on HA1 subunit from the trimer on the pH value. We calculated the force projection onto the axis directed perpendicular to the symmetry axis from the trimer center to the center of mass of HA1 subunit. The values of threshold force for proteins of the influenza virus strains belonging to the same subtypes were considered equal. The calculations were performed using the data on amino acid sequences of the hemagglutinin molecules of influenza virus strains A/NIB/23/89 M (H1N1), A/NIB/23/89 MA (H1N1), A/FM/1/47 (H1N1), A/Aichi/2/86 (H3N2), and A/HK/1/86 (H3N2) and the spatial model of hemagglutinin molecule extracted from the SWISS-PROT database. Figure 4 shows the calculations of the forces acting on HA1 subunit.
Figure 4. Calculated dependence of projections of the forces acting on HA1 subunit from the trimer on pH of the medium. The force is projected onto the axis directed perpendicular to the symmetry axis from the trimer center to the center of mass of HA1 subunit.

Figure 5 shows the experimental measurements of the dependence of kinetic constants of erythrocyte hemolysis on pH of the medium for one of the studied influenza virus strains. It is evident that the rate of erythrocyte hemolysis increases with temperature.

Figure 5. Kinetic constants of erythrocyte hemolysis versus pH of the medium at +27, +32, and +37°C for influenza virus strain A/NIB/23/89 MA.
The theoretically calculated and experimentally measured critical pH values determining the beginning of conformational changes in hemagglutinin molecule for various influenza virus strains are listed in Table 1. The experimental results for pH-dependent erythrocyte hemolysis in the presence of influenza virus strains A/FM/1/47 (H1N1) and A/HK/1/86 (H3N2) were taken from [17] and [18]. The critical pH values for these strains were determined by extrapolating the curves determining the dependence of hemolysis degree on pH value to their intersection with the abscissa. The theoretical calculations for these strains were based on the hemagglutinin amino acids sequences kindly provided by Dr. E. Brown.

Table 1. Theoretically calculated and experimentally measured critical pH values for various influenza virus strains (asterisks indicate the values obtained using the experimental data of [17] and [18]).

| Strain      | Critical pH values |
|-------------|-------------------|
|             | Experimental     | Theoretical  |
| A/NIB/23/89 M | 5.7 ± 0.1      | 5.7          |
| A/NIB/23/89 MA | 5.4 ± 0.1      | 5.1          |
| A/FM/1/47    | 6.0 *          | > 7.0        |
| A/Aichi/2/68 | 5.9 ± 0.1      | 5.9          |
| A/HK/1/68    | 5.2 *          | 5.2          |

All the experimental dependences of kinetic constants for erythrocyte hemolysis decrease monotonically with the increase in pH and are well described with the equation of irreversible chemical reaction. The obtained dependences do not contradict the hypothesis underlying the model in question, namely, the assumption that the conformational changes are initiated by an increase in the electrostatic repulsive forces with the decrease in pH. The calculation of critical pH values is based on the introduced concept of the threshold force, which is a minimal electrostatic repulsive force between hemagglutinin subunits necessary for formation of the cavity with a size sufficient for penetration of water molecules. The penetration of water molecules into the cavity within the trimer leads to hydration of inner regions in hemagglutinin molecules, formation of helical region in HA2 subunits, and irreversible conformational rearrangement of all the three molecules. The critical pH values obtained by computer modeling and experimental measurements fit sufficiently well (see Table 1), thereby convincingly confirming the applicability of the developed model to calculation of protein interactions.

Thus, the main result of this work is the model allowing the function of a virus to be predicted based on the structure of its molecule, i.e., it allows the critical pH value characterizing the fusogenic activity of influenza virus necessary for delivering the virus genome into the host cytoplasm and infecting the host cell to be assessed from the amino acid sequence of its hemagglutinin. Further development of this model will make it possible to assess biological properties of influenza virus strains, in particular, their infectivity, based on the sequences of virus genome.

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