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ARTICLES

Effects of pH on the Adsorption of the Viral Matrix Protein M1

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Abstract—Adsorption of the viral matrix protein M1 on a substrate simulating the lipid membrane surface of the influenza virus was studied by surface plasmon resonance (SPR). It was found that a decrease of pH leads to an increase of the time to reach the saturated level of adsorption, despite the growth of its initial rate. Adsorption of M1 is irreversible in acidic and neutral media, but in the first case the saturated level of adsorption depends on the protein concentration. It was observed that despite the adsorption irreversibility, acidification of the solution down to pH 4 leads to a partial protein desorption from the adsorbed layer that was formed at pH 7. The findings suggest pH-induced changes in shape of the adsorbed M1 molecules. In an acidic medium, elongated protein molecules adsorb mostly laterally in dilute solutions and more orthogonally in concentrated ones. In a neutral medium, protein molecules take on a compact conformation in the adsorption layer, and its thickness does not depend on the concentration. Apparently, flexible C-terminal domain of the adsorbed protein plays a major role in the pH-induced conformational change.

Keywords: influenza A virus, viral matrix protein M1, surface plasmon resonance
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INTRODUCTION

The influenza virus is an enveloped virus which is 80 to 140 nm in diameter [1]. Its outer part is formed by a lipid bilayer containing integrated transmembrane proteins. On the inner side, the lipid bilayer borders with the M1 protein matrix scaffolds associated with ribonucleoprotein (RNP) which is located inside the virion [2–4]. The matrix protein plays a key role in the assembly and budding of its daughter virions [5–7]. M1 has a tendency to oligomerize in neutral medium [8] determines a high density of the protein network [9], which prevents large RNP particles from getting out of the virion [10, 11]. In the neutral medium the protein network protects the genetic material of the virus against external factors, then during the acidification of the intra-endosomal medium down to pH 5 or below that occurs gradually when the virus moves towards the cell nucleus, the matrix frame loses its integrity, allowing the genetic material of the virus to escape into the cell cytoplasm [2, 3, 12, 13].

The structure of the matrix protein largely determines the character of its interaction with the lipid bilayer. At the moment, it is known that M1 is a small molecule weighing 27.8 kDa and consisting of 252 amino acid residues [9, 14]. It is comprised of three domains: N (2–67), M (91–158) and C (165–252) [2]. The M1 protein structure as a whole is not established yet, since all the attempts of its crystallization lead to the detachment of the C-terminal domain [2, 15]. It is known that the N-terminal part of the protein has a globule shape [14, 16] and the M1 protein molecule has an elongated shape, mainly due to the C-domain [14]. According to the data obtained by the methods of circular dichroism, small-angle neutron scattering, tritium planigraphy, and small-angle X-ray scattering in the acidic medium [14, 16, 17], C-domain has a less ordered and flexible structure, in contrast to the N-terminal portion. The conformation of M1 molecule in solution and on the surface of the virus lipid membrane may be different. Thus, according to the estimates based on small-angle neutron and X-ray scattering [14, 17], an estimated length of the M1 molecule is 8–10 nm, while electron microscopy shows that in the protein layer the size of these molecules on the virion surface does not exceed 6 nm [9, 18]. This value corresponds exactly to the estimated size of the protein molecule with most densely packed C-terminal domain [17].

It is still unclear which interactions are most important when the protein binds with the lipid bilayer in the virion. It is known that in M1 protein, 127 of 252 amino acid residues are hydrophobic (alanine, glycine, isoleucine, leucine, phenylalanine, tryp-

tophan, valine, methionine, cysteine, and proline) [19]. This allows the protein to enter into hydrophobic interactions with the membrane. The fact that M1 is positively charged in the neutral medium (48 amino acid residues bear a positive charge and 23, a negative charge) suggests that electrostatic forces play an important role in its interaction with the lipid bilayer. Indeed, a number of papers [9, 20–22] state that the primary role of these forces is generated between positively charged M1 groups and the negatively charged surface of the lipid membrane.

The interaction between the M1 protein and the lipid bilayer is significantly affected by changes in pH. It is known that when the virus enters the acidic medium, RNP molecules are detached from M1 [3, 23]. Recently, by means of cryoelectron microscopy, it was found that the thickness of the protein layer decreases a few minutes after the pH lowering. The authors attribute this phenomenon to the change in the conformation of the M1 molecule [18]. We made a similar conclusion earlier, when studying the M1 adsorption features in the acidic medium [24]. Based on these data, it was suggested that due to the weakly ordered structure (primarily, the C-terminal domain) and strong interaction with the surface simulating the lipid bilayer, M1 spreads on it. However, the study carried out at pH 4 did not investigate the M1 protein behavior at different values of pH and changes in the protein layer formed in the neutral medium as a result of the solution acidification. It is this process that plays a key role in the virus infection. Therefore, the aim of this paper was to investigate the influence of acidity and its changes on the M1 protein adsorption, using the substrate simulating the surface of the cell membrane.

MATERIALS AND METHODS

Reagents. The following reagents were used in the experiments: KCl (chemically pure; Reachem, Russia), MES (Calbiochem, USA; purity ≥99%), mercaptohexadecanoic acid (16-mercaptohexadecanoic acid, 99%; Aldrich, USA), HCl (chemically pure; Reachem, Russia), KOH (chemically pure; Reachem, Russia), chloroform (chemically pure; Reachem, Russia), *n*-hexane (extra pure; Reachem, Russia). All solutions were prepared using deionized water.

Preparation of M1 protein. The M1 matrix protein was obtained from the virions of the A/PR/8/34 influenza strain, using the Zhirnov method [25]. It was isolated by the acid solubilization of the influenza virus membrane, using a non-ionic detergent NP-40 (Igepal CA-630) in the buffer solution containing 50 mM 2-N-morpholinoethanesulfonic acid (MES), 100 mM NaCl, pH 4.0, as described previously [24]. The protein solutions containing 100 mM KCl and 2 mM MES with the desired pH were used in the experiments.

Method of surface plasmon resonance (SPR). The protein adsorption was studied using the SPR refractometer Biosuplar 6 (Mivitec, Germany) [24] that recorded the adsorption kinetics in real time. A flat (about $1 \times 0.5 \times 0.1$ cm^3) two-chamber thermostatic flow cell was attached to a gilded surface of the chip. The test solutions were pumped over it using a peristaltic pump (MasterFlex C/L, Barland Co., USA). Binding of the protein molecules to the sensor surface was accompanied by changes in the optical parameters of the surface layer. Surface plasmon resonance is extremely sensitive to such changes. An increase in the refraction index of the thin near-surface layer due to the adsorption was recorded according to the SPR refractometer signal that was measured in relative units (rel. units). The change of this signal is proportional to the increase in the protein surface concentration.

All measurements were performed at room temperature (24 ± 1°C).

Preparation of thiolated substrates. To study the protein adsorption, we used chips representing glass plates with a gold layer (50 nm) sputtered over an intermediate adhesive layer of chromium (1–1.5 nm). To create a system that simulates the lipid bilayer of the viral envelope, the gold surface was coated with a layer of mercaptohexadecanoic acid molecules that provided a negative surface charge in the neutral medium like the charged lipid heads in the viral envelope. Before coating, gilded plates underwent several stages of purification. First, they were washed with chloroform, and then in an ultrasonic bath (PSB-Gals, Russia) with 96% ethanol for 10–20 s. After that the plates were washed with distilled water and dried at 100°C. For chemical coupling with the gold layer on the glass chip surface of the mercaptohexadecanoic acid molecules, the plates were soaked in 10 mM solution of this substance in hexane (extra pure, not less than 99.85 wt %; Reachem, Russia) for 30 min at 50°C and then left in the same solution overnight at room temperature. After this procedure, the plates were washed three times with hexane and dried under argon atmosphere for 10 min.

RESULTS

Effect of pH on the adsorption kinetics. To determine the effect of acidity on the protein substrate affinity, we investigated the dependence of the initial adsorption rate on the pH value of the medium at a constant concentration of proteins in the solution (250 nM). It was found that within the pH range from 4 to 7, this rate decreased monotonically more than 1.5-fold (Fig. 1), with the most dramatic decrease at low pH values. The resulting dependence apparently reflects the weakening of protein interactions with the surface as the acidity of the solution decreases, which may be caused by the reduction of the M1 charge. A more detailed description of the pH effect on the kinetic curves of adsorption is given below for the pH values of 7 and 4.

Addition of protein at a concentration of 250 nM into the flow cell at pH 7 was followed by an increase

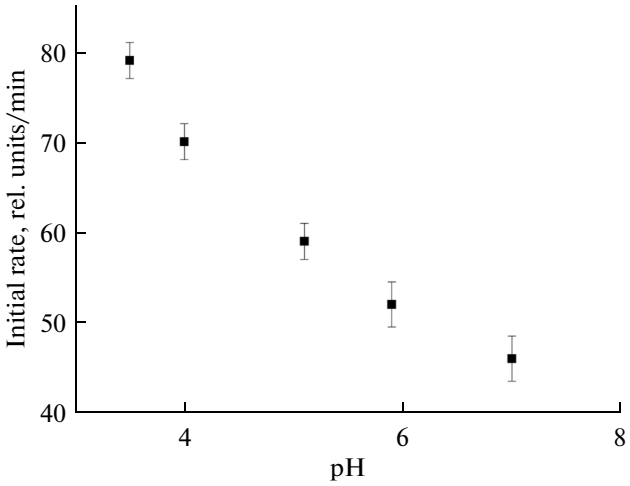

Fig. 1. The dependence of an initial rate of the M1 adsorption signal on the pH value of the medium, when the protein concentration in the solution is 250 nM. Hereinafter, the protein was in the solution containing 100 mM KCl and 2 mM MES. The absorption signal was measured in relative units of the SPR refractometer response. Each point on the curve is an average value obtained from the results of five experiments. Hereinafter, errors indicate the standard deviation of the measurements.

in the adsorption signal that reached the stationary level within 20–30 min (Fig. 2). After the signal growth stopped, the protein was removed from the cell by rinsing with a buffer solution. In response to this, the adsorption signal had virtually no decrease and remained unchanged over time, indicating the irreversibility of the adsorption. Re-introduction of the protein solution with the same concentration did not lead to an additional increase in the signal. Thus, the formation of the protein-saturated adsorption layer was shown. After all these operations, the adsorption signal was 290 ± 15 rel. units.

During the addition of the protein at the same concentration into the acidic medium (pH 4), the adsorption signal first increased faster than in the neutral medium (Fig. 2), but ~2 min later a fast increase changed to a considerably slower one. The second stage of the increase in the adsorption signal continued for a long time, which is why a stationary level was typically reached after about 2 h, rather than in 20 min, as in the neutral medium. Despite this fact, the adsorption signal values reached at pH 4 and pH 7 were almost the same for a given protein concentration. Rinsing with a buffer solution and re-introduction of the protein in the acidic medium also showed the irreversible nature of the adsorption and its saturation. In separate experiments we found that protein desorption is negligible for several hours after the protein removal from the solution. This behaviour of the protein adsorption layer was observed in all further experiments at all used pH and protein concentrations in the solution.

Effect of the medium pH on the M1 adsorption value. As we have shown previously [24], the adsorption signal achieved at pH 4 strongly depended on the protein concentration. In diluted solutions its value was several times lower than in concentrated solutions, and M1 always formed a saturated monolayer. Formation of saturated adsorption layers with a lower surface concentration in diluted solutions actually meant that they had a smaller average thickness. To explain this phenomenon, we made an assumption about a multipoint binding and spreading of weakly ordered protein fragments on the surface. As a result of this process in diluted solutions, the surface can be completely covered with fewer protein molecules. It is known that these processes occur quite slowly, so in diluted solutions, where the distance between the adsorbed molecules is greater, the spreading is stronger than in concentrated solutions, which are characterized by a great mutual competition between neighboring molecules for a place in the adsorption layer [26, 27].

To check whether the properties of the protein layer also depend strongly on the conditions of its formation in the neutral medium, we studied the adsorption of M1 at pH 7 within the range of concentrations from 50 to 500 nM. As in the acidic medium, the protein adsorbed irreversibly and formed a saturated adsorption layer within the entire range of concentrations under study. However, it was found that for all protein concentrations tested, the value of this signal reached the same level of about 300 rel. units (Fig. 3). In contrast to the data for pH 4, this result corresponds to the conventional irreversible adsorption or chemisorption, when due to the strong binding of the protein with the substrate surface, the same monomolecular adsorption layer is formed in the solution at any protein concentration, and a concentration just determines the rate of this process. Note that for the so-called physical adsorption a weak interaction of the adsorbate with the surface caused by van der Waals forces is characterized not only by reversibility, but also by the dependence of the adsorption equilibrium value on the concentration. The adsorption reversibility and concentration dependence is also typical for multilayer coatings.

In the acidic medium at pH 4, as the protein concentration grew from 50 to 500 nM, the achieved adsorption signal level increased steadily, and up to the highest concentrations it was far from reaching the limit value. Interestingly, the curve describing the dependence of the adsorption saturation level on the protein concentration at pH 4 had a form of the Langmuir isotherm (Fig. 3), despite the fact that the protein was irreversibly adsorbed over the entire range under study. When the protein concentration was about 250 nM, the dependencies of saturation levels for pH 4 and pH 7 intersected. In more concentrated solutions the adsorption signal in the acidic medium reached higher values than in the same solutions with neutral pH. When the protein concentration was 1 μM,

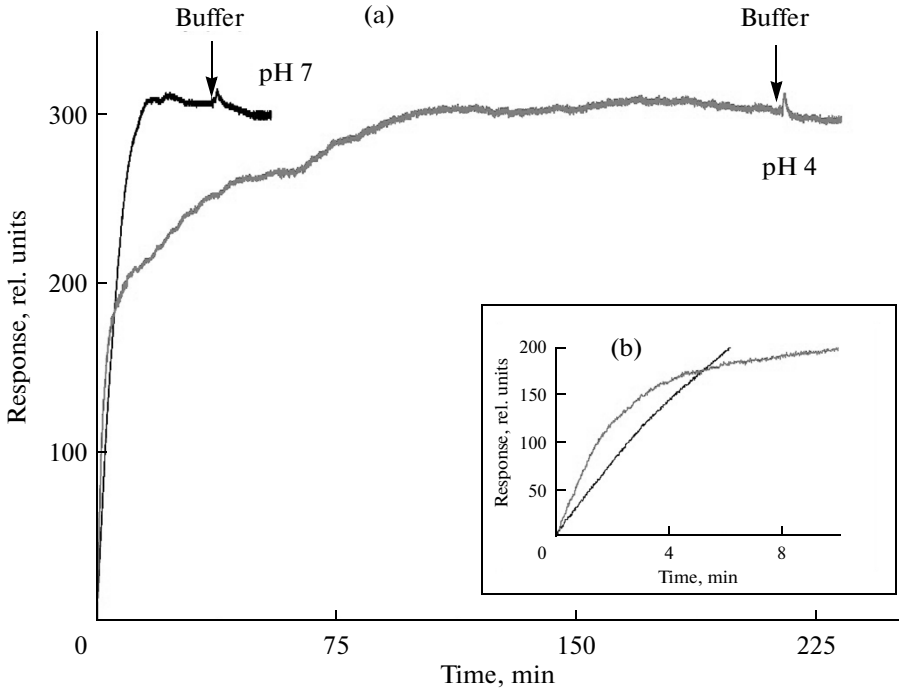

Fig. 2. The M1 protein adsorption kinetics at pH 4 and pH 7 (a). Panel (b) presents an enlarged fragment of the initial section of the curve in (a), showing a strong contrast in the initial adsorption rates in the acidic and neutral media.

the adsorption signal was by one third greater than at pH 7 (data not shown).

Thus, protein M1 behaved differently when adsorbed in acidic and neutral media. In this regard, it was important to determine how the mechanism of adsorption changed as pH increased – continuously or when reaching some critical value. For this purpose, we studied the M1 adsorption within the range of concentrations from 50 to 500 nM, at pH of the medium from 4 to 7.

It was found that at each pH value within the range from 4 to 6, when the protein concentration increased in the solution, the adsorption signal increased just as at pH 4 (Fig. 4). However, at pH 4, when the protein concentration increased from 50 to 500 nM, the adsorption increased approximately 3.5-fold (the overall signal changed from 110 to 370 rel. units), then at pH 5.5 there was only a 1.2-fold increase. At pH > 6, M1 formed the same protein layers within the whole concentration range, and the corresponding signal value was 300 ± 10 rel. units. Thus, as pH increased, the protein surface concentration in the saturated adsorption layer was determined by its concentration in the solution to a lesser and lesser extent. It is interesting that in diluted solutions in the acidic medium, an average thickness of the saturated adsorption layer under the formation (proportional to the protein surface concentration) was smaller than in the neutral medium, and, conversely, in the concentrated solutions it was greater than in the neutral medium. It is difficult to associate this effect with the multilayer filling of the surface, since in the acidic medium the protein was adsorbed irreversibly, and after the formation of the adsorption layers with the lowest surface concentration it showed no propensity to additional adsorption after the repeated application.

Changes in the protein adsorption layer at decreasing pH of the medium. As indicated above, an important step of the viral infection of a cell is the destruction of the protein–lipid envelope of the influenza virus during the acidification of the medium. The cryoelectron microscopy revealed a partial desorption of M1 from the inner surface of the virion lipid membrane when pH decreased [18]. To simulate this process, we examined the change in the protein layer formed at pH 7, when the pH of the solution decreased to 4. The protein was adsorbed at a concentration of 250 nM, at which it forms uniform density adsorption layers throughout the whole pH range studied (Fig. 4). As in the earlier instance (Fig. 2), after the introduction of M1 in the neutral medium, the attainment of saturation by the adsorption signal and removal of protein by rinsing the system with a buffer solution, desorption was virtually absent (Fig. 5). However, the subsequent rinsing with the same solution but at pH 4 resulted in a marked reduction of the signal (roughly by one third). After another introduction of the protein in a concentration of 250 nM at the same pH value (pH 4) no discernible adsorption was observed. Rinsing with a buffer solution at pH 7 did not lead to any changes either. However, in response to the introduction of the protein solution (250 nM) in this medium,

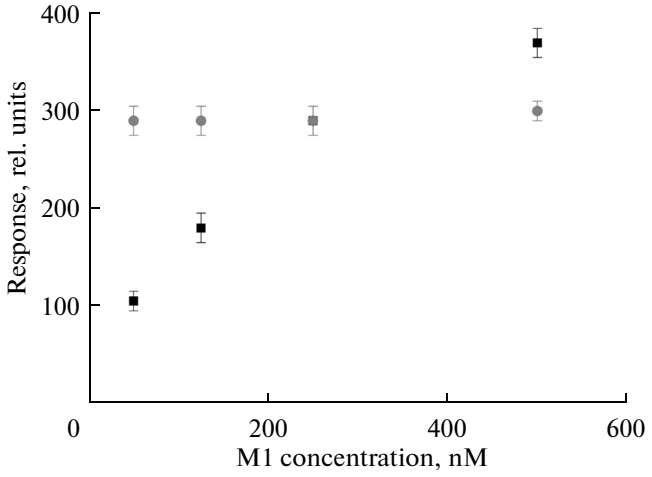

Fig. 3. The dependence of the final signal of the M1 saturated adsorption on the protein concentration at pH 4 (*squares*) and pH 7 (*circles*). Each point on the curve is an average value obtained from the results of five experiments.

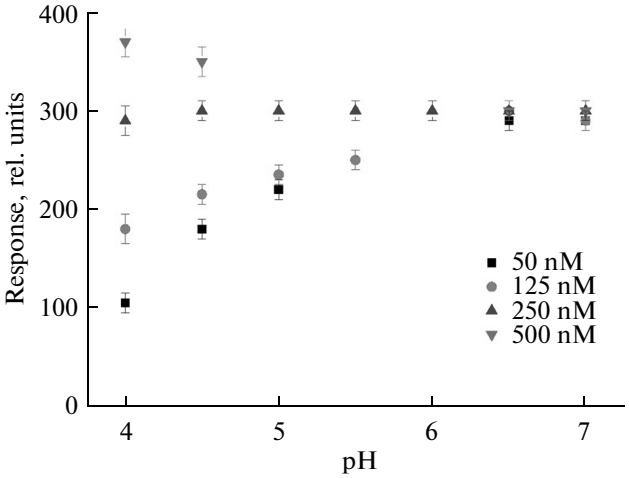

Fig. 4. The effect of pH on the value of the achievable adsorption signal at the M1 concentrations in the solution of 50, 125, 250, and 500 nM (shown in the figure). Each point on the curve is an averaged value from five experiments.

the adsorption signal repeatedly increased almost to a baseline level after the initial addition. This layer also had virtually no decrease after rinsing with a pH 7 buffer solution. Thus, it was shown that the solution acidification causes partial desorption of the protein adsorbed in the neutral medium. In this case, the remaining protein apparently does not fill the free space as a result of spreading or rebuilding of the adsorption layer, which is proved by the fact that it can be fully recovered after another introduction of the protein in the neutral medium. Possibly, after the adsorption, the protein molecules completely establish their shape and position on the surface.

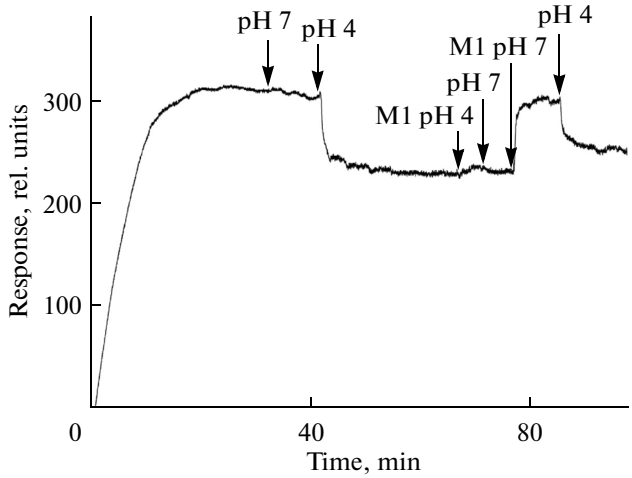

Fig. 5. The effect of pH changes on the properties of the M1 adsorption layer. The initial rise of the signal corresponds to the introduction of the protein solution at pH 7. The subsequent rinsing with buffer solutions with given pH (7 or 4) and addition of the protein at different pH values (M1 and corresponding pH value) are shown on the curve.

It was possible to modify the pH value of the solution in the cell and introduce the protein several times in any sequence. After the adsorption in the neutral medium, the solution acidification down to pH 4 each time led to a partial desorption of the protein. Addition of a new portion of the protein under these conditions did not result in any increase of the signal. The adsorption layer recovered only after the introduction of the protein solution at pH 7. The same effect could be achieved by the addition of the protein directly into the neutral medium.

Completing the construction of the adsorption layer in the neutral medium. We established that lowering the pH value led to a partial desorption of the protein adsorbed at pH 7 and that the freed surface could only be filled with the protein again in the neutral medium. Under such conditions, the charge of the protein molecules and their mutual repulsion reduce significantly, which apparently facilitates the recovery of the adsorption layer.

When the adsorption occurs in the acidic medium, the mutual repulsion of the protein molecules, particularly in diluted solutions, can give rise to blank areas of the surface between them. To test this hypothesis, we formed an adsorption layer from a protein solution with a concentration of 125 nM at pH 4 (Fig. 6). Replacement of the solution with a similar one at pH 7 resulted in a slight signal increase. However, the addition of 125 nM of the protein solution in the neutral medium led to a significant additional adsorption (approximately by 50 rel. units), despite the saturation of the adsorption layer (failure of additional adsorption) at pH 4. This additional adsorption, as in all pre-

vious cases studied, was almost irreversible, i.e., there was no significant desorption when the protein was removed by rinsing with a buffer solution at pH 7. However, in contrast to the adsorption layer initially formed in the neutral medium, in this case the whole additionally adsorbed protein, rather than a third of it, was completely removed by rinsing the system with a solution at pH 4. This showed a significantly weaker protein binding under these conditions.

DISCUSSION

In our model system, the acidity of the medium affected the properties of both the substrate and the protein, and, consequently, their interaction.

By direct measurement of adhesion forces between the carboxyl groups on the thiolated surface similar to that used by us, the dissociation constant of these groups was determined, pKa = 5.2 [28]. Therefore, at pH 4, almost all these groups on the substrate used by us are protonated, and the surface can be regarded as electrically neutral, while at pH 7, in contrast, most of them are ionized and the surface is negatively charged.

The M1 protein charge also varies considerably within the pH range investigated by us. It is known that this protein consists of three domains: N, M, and C [9, 14]. The total charge of the protein as a whole and its individual domains – depending on the pH value – was estimated using Protein Calculator v3.4 [29] on the basis of its amino acid sequence (Fig. 7). The results obtained point to its monotonic decrease with the increasing pH, and this process was most dramatic in more acidic solutions. The largest positive charge within the investigated pH range is borne by the C-terminal domain.

The obtained results in their entirety can be explained in terms of assumptions that are based on the following data.

The respective sizes of the M1 protein molecule – in the solution and on the surface of the virion lipid membrane – differ significantly. According to the independent data obtained from small-angle neutron scattering [14] and small-angle X-ray scattering [17], the M1 molecule has an elongated shape, with a length of about 8 or 10 nm, respectively, due to the presence of the C-domain. At the same time, according to electron microscopy [9, 18], the length of the protein molecule in influenza virus envelope is about 6 nm. The only part of the protein that can significantly change in size is the mobile and relatively weakly structured C-terminal domain [14, 16, 17]. According to [17], its length in the solution ranges from 2 to 10 nm, and the length of the molecule as a whole, from 6 to 13 nm, respectively. The conclusion is that during the adsorption in the neutral medium, the C-terminal domain of the M1 protein is recorded in a compressed conformation.

As noted above, in the neutral medium, most of the protein carboxyl groups and the surface are ionized. At the same time, according to our estimates, the protein

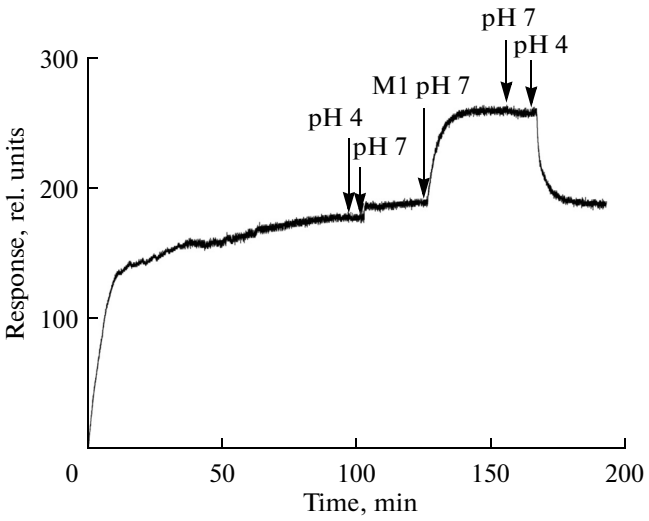

Fig. 6. The reversible completion of the adsorption layer after the protein re-addition in the neutral medium. The initial rise of the signal corresponds to the introduction of protein M1 at a concentration of 125 nM at pH 4. Subsequent changes are shown on the curve: rinsing with buffer solutions at pH 4 and pH 7; introduction of the protein with the same concentration at pH 7 (resulting in the signal increase), and successive rinsing with solutions at pH 7 and 4 (the latter leads to a complete desorption of the protein from the second addition).

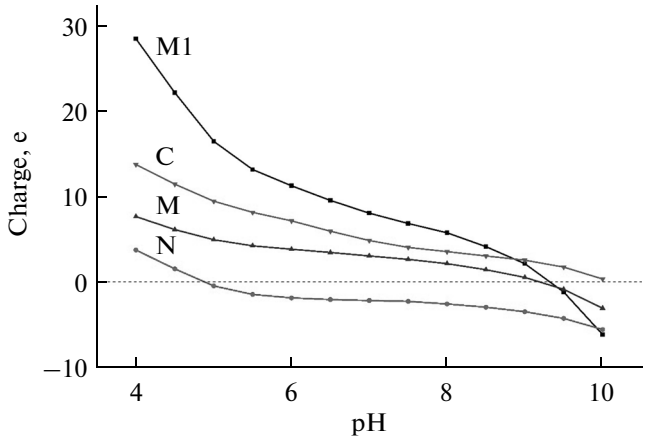

Fig. 7. The net charge of the M1 protein and the charge of its domains M, N, C as a function of the pH value calculated using Protein Calculator v3.4 [29] according to their amino acid sequence.

in general bears a small opposite positive charge (see Fig. 7). According to our data, its most important part is concentrated in the C-terminal domain. The electrostatic interaction of the protein and the substrate should contribute to its rapid absorption. In addition, this interaction may lead to a reduction in size of the M1 molecule, due to compaction of the mobile C-domain structure, which explains the paradox of the difference in size of the protein in the solution and on the surface of the influenza virion lipid membrane

respectively. The compact structure of the adsorbed protein molecules apparently provides a formation of the same adsorption layer in the neutral medium at all concentrations of the protein in the solution.

In the acidic medium (pH 4), surface carboxyl groups are almost completely protonated, as their dissociation is determined by the value pKa = 5.2 [28]. In this case, the protein bears a high positive charge. There are no opposite or similar charges on the surface, but some hydrogen bonds may form between the protein functional groups and the surface, including bonds between protonated carboxyl groups of the protein and the substrate. A possible development of such bonds was mentioned, in particular, in [28]. Moreover, a high charge and mobile structure of the C-domain contribute to its unfolding and extending. All these factors together create conditions for the attraction of various functional groups of the protein in all its domains to the surface. Thus, in the acidic medium, the protein may form more bonds with the substrate surface than in the neutral medium and show a greater affinity to the surface and, therefore, a higher initial rate of adsorption (see Fig. 1).

In diluted solutions, the protein will seek to be positioned laterally on the surface, due to its ability to form hydrogen bonds by functional groups of all its domains. Laying long polymer molecules along the surface is associated with significant steric hindrance, due to which, apparently, more time is needed to reach a level of saturated adsorption in the acidic medium (see Fig. 2). As the concentration increases due to the growing competition between neighboring molecules, the greater part of the protein has to be positioned anglewise, or even perpendicularly to the surface. Apparently, a high charge of the C-terminal part prevents the protein molecules from being converted into a more compact configuration, which can lead to the creation of adsorption layers with greater thickness than in the neutral medium (see Figs. 3 and 4).

The observed partial desorption of the protein adsorbed in the neutral medium due to the decrease in pH (see Fig. 5) is quite interesting. At first glance, it is caused only by the increase of the charge of the protein molecules, leading to their mutual repulsion and displacement out of the adsorption layer. However, the M1 adsorption in both neutral and acidic media is irreversible, and at a sufficiently high concentration of the protein in the solution, its surface concentration during the adsorption in the acidic medium, despite the mutual electrostatic repulsion, may be even higher than during the adsorption from a solution at pH 7. Thus, the observed desorption due to the decrease in pH can serve as the confirmation of the assumptions about different protein orientation and structure during the adsorption in acidic and neutral media. Protein molecules adsorbed in the neutral medium in a compact form, presumably, make a smaller number of bonds with the surface than during the adsorption in the acidic medium, which makes it possible to desorb molecules with the weakest bonds by increasing their mutual electrostatic repulsion due to the pH decrease.

When the medium is neutralized, the adsorption layer formed at pH 4 is not destroyed, since the mutual repulsion of the positively charged protein molecules decreases. However, the possible additional protein adsorption in the neutral medium and its complete desorption due to a subsequent decrease in pH (see Fig. 6) also provides some useful information about the structure of the adsorption layer. Figure 6 illustrates the adsorption of the protein at a concentration of 125 nM in the solution. In this concentration (as in other concentrations) in the neutral medium, it forms an adsorption layer characterized by the absorption signal of about 300 rel. units. (see Figs. 3 and 4), and at pH 4 the surface concentration in the saturated absorption layer is characterized by a signal of about 180 rel. units, which is lower by about 120 rel. units (or 40%). The saturation of the adsorption layer is indicated by the fact that the protein cannot be additionally adsorbed upon its repeated introduction. Apparently, this is not only due to the occupied surface, but also due to the mutual repulsion of the highly charged M1 molecules at pH 4. An attempt to perform an additional adsorption in the neutral medium, where the protein is charged considerably weaker, helps to separate these two effects. As can be seen from Fig. 6, the addition of the protein in the neutral medium leads to an increase in the adsorption signal by approximately 70 rel. units. Apparently, this new protein moves into free spaces between previously adsorbed molecules. However, as was mentioned above, the difference in the saturated absorption signals in the acidic and neutral media for a given protein concentration is 120 rel. units; therefore, only an amount less than 60% of this difference (70 of 120 rel. units) is available for the adsorption in the neutral medium. Moreover, as can be seen from Fig. 6, during the reacidification of the solution, the all the protein (and not just part of it, as in the case of the adsorption on the clean substrate surface) added at pH 7 was completely desorbed. This indicates that the access to the surface is limited for all additionally adsorbed molecules by the previously adsorbed protein, and they do not have enough space for a strong multipoint binding. By that, the described experiment confirms our assumption that in the acidic medium the M1 protein molecules are adsorbed in a much less compact form than in the neutral medium.

Thus, all the results obtained in this study can be explained with an assumption that the M1 protein molecules have different shapes during the adsorption in acidic and neutral media. In the acidic medium, they keep their elongated shape largely because of the weakly ordered C-terminal domain. Depending on the solution concentration, the protein can fill up the adsorption layer more laterally or orthogonally to the surface. In the neutral medium, during the adsorption, the less charged C-terminal domain apparently acquires a compact conformation and the protein M1

forms adsorption layers of the same thickness at all concentrations in the solution.

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