Biochimica et Biophysica Acta 854 (1986) 198–206
Elsevier

BBA 72881

Temperature and pH dependence of the haemolytic activity of influenza virus and of the rotational mobility of the spike glycoproteins

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(Received July 25th, 1985)

Key words: Membrane–virus interaction; Viral envelope; Glycoprotein; Rotational mobility; Hemolysis; (Influenza virus)

Influenza virus (strain X-47) was labeled with the triplet probe, eosin 5-isothiocyanate. Most of the label was found to be associated with haemagglutinin, the major glycoprotein of the viral envelope. Rotational diffusion of the glycoprotein was investigated by measuring flash-induced transient dichroism of the eosin probe. The anisotropy decay curves showed that mobility of haemagglutinin measured at pH 7.3 increased considerably with temperature with the greatest change occurring over the range 20–30°C. However, at pH 5.2 no mobility was detectable over the time range of the experiment. The activity of the virus was determined by assaying haemolysis of human erythrocytes. The haemolytic activity showed an optimum at pH 5.2 and increased markedly with temperature, being negligible below 20°C. In addition, inactivation of the virus by incubation at pH 5.2 was also strongly temperature dependent. A 15 min incubation at pH 5.2 inactivated the virus above 30°C but had no effect below 20°C. On the basis of these results, it is proposed that mobility of haemagglutinin is significant for its functional properties. When the pH is reduced from 7.3 to 5.2, the mobility observed at higher temperatures is required for the molecular rearrangements which accompany the fusion event. In the absence of an apposing membrane, these rearrangements result in irreversible aggregation of haemagglutinin in the viral membrane, and hence loss of mobility and activity.

Introduction

Influenza viruses exhibit their fusogenic and haemolytic activities only in mildly acidic media [1–3]. Haemagglutinin, the major spike glycoprotein of influenza virus, has been identified as the fusogenic agent required for infectivity [3,4]. A variety of biochemical and physical techniques have clearly demonstrated that haemagglutinin undergoes a conformational change when the pH of the medium is reduced from pH 7 to pH 5 [5–8]. This conformational change is thought to be an essential prerequisite for the biological activity of influenza virus.

Biological activity also requires that the haemagglutinin precursor has been cleaved into two disulphide linked polypeptides, haemagglutinin$_1$ and haemagglutinin$_2$, exposing an additional N-terminal peptide on haemagglutinin$_2$. This new N-terminus is both very hydrophobic and very highly conserved between strains of influenza and also shows marked homology with the corresponding apolar F_1 sequences of the paramyxovirus fusion glycoproteins [9]. There is now some evidence that this peptide becomes incorporated into the lipid bilayer of the target membrane [10].

Abbreviations: eosin-SCN, eosin-5-isothiocyanate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulphonic acid.

Incorporation of these viral glycoproteins into a susceptible cell membrane is thought to induce the formation of Ca^{2+}-sensitive 'pores' [11–13], which allow the equilibration of Na^+ and K^+ across the membrane and the leakage of low-molecular-weight-phosphorylated metabolites out of the cells. These permeability changes may, in turn, be responsible for the cytotoxic effects of such viruses including cell lysis and polykaryon formation [13].

The three-dimensional structure of the bromelain-cleaved haemagglutinin, [14], determined at pH 7.5, shows that the N-terminal peptide of haemagglutinin$_2$ is situated 35 nm from the viral membrane and 100 nm from the distal end of the bromelain-cleaved haemagglutinin molecule. Thus low pH must induce a conformational change which is sufficiently large to allow the peptide to reach an apposing membrane.

The molecular events involved in this conformational change and in the fusion process are still ill understood. In view of the considerable structural rearrangements which must occur during fusion, it is very probable that the dynamic properties of the viral glycoproteins are of considerable importance. Indeed, in the related paramyxovirus, Sendai, it was found that increased mobility of the spike glycoproteins with temperature correlated with increased haemolytic activity [15]. In order to understand further the processes involved, this paper investigates the mobility of the spike glycoproteins in influenza virus, strain X-47 (H3N2) both as a function of pH and of temperature and seeks to correlate this mobility with the haemolytic activity and the stability of the virus at low pH. The viral glycoproteins of influenza were labeled with the triplet probe, eosin-5-isothiocyanate (eosin-SCN) which reacts preferentially with amino groups. Rotational motion was measured by observing flash-induced transient dichroism of the triplet probe [16].

Materials and Methods

Virus

Influenza virus, strain X-47, [17] was grown in embryonated chicken eggs and stored at −20°C. Before use the virus was spun at $3000 \times g$ for 5 min to remove aggregated material. The supernatant was pelleted at $30000 \times g$ for 1 h and the viral particles resuspended in phosphate-buffered saline (10 mM phosphate/137 mM NaCl/3 mM KCl, (pH 7.3)) at a protein concentration of 10 mg/ml.

Haemagglutination activity was determined as described by Martin [18]. The haemolytic activity was measured in 0.1 M sodium acetate, 0.1 M NaCl for pH values 4.8–6.0 and in phosphate-buffered saline for pH 6.0 to 7.3. Virus (20–40 μg) was added to a 0.8% suspension of freshly washed human erythrocytes and incubated with gentle shaking for 15 min at 37°C. The suspension was centrifuged at $11000 \times g$ for 15 s and the absorbance of the supernatant measured at 520 nm [6]. The percentage haemolysis was calculated from

$$\frac{100(A - A_0)}{(A_{100} - A_0)} \quad (1)$$

where A_{100} was the absorbance for 100% haemolysis determined in distilled water and A_0 was the base line absorbance measured in the absence of virus.

The stability of the virus at pH 5.2 was investigated by preincubation for 15 min at pH 5.2 at different temperatures in the range 0–37°C. The haemolytic activity was then measured at 37°C and expressed as a percentage of the activity of the control virus kept at pH 7.3.

Labeling of viral particles with eosin-SCN

Influenza virus (4 mg protein/ml phosphate-buffered saline, pH 7.3) was incubated with 24 μg of eosin-SCN (Molecular Probes) for 1 h at room temperature in the dark. The reaction was stopped by adding 1/10th volume of 1 M Tris-HCl, pH 7.3. Unreacted label was removed by centrifugation at 4°C, the labeled virus particles being washed respectively with phosphate-buffered saline containing 0.2% and 0.1% bovine serum albumin and then twice with phosphate-buffered saline.

Characterisation of labeled virus

Labeled virus was solubilised in 1% SDS before the eosin content was measured spectrophotometrically [16] and the protein estimated by the method of Lowry et al. [19]. A molar ratio of eosin/glycoprotein was estimated assuming the glycoprotein content to be 37% by weight of the total viral protein [20,21].

Labeled virus (2 mg/ml) was digested with pineapple stem bromelain (Sigma Chemical Co.) (1 mg/ml) by incubation at 35°C for 14 h [22]. The cleaved glycoprotein was removed by centrifugation at 100000 × g for 1 h. The eosin content of the supernatant and the pellet was determined spectrophotometrically. SDS-polyacrylamide gel electrophoresis [23] was performed on labeled virus and on bromelain digested virus. Samples (20–100 μg) were solubilised in buffer containing 0.23 M Tris-HCl/20% glycerol/10% SDS/160 mM dithiothreitol and boiled for 2 min before applying to 2 mm slab gels. Gels were examined under ultraviolet illumination to detect eosin fluorescence prior to staining with Coomassie brilliant blue.

Labeled virus was extracted with chloroform/methanol [24] in order to determine the amount of free label and label bound to lipids.

Measurement of rotational motion

The rotational motion of the viral glycoproteins was measured using a transient dichroism apparatus similar to that described by Cherry [16]. The sample was excited at 532 nm by approx. 15 ns pulses of vertically polarised light from a Nd-YAG laser (J.K. Lasers Ltd.). Excitation gives rise to transient absorption changes due to excitation of the eosin triplet state. Ground state depletion signals were recorded at 515 nm for light polarised parallel and perpendicular to the polarisation of the exciting flash. Typically 512 nm signals were collected per sample and averaged by a Datalab DL 102A signal averager. The absorption anisotropy was calculated from the expression

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (2)$$

where $A_{\parallel}(t)$ and $A_{\perp}(t)$ are the absorption changes at time t after the flash for light polarised parallel and perpendicular, respectively, to the polarisation of the exciting flash. To obtain a better signal to noise ratio, results from 3–4 samples at the same temperature were averaged using a BBC microcomputer.

The analysis of anisotropy decays for membrane proteins has been discussed in detail elsewhere [25,26]. In the present case, the data only justified fitting by a single exponential decay of the form

$$r(t) = r_1 \exp(-t/T_1) + r_3 \quad (3)$$

The time constant T_1 may be regarded as an estimate of the relaxation time for rotation about the membrane normal. The constant term r_3 is a consequence of the inability of membrane proteins to tumble across the membrane, it may also be increased by contributions from immobile species. Experiments in which the values of r_3 were compared at different temperatures were performed on the same day to avoid variations due to changes in laser intensity.

Measurements were made in 5 mM Hepes/5 mM Mes/150 mM NaCl containing glycerol (50% w/v) to reduce light scattering and virus particle tumbling. The final eosin concentration was 3–5 μM. Samples were flushed for 10 min with argon to remove oxygen and preincubated at the required temperature for 10–15 min.

Results and Analysis

Characterisation of eosin-labeled viral glycoproteins

SDS-polyacrylamide gel electrophoresis of the eosin-labeled influenza virus showed weak eosin fluorescence at the position of the two main protein bands, subsequently visualised by Coomassie blue staining, but not at the position of the neuraminidase glycoprotein (the band directly above position 1, Lane A, Fig. 1). This observation, however, does not permit identification of the eosin-labeling sites as the haemagglutinin$_1$ peptide runs at the same position as the nucleoprotein and the haemagglutinin$_2$ peptide does not separate readily from the matrix protein (Fig. 1, Lane A, 1 and 2). In order to ascertain the position of the label, the virus was digested with bromelain which solubilises the haemagglutinin glycoprotein leaving the C-terminal anchoring peptide of haemagglutinin$_2$ (M_r 5400) in the viral membrane [22,27]. Two peptides were released, one corresponding to haemagglutinin$_1$ and the other to the bromelain-cleaved haemagglutinin$_2$ peptide (Fig. 1, lane C, 1 and 3). 40% of the eosin label was cleaved from the virus particle as measured spectrophotometrically, 60% remaining associated with the viral par-

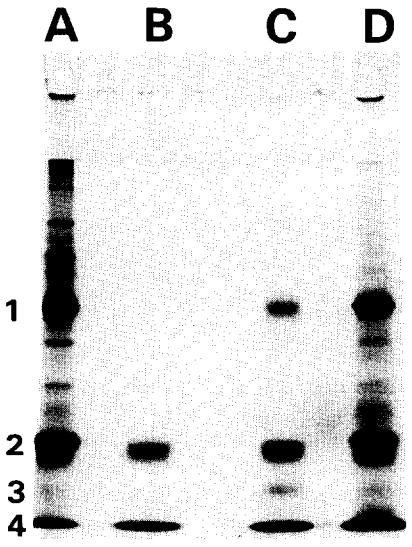

Fig. 1. SDS-polyacrylamide gel electrophoresis of eosin-labeled influenza virus before and after bromelain digestion. Lane (A), eosin-labeled influenza virus (1. haemagglutinin$_1$ and nucleoprotein; 2. haemagglutinin$_2$ and matrix protein); lane (B), Bromelain after incubation for 14 h at 35°C (2. bromelain); lane (C), 100000 × g supernatant of eosin-labeled virus digested for 14 h by bromelain at 35°C (1. haemagglutinin$_1$; 2. bromelain; 3. bromelain cleaved haemagglutinin$_2$); lane (D), 100000 × g pellet after bromelain digestion (1. nucleoprotein; 2. matrix protein; 4. low M_r fragments associated with virus particle).

ticles. When the pellet remaining after bromelain digestion was run on the SDS-polyacrylamide gels, no fluorescence was observed in the position of the nucleoprotein and matrix proteins. Fluorescence was visible only at the solvent front and would thus be associated with the C-terminal 'anchor' peptide of haemagglutinin$_2$.

No more than 6% of the eosin could be extracted from the labeled virus by chloroform/methanol indicating that no significant amount of nonconjugated or lipid conjugated eosin was present.

For transient dichroism measurements the molar labeling ratio of eosin to glycoprotein was in the range 0.6–1.2. Eosin labeling had no significant effect on the haemmaglutination activity of the virus, the activity remaining at $2–5 \cdot 10^4$ haemagglutinating units/mg. Haemolytic activity, however, increased by up to 3-fold over the unlabeled control and the untreated virus. The labeling procedure could have resulted in a more 'leaky' viral membrane, thereby increasing haemolytic activity just as sonicated virus is more haemolytically active [1].

pH dependence of haemolytic activity and rotatational mobility

The haemolytic activity of influenza virus, X-47 (Fig. 2) shows the marked pH dependence characteristic of other strains of influenza virus (see, for example, Ref. 2). Maximal activity was observed at pH 5.2, falling to negligible haemolysis for pH values 6.0 to 7.5.

Fig. 3 shows the time-dependent absorption anisotropy $r(t)$ for eosin-labeled influenza virus at 36°C. At pH 7.3, $r(t)$ decayed to a constant level within about 0.2 ms. The time constant, T_1, obtained by fitting the data by Eqn. 2 was found to be about 30 μs.

At pH 5.2, the decay of the absorption anisotropy was no longer observed. In fact, $r(t)$ exhibited a slight upward drift with time (Fig. 3). This effect has been observed previously and can be explained by multiple binding sites for the eosin probe [28]. Alternatively, it could be due to a small baseline drift which will be most evident when anisotropies are low, as in the present case. In any event, the rotational mobility responsible for the decay of $r(t)$ at pH 7.3 has clearly been abolished at pH 5.2.

Effect of temperature on the rotational motion of influenza glycoproteins and on viral activity

The time dependence of the absorption ani-

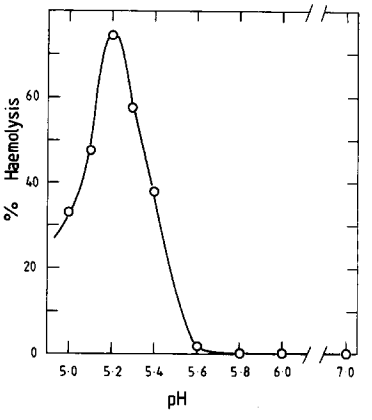

Fig. 2. pH dependence of the haemolytic activity of influenza virus, strain X-47, at 37°C, measured as described under Materials and Methods.

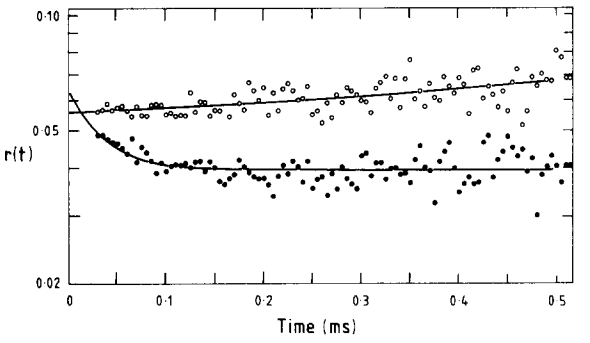

Fig. 3. The time-dependent absorption anisotropy, $r(t)$, at 35°C of eosin-labeled influenza virus at pH 7.3 (●) and at pH 5.23 (○). Molar labeling ratio eosin/glycoprotein was 0.6. Data were fitted using Eqn. 3.

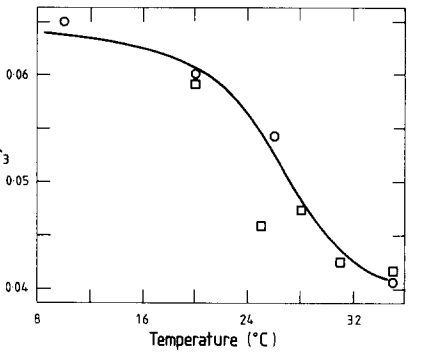

Fig. 4. The time-dependent absorption anisotropy, $r(t)$, of eosin-labeled influenza virus measured at pH 7.3 for the following temperatures (a) 10°C (b) 20°C (c) 28°C (d) 35°C. The molar labeling ratio eosin/glycoprotein was 1.0. Data were fitted using Eqn. 3. For clarity the data points are shown only for (c).

sotropy shows a strong temperature dependence (Fig. 4). At 20°C the curve is relatively flat indicating little rotational motion of the glycoprotein over the measured time-range. When the temperature was raised above 25°C, the glycoprotein became more mobile as shown by the decreasing values of the anisotropies. Analysis of the decays indicated that there is a decrease in T_1 and an increase in r_1 with increasing temperature consistent with increasing mobility with temperature in the microsecond time range. Similarly, the decrease in the initial values of anisotropy indicates an increase in the contributions from motions too fast to be detected in the present experiments. However, the data are such that it would be unwise to attempt any detailed interpretation of the mobility changes. For present purposes, the most reliable parameter is r_3 which is a measure of the degree of rotational randomization occurring within a time of 0.5 ms. The values of r_3 were plotted as a function of temperature (Fig. 5). These results clearly show that there is a marked increase in overall mobility of haemagglutinin with temperature. The maximum rate of change occurs between 20°C and 30°C.

The temperature dependence of viral activity at pH 5.2 is shown in Fig. 6. The activity is negligible below 20°C, but rises sharply between 25°C and 35°C.

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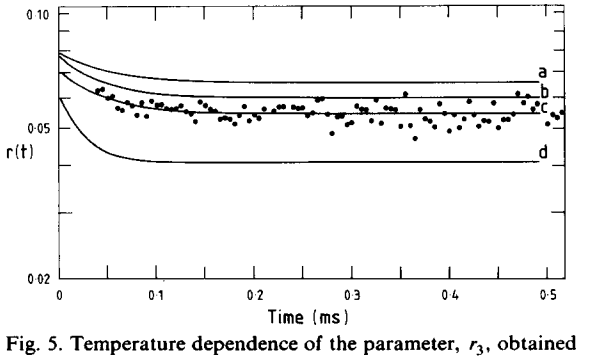

Fig. 5. Temperature dependence of the parameter, r_3, obtained by fitting the anisotropy decay curves using Eqn. 3. The symbols (○) and (□) refer to two separate samples measured on different days. One set of values has been adjusted to take account of different absolute values of anisotropy probably due to variation in the laser intensity [26].

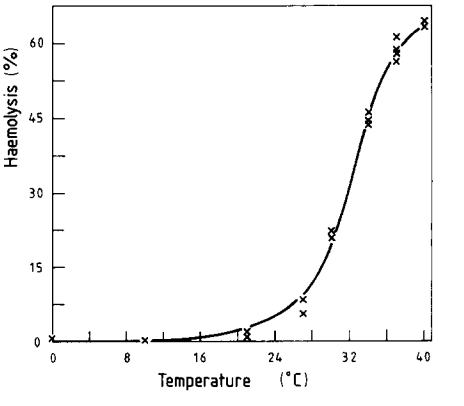

Fig. 6. Temperature profile for the haemolytic activity of influenza virus, X-47, at pH 5.2, measured as described under Materials and Methods.

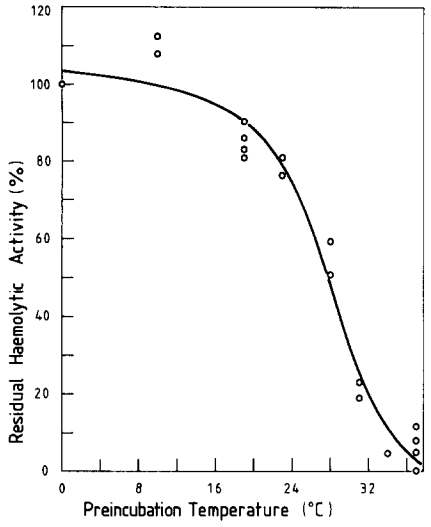

Fig. 7. The temperature dependence of the stability of influenza virus, X-47, to preincubation at pH 5.2 for 15 min. The haemolytic activity remaining after 15 min was expressed as a percentage of that of the virus kept at pH 7.3.

virus to maintain its haemolytic activity when incubated at pH 5.2 are shown in Fig. 7. At temperature less than 20°C, influenza virus is not inactivated by a 15 min exposure to pH 5.2. However, incubation under the same conditions but above 30°C leads to almost total inactivation.

Discussion

Labeling of influenza virus with eosin-SCN

An advantage of working with influenza virus is that haemagglutinin is the predominant glycoprotein exposed at the viral surface [20,21]. The other major surface glycoprotein, neuraminidase, accounts for no more than 10% of the total glycoprotein [20]. It thus would appear likely that the non-specific probe, eosin-SCN, would predominantly label haemagglutinin. This prediction is borne out by SDS-polyacrylamide gels which show no detectable fluorescence in the neuraminidase region. The major eosin fluorescent bands occur in the region of haemagglutinin$_1$ and haemagglutinin$_2$. Upon bromelain treatment, 40% of the eosin label was released together with the hydrophilic moiety of the haemagglutinin glycoprotein, while the remaining membrane label appeared to be associated with low M_r peptides. Thus it is probable that eosin in part labels one or more of the three lysine residues which remain on the external surface of the membrane anchoring peptide of haemagglutinin from influenza X-47 after bromelain digestion [29]. After bromelain treatment, eosin fluorescence was not detected in the region of the matrix protein, which runs close to the haemagglutinin$_2$ band in gels of the untreated virus, indicating that the eosin probe does not penetrate the viral envelope membrane. Little label was free or bound to lipid as judged by chloroform-methanol extraction. Overall we conclude that the eosin label is mostly attached to haemagglutinin$_2$ and that a significant fraction binds close to the hydrophobic 'anchor' of the glycoprotein. We therefore discuss the rotational diffusion experiments in terms of the mobility of the haemagglutinin glycoprotein.

Rotational mobility of haemagglutinin

The influenza virus particle has a diameter of approx. 100 nm [20]. The rotation of the whole particle in 50% glycerol is negligible over the time range of the present experiments. Thus the measured anisotropies can be related to the rotational mobility of haemagglutinin molecules within the virus.

At 36°C and pH 7.3, the anisotropy exhibits a weak decay, with a decay time, T_1, of about 30 μs (Fig. 3). Rotational relaxation times of membrane proteins are inversely proportional to the square of the diameter of the membrane spanning segment [30]. Bacteriorhodopsin, for example, has seven membrane-spanning helices [31] and a rotational relaxation time of about 15 μs in fluid lipid bilayers [25]. The membrane-spanning segment of haemagglutinin probably consists of a single α-helix although, on the basis of the bromelain-cleaved haemagglutinin crystal structure, it is generally assumed that haemagglutinin trimers are present in the viral membrane. The rotation of the haemagglutinin trimer should thus be faster than bacteriorhodopsin and that of the monomer, much faster. The value of T_1 obtained for haemagglutinin is only an approximate relaxation time due to the nature of the data and the use of the simplified Eqn. 3 for curve fitting. In view of the uncertainties involved, it is not out of the question that the 30 μs decay corresponds to haemagglutinin tri-

mers. It is, however, very unlikely to correspond to haemagglutinin monomers.

A noteworthy feature of the results at 36°C is the low absolute values of the anisotropy. The initial anisotropy (r_0) is about 0.06, compared for example with r_0 of about 0.15 for eosin-labeled band 3 [32]. This low value of initial anisotropy is likely to be due to segmental motion [15,33]. In addition, as discussed above, the rotation of haemagglutinin monomers and possibly haemagglutinin trimers could be sufficiently fast to cause significant loss of anisotropy in a time shorter than the time resolution of the present experiments.

Temperature dependence of rotational mobility

Influenza virus haemagglutinin shows a marked increase in rotational mobility with increasing temperature, with the largest increase occurring between 20° and 30°C. It is unlikely that the effect is determined by membrane viscosity, since the viral membrane lipids only exhibit a small and gradual change in fluidity over this temperature range [15]. The temperature dependence of haemagglutinin mobility is qualitatively similar to that previously observed with Sendai virus glycoproteins [15], although the major increase in mobility occurs at a somewhat lower temperature for the influenza virus.

Although it is difficult to make a precise interpretation of the mobility changes from the present experiments, it is reasonable to suppose that we are observing a temperature-dependent association–dissociation equilibrium. This has been shown to occur with several other membrane proteins [25,34,35]. It is not clear whether this would involve dissociation of haemagglutinin trimers into monomers, although it would be of considerable functional interest if this were so. Dissociation at higher temperature of either trimers or larger aggregates could increase both the whole body rotation and the segmental flexibility of haemagglutinin.

pH dependence of haemolytic activity and rotational mobility

The sharp rise in haemolytic activity between pH 5.6 and pH 5.2 (Fig. 2) agrees with the hypothesis that the protonation of a carboxyl group(s) presumably with a high pK_a, is essential for viral activity [3]. Possible sites of protonation could be (i) the aspartic residue which forms a salt link with the NH_2-terminal glycine of the haemagglutinin$_2$ peptide, thereby holding it in the interior of the bromelain-cleaved haemagglutinin trimer, or (ii) other polar groups present nearby which stabilise the lower section of the triple stranded coiled-coil of the haemagglutinin trimer [14]. Breakage of several salt-bridges in the interior of the trimer could allow the conformational change which exposes nonpolar regions of the haemagglutinin molecule (i.e., the N-terminus of the haemagglutinin$_2$ polypeptide and the hydrophobic residues which interact at the distal end of the coiled-coil).

The pH conformational change of haemagglutinin is also detected by the rotational mobility experiments, which show that haemagglutinin is far less mobile at pH 5.2 than at pH 7.3 (Fig. 3). This could be explained by interaction of the exposed hydrophobic regions. As previously suggested the function of such regions may be to initiate fusion by interaction with an apposing membrane. In the absence of such a membrane, it would be thermodynamically favourable for them to interact with one another, with a subsequent aggregation of the haemagglutinin molecules. 'Clumping' as a result of acid treatment has been observed by electron microscopy for isolated haemagglutinin [6] and for bromelain-cleaved haemagglutinin [5]. Skehel and coworkers have also proposed that the hydrophobic N-terminal sequence of haemagglutinin$_2$ is responsible for the aggregation [37].

In contrast to the temperature effects, there is little change in the initial anisotropy on lowering the pH. This indicates that faster motions, such as segmental motion, are little affected by the aggregation.

Significance of mobility for viral activity

In a previous study the mobility of the glycoproteins of Sendai virus as a function of temperature showed a good correlation with haemolytic activity [15]. It was postulated that mobility is important for the extensive molecular rearrangements which must occur during fusion. In the present study, the mobility of haemagglutinin showed a similar temperature dependence to that

found for Sendai glycoproteins. Moreover, like Sendai virus, the haemolytic activity of influenza virus X-47 is strongly temperature dependent. No activity is observed below 20°C, with a rapid rise occurring above 25°C (Fig. 6).

The situation, however, is not so simple in the case of influenza virus. The fusion activity is only detected at pH 5.2, whereas our data on the increase in mobility with temperature were obtained at pH 7.3. Indeed, we find that at pH 5.2 mobility is decreased even at high temperature. An explanation of this result is indicated by the results of low pH inactivation of the influenza virus shown in Fig. 7. At higher temperatures, the virus is inactivated at pH 5.2 in a time less than it takes to set up the sample for mobility measurements. Our mobility data at low pH and high temperature are therefore obtained with the virus in an inactivated state.

In view of the rapid inactivation at pH 5.2, it is necessary to make an additional assumption in order to relate mobility of influenza virus to function. This assumption is that a similar temperature dependence of mobility to that measured at pH 7.3 is also present at pH 5.2 immediately after lowering the pH. This could occur if the conformation of the 'active' virus at pH 5.2 was similar to that of the pH 7.3 structure. Evidence which supports this view comes from studies with monoclonal antibodies by Webster et al. [8]. Monoclonal antibodies raised against the pH 7 conformation inhibited the viral haemolytic activity and infectivity at pH 5 although haemagglutination still occurred. In contrast antibodies raised against acid-treated virus were poor inhibitors of haemolysis. It is also apparent that although both mobility and activity are strongly temperature dependent, the most rapid change in activity occurs at a slightly higher temperature than that of mobility. This does not necessarily count against the hypothesis that mobility is important for activity, since the relationship between the two is likely to be complex.

Sato et al. [6] previously showed that the pH dependence of viral inactivation is similar to that of the haemolytic activity. This led them to propose that both effects are a consequence of the same conformational change. In the present study, we show that the pH inactivation of the virus is also temperature dependent. The pH inactivation displays essentially the same temperature profile as the mobility changes in haemagglutinin at pH 7.3. This result, together with those of Sato et al. [6] suggests that temperature-induced mobility of haemagglutinin is required for the pH-dependent inactivation of the virus.

In conclusion, the results presented here together with the known properties of the virus lead us to postulate that mobility of haemagglutinin is important in viral activity according to the following scheme.

(1) The increased haemolytic activity of influenza virus with increasing temperature is a consequence of increasing mobility of the viral glycoproteins. It is assumed that a similar temperature dependence of mobility to that measured at pH 7.3 is also present at pH 5.2 immediately after lowering the pH.

(2) Acidic pH induces a conformational change in haemagglutinin which exposes hydrophobic regions suitable for interaction with an apposing membrane.

(3) In the absence of an apposing membrane these hydrophobic regions cause aggregation of haemagglutinin with irreversible loss of mobility and of activity.

(4) The aggregation phenomenon also requires mobility of haemagglutinin and is therefore, like the haemolytic activity, strongly temperature dependent.

The results obtained in the present study are consistent with the above postulates. Further work is clearly required to firmly establish their validity.

Acknowledgement

We thank Professor C.A. Pasternak and Kalperna Patel for helpful discussions and for the generous gift of purified influenza virus; also Ian Morrison for help with the transient dichroism measurements and for the curve fitting. This work was supported by grants from the Wellcome Trust and the SERC.

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