Eur Biophys J (1994) 23:105–113

European Biophysics Journal

pH-Dependent binding of the fluorophore bis-ANS to influenza virus reflects the conformational change of hemagglutinin

Thomas Korte, Andreas Herrmann

Institut für Biophysik, Fachbereich Biologie, Humboldt-Universität zu Berlin, Invalidenstrasse 43, D-10115 Berlin, Germany

Received: 16 August 1993 / Accepted in revised form: 6 January 1994

Abstract. Binding of the fluorophore 1,1′-bis(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS) to influenza virus A/PR 8/34 is strongly enhanced at low pH. Binding is accompanied by a significant increase in fluorescence intensity. The binding and the fluorescence increase are associated with the low-pH induced conformational change of the viral spike protein, hemagglutinin, exposing hydrophobic binding sites. The data indicate that in addition to the hydrophobic N-terminus of HA2 other hydrophobic sequences of the HA ectodomain become accessible to bis-ANS at low pH. It is shown that the time course of the fluorescence increase of bis-ANS at low pH is determined by the conformational change of HA. The application of this assay for continuously monitoring the kinetics of the structural alteration in HA is discussed and its relevance for elucidating the temporal relationship between the conformational change of HA and virus-membrane fusion is outlined.

Key words: Influenza virus – Hemagglutinin – Conformation – Fusion – Fluorescence – Dequenching

Introduction

Influenza virus enters host cells via receptor-mediated endocytosis. Both attachment of the viral envelope to cell surface receptors and fusion with the endosomal membrane subsequent to acidification of the endosomal lumen are mediated by the influenza virus spike protein, the hemagglutinin (HA) (Gething and Sambrook 1981; White et al. 1983)).

Influenza hemagglutinin is an integral membrane protein which consists of two disulfide-linked subunits, HA1 and HA2 (Klenk et al. 1975). It is anchored to the viral membrane only by the HA2 subunit. The molecular structure of the bromelain-solubilized ectodomain of HA (BHA) has been resolved by X-ray crystallography with a resolution of 3 Å (Wilson et al. 1981). The HA is arranged as a homotrimer extending 135 Å from the viral membrane. The sialic acid binding pocket is localized on HA1 in the top region of the globular head of the trimer (Weis et al. 1988).

Fusion between influenza virus and the target membrane, which is triggered at low pH, has been associated with an irreversible conformational change in HA leading to the exposure of a hydrophobic segment of the HA2 subunit and promotion of the fusion reaction (Skehel et al. 1982; Doms et al. 1985; White and Wilson 1987). However, while many details of the molecular rearrangement of HA at low pH have been described, the fusogenic structure of HA and the molecular mechanism of subsequent membrane fusion remain obscure.

Over recent years, many studies on the kinetics of lipid mixing as a result of fusion of enveloped viruses with target membranes have been undertaken in order to elucidate the structure of the fusion site and the kinetics of the fusion process. Fluorescence assays have become a valuable tool for continuously monitoring membrane fusion e.g. octadecylrhodamine (R18) fluorescence dequenching (Hoekstra et al. 1984). The plasma membrane of human erythrocytes, as well as liposomes bearing sialic acid receptors, have been widely used as model target membrane to investigate fusion between influenza virus and biological membranes. The fusion process is regulated by the pH of the suspension medium (Stegmann et al. 1985; Clague et al. 1991). From those studies it has been concluded that the HA-mediated fusion at low pH is a complex multistep process which comprises a conformational change of the HA, the association of several HA-trimers to form a fusion site and destabilization of the target membrane (Bentz et al. 1990, Bentz 1992; Stegmann et al. 1990; Blumenthal et al. 1991). Competing inactivation processes lead to a loss of those fusion intermediates and, hence, to an impairment of viral fusion activity (Steg-

Abbreviations: HA, hemagglutinin; BHA, bromelain-solubilized ectodomain of HA; N-HA2, N-terminus of the HA2 subunit; PBS, phosphate buffered saline; bis-ANS, (1,1′-bis(4-anilino)naphthalene-5,5′-disulfonic acid); R18, octadecylrhodamine B chloride; FDQ, fluorescence dequenching; RBC, red blood cell

Correspondence to: A. Herrmann

mann et al. 1986; Nir et al. 1990). However, to deduce from lipid mixing experiments the precise nature of those intermediates and the time-dependence of their appearance remains a difficult task. Recently, Bentz (1992) has concluded from theoretical considerations of virus fusion kinetics that the intermediates of protein mediated fusion can be studied only by using assays sensitive to the formation of each proposed intermediate. For instance, several assays have been developed to assess the conformational change, e.g. antibodies (White and Wilson 1987), proteinase K sensitivity (Doms et al. 1985), and binding to liposomes (Stegmann et al. 1987, 1990). However, these assays are not able to continuously monitor the rapid kinetics of the conformational change.

In the present study we have employed a fluorescence assay which allows us to follow the low pH-induced conformational change of HA continuously. We have used the water soluble fluorophore 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS) which is sensitive to the polarity of its environment. It has been shown that bis-ANS is virtually non-fluorescent in aqueous solutions, but becomes strongly fluorescent in apolar solvents or when it is bound to hydrophobic sites in proteins (Rosen and Weber 1969). Very recently a similar fluorescence probe (ANS) has been used to characterize an insulin analogue before and after is thermal unfolding (Hua et al. 1993). In the presence of influenza virus we observed a dramatic increase of bis-ANS fluorescence intensity at low pH, which is mainly attributed to an enhanced binding of the fluorophore to hydrophobic sites of the HA ectodomain. The extent of bis-ANS fluorescence at low pH parallels the pH-dependence of fusion between influenza virus and red blood cell membranes.

Furthermore, we could show that the time-dependent increase of the bis-ANS fluorescence upon lowering the pH is determined by the structural change in HA. Thus, the assay may allow one to elucidate the relation between the kinetics of the conformational change and the onset of virus-membrane fusion under various conditions.

On the basis of our results we want to suggest that this fluorophore may be useful to follow the kinetics of structural alterations of other proteins.

Material and methods

Materials

Octadecylrhodamine B chloride (R18) and bis-ANS were purchased from Molecular Probes (Junction City, OR) Bromelain was from Calbiochem Corporation, melittin from Sigma and 2-mercaptoethanol from FERAK Berlin. Fresh blood from healthy donors was obtained from the Blood Bank, Berlin-Lichtenberg, and was used within 3 days of sampling (ACD storage medium). Purified influenza virus A/PR 8/34 (Hils et al. 1986) was kindly provided by Dr. A. Lesnau from the Zentralinstitut für Hygiene, Mikrobiologie und Epidemiologie (Berlin). The 20 amino acid peptide N-HA2 (N-HA2) of A/PR 8/34 was synthesized by Dr. Henklein (Charite, Humboldt-University, Berlin).

Preparation of egg-PC- and PS-liposomes. Egg-PC (Sigma) and PS (Sigma) were dissolved in chloroform, the solvent was evaporated with N_2 and PBS (pH 7.4) was added to give a concentration of 2 mg lipid/ml. Liposomes were obtained by sonification with a Branson sonifer (3–4 min, 50 W).

Red blood cell and ghost preparation. After removal of buffy coat and plasma, red blood cells (RBC's) were washed three times in phosphate-buffered saline (PBS, pH 7.4). Erythrocyte ghosts were prepared according to Dodge et al. (1963).

Preparation of bromelain treated viruses. Removal of the HA-ectodomain was performed by bromelain-digestion of A/PR 8/34 according to Brand and Skehel (1972) with minor modifications described by Harter et al. (1989). The remaining viral particles were sedimented by centrifugation (100 000 × *g*, 60 min, 4 °C, UP 65-MLW Leipzig), washed, resuspended and stored in PBS, pH 7.4.

Proteinase K digestion of virus. Virus A/PR 8/34 was incubated at different pH values for 15 min (37 °C) or at pH 5.0 for different times, respectively, neutralized, digested with proteinase K (Boehringer Mannheim) (30 min, 37 °C), precipitated with tricloroacetic acid and the HA bands were quantitated after SDS-PAGE (Doms et al. 1985).

Labeling of virus with R18. 1.25 µl of a 2 mM stock solution of R18 in ethanol was added with rapid vortexing to 0.25 ml of A/PR 8/34 (1 mg virus protein/ml). After incubation for 30 min at room temperature (in the dark) virus was washed by high speed centrifugation with ice-cold PBS to remove unbound R18, and resuspended to a concentration of 1 mg virus protein/ml (Herrmann et al. 1993 a, b).

Binding of bis-ANS to influenza virus. 20 µl of the virus suspension were added to 2 ml of buffer at pre-set temperature and pH (37 °C, pH 4.5–7.4) containing 3.25 µM of bis-ANS. In some experiments bis-ANS was added after virus resuspension. Increase of bis-ANS fluorescence in the presence of influenza virus was measured at $\lambda_{em} = 490$ nm ($\lambda_{ex} = 400$ nm) using a SHIMADZU RF-5001 PC spectrofluorometer equipped with a thermostated sample chamber. The suspension was stirred continuously with a 2 × 8 mm teflon-coated magnetic stir bar. (Similar experiments were performed in the presence of melittin, egg-PC liposomes, bromelain treated viruses and in the presence of the peptide corresponding to N-HA2, for concentrations and conditions see Results and legends to the graphs.) The amount of bis-ANS noncovalently bound to virus was measured by a centrifugation assay. To easily quantify the virus concentration, we have also used R18-labeled influenza virus. In independent experiments we have verified that the binding of bis-ANS is not affected by R18 and that the fluorescence properties of both fluorophores are not changed. 60 µl of R18-labeled virus were added to 6 ml of prewarmed buffer (37 °C, pH 4.7–7.4) containing 3.25 µM bis-ANS and

incubated with gently vortexing. After 8 min the virus suspension was centrifuged at 100 000 × *g* (60 min, 4 °C, UP 65-MLW Leipzig). The amount of fluorophore bound to virus was obtained by spectroscopic determination of the remaining bis-ANS concentration in the supernatant in the presence of Triton X-100.

Virus binding to ghost membranes. Viruses were prebound to ghost membranes by incubation of 100 µl R18-labeled A/PR 8/34 and 200 µl ghosts (protein concentration of 6 mg/ml) for 30 min on ice with gently vortexing. Protein concentration was determined using the lowry method.

Fusion analysis. Membrane fusion was measured with the octadecylrhodamine (R18) fluorescence dequenching assay (Hoekstra et al. 1984). Fusion was triggered by transferring 30 µl of ice-cold virus-ghost suspension to a quartz cuvette containing 1.8 ml of prewarmed (37 °C) buffered saline at the appropriate pH (pH 4.5–7.4). The suspension was stirred continuously with a 2 × 8 mm teflon-coated magnetic stir bar. Fusion was monitored continuously by measuring fluorescence dequenching for 8 min (λ_{ex} = 560 nm, λ_{em} = 590 nm, cut-off filter 570 nm), after which Triton X-100 (0.5% final concentration) was added to obtain maximum R18 fluorescence, F (max). The percentage of fluorescence dequenching FDQ was calculated as described previously (Blumenthal et al. 1987):

$$\%FDQ = 100 \times [F(t) - F(0)]/[F(max) - F(0)] \quad (1)$$

with F (0) and F (t) corresponding to the fluorescence intensity of the virus before starting fusion and the fluorescence intensity at a given time t, respectively. Values of FDQ presented in Results refer to the FDQ at 8 min.

Results

pH-dependent binding of bis-ANS to influenza virus

bis-ANS is virtually non-fluorescent in aqueous solutions in the pH-range investigated (not shown). However, upon addition of influenza virus A/PR 8/34 a rapid increase of the fluorescence of bis-ANS was observed at pH 7.4, 37 °C (Fig. 1) due to binding of the fluorophore to the virus (Fig. 2, inset, and below). While the fluorescence intensity and binding were only slightly enhanced by reducing the pH to 6.0, we measured a dramatic increase of both parameters at lower pH (< pH 6.0) (Figs. 1, 2). Maximal fluorescence extent was achieved in the pH range 5.0 to 4.7, while a further decrease of the pH resulted in a decrease of the fluorescence change (Fig. 2). Fluorescence intensity measured at pH 5.0 was about 5 times higher than the value at pH 7.4 (control). Fluorescence extent almost reaches a plateau within 3 min after addition of virus[1].

[1] Similar results were obtained with a different influenza A strain of type H1N1, A/Brazil 11/78 (data not shown)

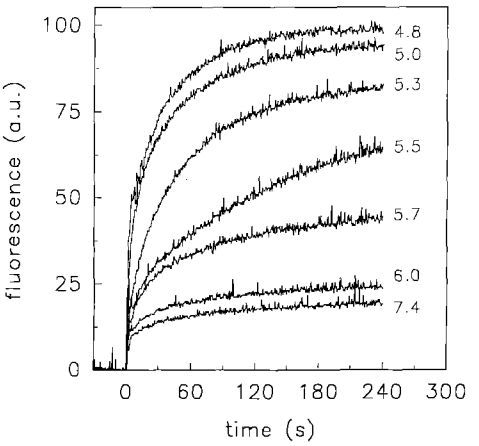

Fig. 1. Kinetics of the increase of bis-ANS fluorescence in the presence of influenza virus A/PR 8/34 at different pH (indicated), 37 °C. At time $t = 0$ 20 µl of virus stock suspension (1 mg virus protein/ml) were injected into a cuvette of 2 ml prewarmed buffer (145 mM NaCl; 20 mM *natrium acide*) containing 3.25 µM bis-ANS. Fluorescence (λ_{ex} = 400 nm, λ_{em} = 490 nm) was measured with a time resolution of 1 s.

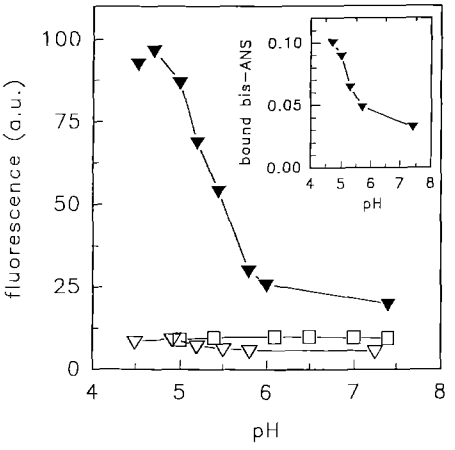

Fig. 2. pH-dependent fluorescence intensity of bis-ANS in the presence of intact influenza virus A/PR 8/34 (10 µg/ml) (▼), bromelain treated influenza virus (10 µm/ml) (▽) and egg-PC liposomes (3.3 µg lipid/ml) (□), respectively, at 37 °C. Fluorescence was measured 6 min after addition of virus or liposomes, respectively, to prewarmed buffer containing 3.25 µm bis-ANS. Inset: Binding of bis-ANS to influenza virus (in [nmol/µg virus protein]) as a function of pH (3.25 µM bis-ANS; 10 µg virus protein/ml). For details see Materials and methods

The pH-dependence of bis-ANS fluorescence in the presence of influenza virus was eliminated upon removal of the HA ectodomain of influenza virus by digestion with bromelain (Fig. 2) In this case, we observed over the entire pH range investigated a fluorescence intensity which was of the same order as that measured for the control at pH 7.4. No increase in bis-ANS fluorescence was seen at acidic pH. The fluorescence increase upon binding of bis-ANS to liposomes is similar to that of bromelain-treated influenza virus and is pH independent (Fig. 2) The concentration of egg-PC was chosen to match the concentration of virus lipids by assuming that virus protein corresponds to about 75% of total weight (Compans and Choppin 1975). It is important to note

that the fluorescence increase upon binding of bis-ANS to liposomes containing PS was also pH independent, but significantly lower than that found for egg-PC (not shown). This suggests, that (i) binding of bis-ANS to lipid sites is independent of pH, (ii) the low fluorescence at pH 7.4 in the presence of intact virus can be assigned to a significant extent to binding to lipid sites, and (iii) the increase of bis-ANS fluorescence at low pH is mainly attributable to binding of the fluorophore to HA.

The increase of bis-ANS fluorescence at low pH in the presence of influenza virus is accompanied by a blue shift of the wavelength of maximal fluorescence. λ_{max} is shifted from 505 nm in aqueous solution to about 484 nm upon addition of influenza virus. Binding of bis-ANS to egg-PC-liposomes also results in a blue shift of maximal fluorescence, but to a smaller extent ($\lambda_{max} = 492$ nm).

Low pH-induced fusion of influenza virus with erythrocyte membranes

In Fig. 3 we have compared the pH-dependence of fusion between influenza virus and erythrocyte membranes with that of bis-ANS fluorescence in the presence of intact virus. Fusion was assessed by the dequenching assay using the fluorophore R18 incorporated into the virus membrane at a self-quenching concentration. The pH-dependence of fusion expressed as %fluorescence dequenching (FDQ) is in agreement with previous reports (Stegmann et al. 1986; Herrmann et al. 1993). It is obvious that the increase of bis-ANS fluorescence at low pH occurs in the same pH range where extensive fusion is observed (< pH 6.0).

However, the dependence of the bis-ANS fluorescence in the pH range between 6.0 and 5.0 is different from that of fusion; the increase of bis-ANS fluorescence is almost linear, while the fusion extent exhibits a steep slope between pH 6.0 and 5.5, but changes only moderately between 5.5 and 5.0. Maximal extent of both fusion and bis-ANS fluorescence were observed between pH 5.0 and 4.7.

Enhanced binding of bis-ANS at low pH is related to the conformational change of HA

We have investigated whether the enhanced affinity of bis-ANS for HA at low pH reflects the conformational change of the HA and associated exposure of hydrophobic binding sites. It is already known that this conformational transformation of HA of A/PR 8/34 is irreversible after a short exposure to low pH, 37 °C (Brunner et al. 1991). Therefore, if the strong increase of bis-ANS binding and fluorescence at low pH is caused by the irreversible alteration of the HA structure, one may expect that the fluorescence intensity is – at least partially – retained after switching from low pH to pH 7.4. Indeed, comparing the fluorescence at pH 7.4 before and after exposure of intact virus to low pH, 37°C, we found that the fluorescence extent could only be partially reversed upon changing the pH from 5.0 to 7.4.

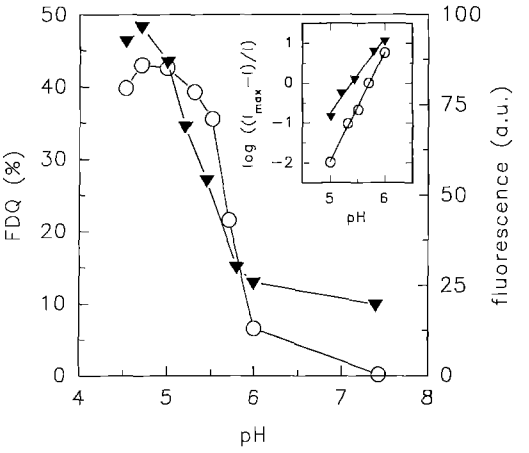

Fig. 3. Comparison of the pH dependence of the fluorescence intensity of bis-ANS bound to influenza virus (▲) with that of the low pH-induced fusion of influenza virus with erythrocyte ghost membranes (o). Fusion was measured using the octadecylrhodamine (R18) fluorescence dequenching assay. R18 labeled influenza virus was prebound to erythrocyte ghost membranes at 0 °C, pH 7.4. Fusion was triggered by injecting R18-labeled virus-erythrocyte complexes into a fluorescence cuvette containing 2 ml of prewarmed buffer of the desired pH. %FDQ was calculated according to Eq. (1). For details see Materials and methods. Kinetics of FDQ were monitored with a time-resolution of 1 s. Fluorescence of bis-ANS was measured as described in legend of Fig. 1. Inset: Hill plot of the data in Fig. 3. I and I_{max} are the extent at a given pH and the maximum of FDQ (or bis-ANS fluorescence), respectively. Maximal values correspond to a pH of 4.7. Linear regression yields a Hill coefficient of 2.8 (FDQ) and 1.7 (bis-ANS fluorescence) (see Discussion)

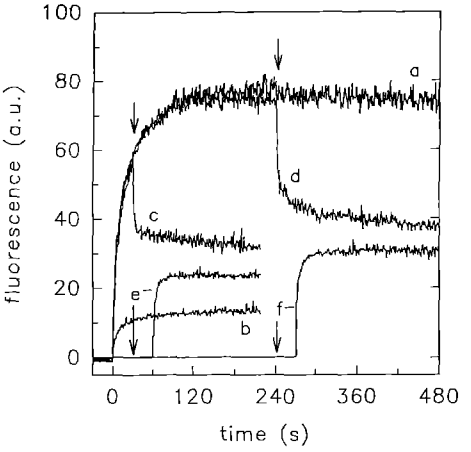

Fig. 4. Reversibility of bis-ANS binding to influenza virus. Kinetics of fluorescence increase upon binding of bis-ANS to influenza virus is measured at 37 °C. At time $t = 0$ virus was added to 2 ml of prewarmed buffer in the presence (a–d) or abscence of bis-ANS (e, f) at pH 5.0 (a, c–f) or pH 7.4 (b, control). pH was readjusted to pH 7.4 by injecting appropriate amounts of 1 M NaOH into the cuvette at different times (arrows, c, e – 30 s, and d, f – 240 s). bis-ANS was added 30 s after shifting the pH from 5.0 to 7.4 (e, f)

The extent of reversibility declined with the duration of low pH exposure. Longer incubation at low pH, 37 °C, leads to an enhanced fluorescence at neutral pH. This is based on an increase of both the amount of bis-ANS remaining associated at neutral pH and its quantum yield (data not shown). Similarly, when bis-ANS was added at

neutral pH to influenza virus preincubated at pH 5.0, 37 °C, the fluorescence extent was significantly enhanced with respect to the control (no preincubation) (Fig. 5). These results give evidence that enhanced binding and fluorescence of bis-ANS upon lowering the pH are due to hydrophobic binding sites of the ectodomain of HA which become exposed by the conformational change. They clearly show that the increase of fluorescence at low pH cannot be explained solely by protonation of negatively charged groups facilitating binding of bis-ANS although this mechanism might be partially involved (see below). Moreover, the data preclude the possibility that the enhanced fluorescence at low pH is provoked by an influx of the fluorophore binding to intraviral hydrophobic sites not accessible at neutral pH. Experiments with non-permeant quenchers such as KI and acrylamide support this conclusion (data not shown).

In order to give more evidence for the correlation of the conformational change of HA and the extent of the bis-ANS fluorescence by an independent method, we have probed the proteinase K susceptibility of HA of intact viruses after a preincubation of 15 min at different pH (Doms et al. 1985). We found that the main increase of the sensitivity of HA to proteinase occurs in the pH-range of 6.0 to 5.3 (Data not shown). In the same range we observed the pronounced enhancement of bis-ANS fluorescence.

Furthermore, the kinetics of the conformational change at pH 5.0 (37 °C) were also assessed by proteinase K. Most of the HA became proteinase-sensitive within the first minute of preincubation and conversion into the proteinase K sensitive form was almost completed after a preincubation of 3–5 min (data not shown). These results correlate with the kinetics detected by the bis-ANS fluorescence (see Fig. 1).

Characterization of bis-ANS binding to influenza virus

In Fig. 5 the amount of bis-ANS bound to influenza virus is shown as a function of the total bis-ANS concentration in the suspension. Binding was assessed by the centrifugation assay as described in Materials and methods.

As can be seen, binding of bis-ANS is considerably enhanced at low pH. Taking into account the fact that bromelain treatment of influenza virus abolished the pH-dependence of the bis-ANS fluorescence we ascribe the difference of bis-ANS binding between low (pH 5.0) and neutral pH (7.4) (open symbols in Fig. 5) to fluorophore bound to hydrophobic binding sites of HA exposed upon its conformational change. A Scatchard plot of the data may give an estimate of the number of binding sites. As can be seen an upward concave curve is obtained (Fig. 6).

Since we have no indication that this non-linear Scatchard-plot might be associated with negative cooperativity in binding of bis-ANS we have fitted these data by assuming two independent binding sites, n_1 and n_2, with dissociation constants k_1 and k_2, respectively. We obtained $n_1 = 0.023$ nmol, $k_1 = 0.087 \cdot 10^{-6}$ M, $n_2 = 0.0864$ nmol and $k_2 = 1.904 \cdot 10^{-6}$ M suggesting that about 0.1 nmol of binding sites for bis-ANS per 1 μg virus protein exist, which differ significantly in their affinity. This corresponds roughly to about 7–12 binding sites of bis-ANS per HA at low pH, assuming between 500 (Klenk 1991) and 1000 trimers/virion (White et al. 1983) and about $2.5 \cdot 10^{12}$ virus particle/mg virus protein. It is worth noting that a simple Hill plot gives a straight line with a Hill coefficient of about 1 on assuming 0.1 nmol binding sites per 1 μg virus protein (inset of Fig. 6). This may suggest that binding is a non-cooperative process.

Exposure of hydrophobic binding sites for bis-ANS at low pH is not only indicated by the pronounced blue shift, but also by the increase in quantum efficiency. The fluorescence intensity normalized to the amount of bound bis-ANS is taken as a parameter reflecting the

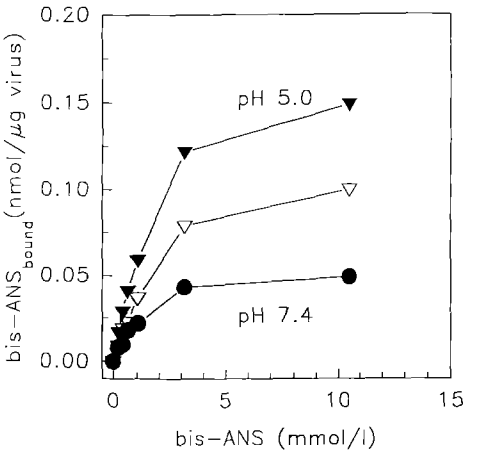

Fig. 5. Binding of bis-ANS to influenza virus (per 1 μg virus protein) at pH 7.4 (●) and pH 5.0 (▼), respectively, 37 °C (▽ – difference between bis-ANS bound at pH 5.0 and pH 7.4). Influenza virus (10 μg/ml) was incubated at the indicated pH for 8 min. After pelleting virus the amount of bis-ANS bound to virus was estimated from the fluorophore remaining in the supernatant. For details see Materials and methods

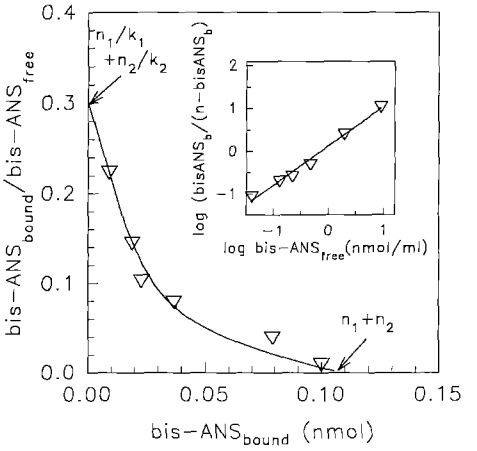

Fig. 6. Scatchard plot of binding of bis-ANS to influenza HA at pH 5.0. Points correspond to the difference between bound bis-ANS at pH 5.0 and pH 7.4 (see Fig. 5). Binding of the fluorophore is refered to 1 μg virus protein. Data were fitted by assuming two independent binding sites n_1 and n_2 with the dissociation constants k_1 and k_2, respectively: $n_1 = 0.0233$ nmol; $n_2 = 0.0864$ nmol; $k_1 = 0.087 \cdot 10^{-6}$ M; $k_2 = 1.905 \cdot 10^{-6}$ M. Inset: Hill plot of bis-ANS binding to influenza virus ($n = n_1 + n_2$)

Table 1. pH-dependence of fluorescence quantum efficiency as a function of pH. Maximum of fluorescence intensity (F_{max}) of bis-ANS bound to influenza virus membrane (10 μg virus protein/ml) and the amount of bound bis-ANS were measured at different pH, 37 °C (total concentration of bis-ANS 3.25 μM). The fluorescence intensity normalized to the amount of bound bis-ANS is taken as a measure of the quantum efficiency. Data represent mean values of three independent measurements, standard error of estimate was less then 5.8% of the average in each case

pH	$(bis)_{bound}$ [%]	$(bis)_{bound}$ [μM]	F_{max} [a.u.]	$F_{max}/(bis)_{bound}$
7.4	9.3	0.30	21.4	71
5.7	14.9	0.49	48.6	100
5.3	19.9	0.65	63.6	98
5.0	26.5	0.86	89.4	104
4.7	31.2	1.01	99.6	98

quantum efficiency. It can be seen that when the pH is lowered the normalized fluorescence is increased, suggesting a more hydrophobic environment for the bound fluorophore.

In order to characterize possible binding sites of the HA-ectodomain for bis-ANS we have investigated the pH-dependence of bis-ANS fluorescence in the presence of the 20 amino acid sequence N-HA2 of A/PR 8/34, the so-called fusion sequence. This terminus has been suggested to play a crucial role in triggering membrane fusion upon its exposure at low pH (White et al. 1983) mainly due to its pronounced hydrophobic properties (Lear and DeGrado 1987; White 1990). Indeed, on lowering the pH we observed an increase of fluorescence extent of bis-ANS in the presence of the fusion sequence (Fig. 7) which was reversible when shifting the pH to neutral.

However, rather high concentrations of the peptide were required to get a fluorescence increase which is of the order of that observed for intact influenza virus. Even at 4 μM of fusion peptide we established only a fourth of the fluorescence extent measured for influenza virus at 10 μg virus protein/ml, pH 5.0 (see above, Fig. 2). This virus concentration corresponds to about 10^{-1} μM of HA on taking the conversion factors given above. This suggests that the fusion sequence exposed after the conformational change of HA cannot alone account for the enhanced binding and fluorescence of bis-ANS at low pH in the presence of intact virus even if we suspect that the peptide may form self-aggregates in aqueous solutions (Takahashi 1990)[2]. Note, that the pH dependent bis-ANS fluorescence of the fusion peptide is not a general feature of membrane active peptides. In Fig. 7 we have included data obtained with the 26 amino acid peptide melittin, which is known to be membrane active (Dempsey 1990). Although bis-ANS fluorescence was enhanced upon addition of melittin, fluorescence was independent of pH in the range investigated. This implies that the pH dependence of bis-ANS fluorescence is dependent on the amino acid composition.

[2] For instance, assuming that (i) the effective concentration of available bindings site is reduced four-fold by tetramer formation and (ii) the quantum efficiency of peptide bound bis-ANS is similar to that of the fluorophore bound to HA, this sequence would account for less than one binding site per HA

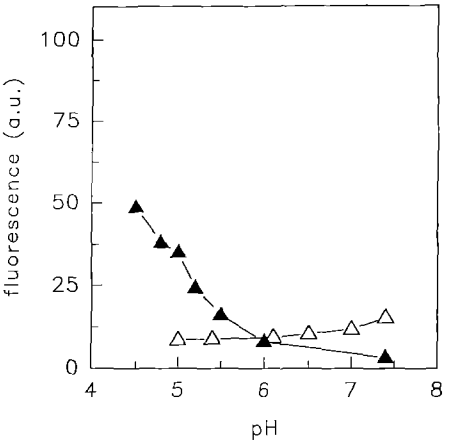

Fig. 7. pH-dependence of bis-ANS fluorescence in the presence of 4.6 μg/ml peptide corresponding to the 20 amino acid long N-terminus of HA2 of A/PR 8/34 (▲) and 4.6 μg/ml melittin (△)

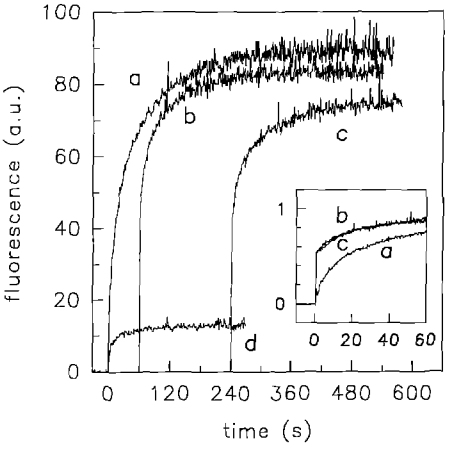

Fig. 8. Kinetics of fluorescence increase of bis-ANS bound to influenza virus after different times of preincubation of virus at pH 5.0, 37 °C. Fluorophore was added after (a) – 0 s; (b) – 60 s and (c) – 240 s of preincubation. (d) – control at pH 7.4. Inset: Comparison of the inital phase of bis-ANS fluorescence (curves from Fig. 8). Curves were normalized to the final extent of fluorescence intensity

As shown in Fig. 1, the fluorescence increase upon addition of influenza virus proceeds rapidly even at low pH. To follow the time course of the conformational change by this assay a main prerequisite is to exclude the possibility that the kinetics are determined solely by the rate of binding of bis-ANS to hydrophobic sites. To elucidate this point we have imployed two strategies. (1) We could show that the kinetics of the fluorescence increase of bis-ANS in the presence of virus at low pH were not affected by variation of the marker concentration between 0.5 μM and 5 μM (10 μg virus protein/ml) (data not shown). (2) We have investigated, at pH 5.0, the kinetics of the increase of bis-ANS fluorescence after different times of virus preincubation at pH 5.0, 37 °C (Fig. 8). The maximal extent of fluorescence was almost equal when the fluorophore was added between 0 and 90 s of preincubation at pH 5.0. However, the kinetics of fluorescence enhancement are

slower when the conformational change of HA was triggered in the presence of bis-ANS (no preincubation) compared to the time course of fluorescence when the fluorophore was added after ≥ 30 s of low pH preincubation of influenza virus (shown for 60 and 240 s of preincubation, Fig. 8). The kinetics of bis-ANS fluorescence after 60 s of preincubation of influenza virus (pH 5.0, 37 °C) will be determined mainly by the rate of binding but not by the conformational change which has already occurred. The latter can be concluded from the fusion kinetics at 37 °C, pH 5.0, which attains almost 80% of its plateau value within 1 min after triggering fusion (not shown, see Herrmann et al. 1993 a). Moreover, it has been suggested that the half time of the conformational change of HA is of the order of seconds (Stegmann et al. 1990). In summary, these results suggest that the conformational change of HA at low pH determines the time course of bis-ANS fluorescence.

Another conclusion which can be derived from these experiments is that the extent of HA-associated bis-ANS fluorescence does not depend on its presence during the conformational change. Thus, one may suspect that the fluorophore does not alter the conformational change of HA.

It is important to note that prolonged preincubation at pH 5.0 results in a lower final extent for the fluorescence intensity of the fluorophore. We suggest that the decrease of bis-ANS fluorescence upon prolonged preincubation of influenza virus at low pH might reflect aggregation of hydrophobic sites (sequences) of the HA ectodomain which makes them inaccessible to the fluorophore.

Discussion

The present investigation was designed to assess continuously the conformational change of HA by fluorescence spectroscopy using the fluorophore bis-ANS. Although several assays have been developed to detect the conformational change of HA, none of them allow one to follow continuously the structural alterations of HA at low pH. We have shown that binding of bis-ANS to influenza virus at low pH is mainly caused by hydrophobic binding sites of HA becoming exposed by the conformational change of the HA. This conclusion is based on several observations:

(a) pH-dependent binding and fluorescence extent of bis-ANS in the presence of influenza virus parallels the low-pH induced fusion of influenza virus with erythrocyte ghosts mediated by the conformational change of HA.
(b) Upon removal of the ectodomain of HA the increase of the fluorescence of bis-ANS at low pH is abolished. Influenza virus associated fluorescence intensity becomes pH-independent and similar to that found for liposomes of egg-PC.
(c) The strong increase of the fluorescence extent at low pH could only be partially reversed when shifting the pH from low to neutral. Even after only a short exposure of influenza to low pH, 37 °C, restoration to neutral pH did not reverse the fluorescence intensity to the low level observed when virus was exclusively incubated at pH 7.4. This irreversible change of bis-ANS fluorescence coincides with the irreversibility of the low pH-triggered conformational change of HA (Brunner et al. 1991).
(d) We could show that the enhancement of the bis-ANS fluorescence in the presence of influenza virus at low pH correlates for both, pH and time dependence, with the conformational change of HA, assessed by the proteinase K susceptibility of the viruses.
(e) We did not find any evidence for a high (pH-dependent) affinity of the fluorophore for lipid sites. A low affinity for lipid binding sites has also been reported by Lambers et al. (1984).
(f) Preliminary results (M. Krumbiegel, personal communication) indicate that the application of the bis-ANS assay to purified influenza HA yields comparable results to those for intact viruses. The bis-ANS fluorescence in the presence of purified HA of influenza virus A/PR 8/34 is significantly enhanced after lowering the pH.

The HA-associated binding of the fluorophore argues for a pronounced affinity for proteins, in particular for hydrophobic sites. This is supported by (i) a blue shift of the bis-ANS emission spectrum upon binding and (ii) an increase of quantum efficiency. The strong affinity of the fluorophore for hydrophobic binding sites of proteins in comparison to lipids agrees with previous results on other systems (Rosen and Weber 1969; Lambers et al. 1984).

The Scatchard plot (Fig. 6) indicates that the ectodomain of HA bears at least two types of binding sites differing in their affinity for bis-ANS. However, one has to consider also that bis-ANS binds via binding pockets rather than by a 1 : 1 interaction. Although incomplete we have tried to characterize possible binding sites on the ectodomain of HA. The exposure of the hydrophobic fusion sequence of N-HA2 at low pH accounts only partially for the observed increase of binding and fluorescence extent of bis-ANS (see Figs. 2 and 7). This suggests that apart from the hydrophobic N-terminus of HA2 several other hydrophobic sequences of the HA become exposed at low pH and, thus, accessible to bis-ANS. Recently, we have analyzed the hydropathy profile of hemagglutinin (HA) subunits HA1 and HA2 of influenza virus A/PR 8/34 at different pH by taking into account the pH-dependent protonation of the amino acids GLU and ASP (Korte et al. 1992). We have predicted that, besides N-HA2, several other parts of the ectodomain of HA develop hydrophobic properties, in particular under acidic conditions. Different hydrophobic sequences in the ectodomain exist which are comparable in both hydrophobicity and length to the N-terminus of HA2. The existence of several hydrophobic stretches in the ectodomain of HA – apart from the N-terminus of HA2 – might explain the pronounced binding of bis-ANS to HA subsequent to the conformational change. Recently, Burger et al. (1991) have found that several fragments of the HA ectodomain other than the N-terminus of HA2 develop membrane surface activity by hydrophobic interaction. The enhanced affinity of bis-ANS for influenza virus at pH 7.4 after low pH exposure of virus fits with our suggestion that even at neutral pH hydrophobic se-

quences exist in the HA1 subunit besides the N-terminus and the transmembrane spanning sequences of HA2 (Korte et al. 1992). Presumably, these stretches are effectively shielded in the compact structure of the HA trimer which is not altered by low pH-treatment.

As mentioned, a main consideration of developing assays sensitive to fusion intermediates should be their ability to monitor also the kinetics of their formation. Therefore, we examined whether the bis-ANS assay would allow one to follow the time course of the conformational change of HA. One might argue that although sensitive to exposed hydrophobic sites of HA the rate limiting step in the increase of bis-ANS fluorescence is the binding itself. The latter case would preclude the use of the assay for kinetic studies of the conformational change. We were able to show that the increase of the fluorescence intensity of bis-ANS added subsequent to the conformational change is significantly faster than that observed when the conformational change was triggered in the presence of the fluorophore (Fig. 8, curve a). This suggests that the time course of the fluorescence increase of bis-ANS in the presence of influenza virus is sensitive to the kinetics of the low pH triggered structural alteration of HA. We have estimated a half time of the conformational change of about 14–19 see (37 °C, pH 5.0). The half time of binding is less than 2 s. In order to resolve the kinetics adequately stoped-flow measurements are required and these are in progress.

The half time of the conformational change deduced from bis-ANS binding is in agreement with data previously reported. White and Wilson (1987) probing the conformational change of BHA (X31) with anti-HA-peptide antibodies found that the half times of the exposure of the N-terminus of HA2 and the COOH-terminus of HA1 are 15 s and 30 s, respectively (37 °C, pH 5). Stegmann et al. (1990) have estimated from strongly enhanced binding of influenza virus X31 to liposomes upon exposure of hydrophobic sequences of HA at low pH (0 °C), that the half time of the conformational change of HA is in the order of seconds (< 15 s).

HA mediated fusion of influenza virus has been previously discussed and analyzed in terms of cooperativity between viral spike proteins and their ability to trigger fusion (Blumenthal 1988; Bentz 1992). However, we have to be aware that cooperative interactions can occur at several stages of the fusion process. For example, the conformational change of HA might be affected by interactions with other spike proteins. Assuming that binding of the ligand H to HA induces a conformational change (Blumenthal 1988; Morris et al. 1989) we obtained from the Hill plot of the bis-ANS fluorescence extent as a function of pH a Hill-coefficient of 2 (Fig. 3, inset). This might indicate that the conformational change of HA involves some cooperativity. For instance, the exposure of hydrophobic sequences of one HA in the trimer due to the conformational change may facilitate the structural alteration in the remaining HA's. However, the Hill plot is only a crude approximation to study cooperativity and may only be taken as indicative of interacting units. Another aspect of cooperative phenomena may concern the steps forming fusion intermediates and, eventually, the fusion site subsequent to the conformational change. Several efforts have been made to estimate the number of activated trimers required to form a fusion pore (Morris et al. 1989; Ellens et al. 1990). We have followed the approach of Blumenthal (1988) to deduce the minimal fusion units by variation of the surface concentration of active HA by pH. A Hill plot of the fusion extent (expressed as %FDQ) versus pH gives a significantly higher Hill coefficient of about 3 (Fig. 3, inset). Since it is known that even after the conformational change of HA the trimers do not dissociate in the viral membrane we may speculate that at least three trimers are required to form a fusion site. This estimate is in agreement with that of Ellens et al. (1990). They have determined that the minimal fusion site is 2 or larger. This conclusion was based on fusion experiments, using two fibroblast cell lines differing in their surface density of HA.

While we hope to get detailed insights into the kinetics of the conformational change of HA by using this assay in conjunction with the stoped-flow technique, a main task will be to assay the conformational change when HA is bound to the target membrane in the presence of sialic acid receptors. So far, structural alterations of HA in the vicinity of the target membrane and/or when bound to cell surface receptors have not been characterized. The main problem to be tackled is that only a minor part of the HA of a virion will be in the vicinity of the target membrane (or even less will be in contact with virus receptors), the overwhelming part of HA will behave as demonstrated in this paper. To overcome this problem, one future prospect might be the application of this bis-ANS assay to BHA fragments in the presence of receptor analogues or liposomes bearing gangliosides.

Acknowledgement. A.H. was supported by grants from the Deutsche Forschungsgemeinschaft (He1928/1-1; SFB 312 D9/YE3) and the Fritz-Thyssen-Stiftung. T.K. is a recipient of a fellowship from the Boehringer-Ingelheim-Fonds. We are indebted to Dr. M. Clague (EMBL, Heideberg) for helpful comments.

References

Bentz J (1992) Intermediates and kinetics of membrane fusion. Biophys J 63:448–459

Bentz J, Ellens E, Alford D (1990) An architecture for the fusion site of influenza hemagglutinin. FEBS 276:1–5

Blumenthal R (1988) Cooperativity in viral fusion. Cell Biophys 12:1–12

Blumenthal R, Bali-Puri A, Walter A, Covell D, Eidelman O (1987) pH-dependent fusion of vesicular stomatitis virus with vero cells: measurement by dequenching of octadecylrhodamine fluorescence. J Biol Chem 262:13614–13619

Blumenthal R, Schoch C, Puri A, Clague MJ (1991) A dissection of steps leading to viral envelope protein-mediated membrane fusion. Ann NY Acad Sci 635:285–296

Brand CM, Skehel JJ (1972) Crystaline antigen from the influenza virus envelope. Nature 238:145–147

Brunner J, Zugliani C, Mischler R (1991) Fusion activity of influenza virus PR 8/38 correlates with a temperature-induced conformational change within the hemagglutin ectodomain detected by photochemical labeling. Biochemistry 30:2432–2438

Burger KNJ, Wharton SA, Demel RA, Verkleij AJ (1991) Interaction of influenza virus hemagglutinin with a lipid monolayer. A com-

parison of the surface activities of intact virions, isolated hemagglutinins and a synthetic fusion peptide. Biochemistry 30:11173–11180

Clague MJ, Schoch C, Blumenthal R (1991) Delay time of influenza virus hemagglutinin-induced membrane fusion depends on hemagglutinin surface density. J Virol 65:2402–2407

Compans RW, Choppin PW (1975) Reproduction of myxoviruses. In: Fraenkel-Conrad H, Wagner RR (eds) Comprehensive virology. Plenum Press, New York, London, pp 179–252

Dempsey CE (1990) The actions of melittin of membranes. Biochim Biophys Acta 1031:143–161

Dodge JT, Mitchell C, Hanahan DJ (1963) The preparation and chemical characteristics of hemoglobin free ghosts of human erythrocytes. Arch Biochem Biophys 100:119–130

Doms RW, Helenius A, White J (1985) Membrane fusion activity of the influenza virus hemagglutinin: The low pH-induced conformational change. J Biol Chem 260:2973–2981

Ellens H, Bentz J, Mason D, Zhang F, White JM (1990) Fusion of influenza hemagglutinin-expressing fibroblasts with glycophorin-bearing liposomes: role of hemagglutinin surface density. Biochemistry 29:9697–9707

Gething MJ, Sambrook J (1981) Cell-surface expression of influenza hemagglutinin from a cloned DNA copy of the RNA gene. Nature (London) 293:620–625

Harter C, James P. Bächi T, Semenza G, Brunner J (1989) Hydrophobic photolabeling identifies BHA2 as a subunit mediating the interaction of bromelain-solubilized influenza virus hemagglutinin with liposomes at low pH. J Biol Chem 264:6459–6464

Herrmann A, Clague MJ, Blumenthal R (1993a) Enhancement of viral fusion by nonadsorbing polymers. Biophysical J 65:528–534

Herrmann A, Clague MJ, Blumenthal R (1993b) Role of target membrane structure in fusion with influenza virus: effect of modulating erythrocyte transbilayer phospholipid distribution. Membr Biochem 10:3–15

Hils J, May A, Sperber M, Klöcking R, Helbig B, Sprössig M (1986) Hemmung ausgewählter Influenzastämme der Typen A und B durch Phenolkörperpolimerisate. Biomed Biochim Acta 45:1173–1179

Hoekstra D, de Boer T, Klappe K, Wilschut J (1984) Fluorescence method for measuring the kinetics of fusion between biological membranes. Biochemistry 23:5675–5681

Hua QX, Ladbury JE, Weiss A (1993) Dynamics of a monomeric insulin analogue: Testing the molton-globule hypothesis. Biochemistry 32:1433–1442

Klenk HD (1991) In: Francki RIB, Fanquet CM, Kundson DL, Bron F (eds) Classification and nomenclature of viruses. Springer, Berlin Heidelberg New York, pp 263–272

Klenk HD, Rott R, Orlich M, Blodorn J (1975) Activation of influenza A viruses by trypsin treatment. Virology 68:426–439

Korte T, Ludwig K, Herrmann A (1992) pH-dependent hydrophobicity profile of hemagglutinin of influenza virus and its possible relevance in virus fusion. Biosci Rep. 12:(5)397–406

Lambers JJW, Terpstra W, Levine YK (1984) Studies on the action mechanism of the membrane enzyme chlorophyllase. Biochim Biophys Acta 789:182–196

Lear JD, DeGrado WF (1987) Membrane binding and conformational properties of peptides representing the NH2 terminus of influenza HA-2. J Biol Chem 262:6500–6505

Morris SJ, Sarkar DP, White JM, Blumenthal R (1989) Kinetics of pH-dependent fusion between 3T3 fibroblasts expressing influenza hemagglutinin and red blood cells: measurement by dequenching of fluorescence. J Biol Chem 264:3972–3978

Nir S, Düzgünes N, Pedroso de Lima M, Hoekstra D (1990) Fusion of enveloped viruses with cells and liposomes: activity and inactivation. Cell Biophys 17:181–201

Rosen CG, Weber G (1969) Dimer formation from 1-Anilino-8-naphthalenesulphonate catalyzed by bovine serum albumin. A new fluorescent molecule with exceptional binding properties. Biochemistry 8:3915–3920

Skehel JJ, Bayley PM, Brown EB, Martin SR, Waterfield MD, White JM, Wilson IA, Wiley DC (1982) Changes in conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc Natl Acad Sci USA 78:968–972

Stegmann T, Hoekstra D, Scherphof G, Wilschut J (1985) Kinetics of pH-dependent fusion between influenza virus and liposomes. Biochemistry 24:3017–3113

Stegmann T, Hoekstra D, Scherphof G, Wilschut J (1986) Fusion activity of influenza virus. A comparison between biological and artificial target membrane vesicles. J Biol Chem 261:10966–10969

Stegmann T, Booy FP, Wilschut J (1987) Effects of low pH on influenza virus. Activation and inactivation of the membrane fusion activity of the hemagglutinin. J Biol Chem 262:17744–17749

Stegmann T, White JM, Helenius A (1990) Intermediates in influenza induced membrane fusion. EMBO J 9:4231–4241

Takahashi S (1990) Conformation of membrane fusion-active 20-residue peptides with or without lipid bilayers. Implication of α-helix conformation for membrane fusion. Biochemistry 29:6257–6264

Weiss WI, Brown JH, Cusack S, Paulson JC, Skehel JJ, Wiley DC (1988) Structure of influenza virus hemagglutinin complexed with its receptor, sialic acid. Nature 333:426–431

White JM (1990) Viral and cellular membrane fusion proteins. Annu Rev Physiol 52:675–697

White JM, Wilson IA (1987) Anti-peptide antibodies detect steps in a protein conformational change: Low-pH activation of the influenza virus hemagglutinin. J Cell Biol 105:2887–2896

White J, Kielian M, Helenius A (1983) Membrane fusion proteins of enveloped animal viruses. Q Rev Biophys 16:151–195

Wilson IA, Skehel JJ, Wiley DC (1981) Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. Nature 289:366–373