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Analysis of delay times of hemagglutinin-mediated fusion between influenza virus and cell membranes

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Abstract. We have studied the kinetics of low pH-induced fusion between influenza virus A/PR 8/34 and human erythrocyte membranes in suspension by using an assay based on fluorescence dequenching (FDQ) of the lipophilic dye octadecylrhodamine B chloride (R 18). As shown previously (Clague et al. 1991) the onset of FDQ is preceded by a characteristic lag time (t_{lag}) following pH reduction. Whereas t_{lag} represents only a subpopulation of fusing viruses with the shortest delay time we suggest here that a representative mean lag time μ_{lag} of virus-cell fusion can be deduced from the R 18-assay. Kinetics of FDQ reflects the cumulative distribution function of lag times τ_{lag} of single fusion events with the mean value μ_{lag}. We show that t_{lag} obtained from the onset of FDQ does not always reflect the fusion behaviour of the whole population of fusing viruses. While both lag times, t_{lag} and μ_{lag}, exhibit a similar temperature dependence we found a significantly different dependence of both delay times on virus inactivation by low pH-pretreatment. We conclude that the mean lag time μ_{lag} appears to be a more appropriate parameter describing the kinetics of virus-cell fusion. The analysis of delay times offers a new approach to test the validity of different kinetic models of HA-mediated fusion and to gain valuable information about HA-mediated fusion. The analysis confirms that the inactivation process proceeds via steps of the formation of the fusion pore. Although the increase of lag times can be explained by a depletion of fusion competent HA's, our data suggest that intermediate structures of HA along the inactivation pathway can still transform into a fusion site.

Key words: Influenza virus – Membrane fusion – Inactivation – Fluorescence – Dequenching

Abbreviations: FDQ, fluorescence dequenching; HA, hemagglutinin; PBS, phosphate buffered saline; R 18, octadecylrhodamine B chloride; t_{lag}, lag time; μ_{lag}, mean lag time; τ_{lag}, individual delay time

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Introduction

Binding of influenza virus to the target membrane and the subsequent fusion are mediated by the viral spike protein hemagglutinin (HA) which is organized as a homotrimer within the virus membrane (Gething and Sambrook 1981; White et al.1983). The HA-mediated fusion is triggered at acidic pH with a maximum extent at about pH 5. Fusion correlates with a conformational change of HA. The unfolding of the trimer and the exposure of hydrophobic sites, e.g. the highly conserved N-terminus of the subunit HA2, at low pH have been associated with the fusion activity (Skehel et al. 1982; Doms et al. 1985; White and Wilson 1987). Although direct evidence is lacking it has been suggested that several trimers aggregate to form a fusion pore (Bentz et al. 1990; Bentz 1992; Stegmann et al. 1990). Viral fusion activity may be impaired by competing inactivation processes at acidic pH (Stegmann et al. 1986; Nir et al. 1990). Recently, Ramalho-Santos et al. (1993) have suggested that the rate-limiting step in HA-mediated fusion, the rearrangement of HA at the attachment site with target membrane, is similar to that involved in fusion inactivation. Thus, investigation of inactivation processes may be help to illuminate the mechanism of HA-mediated fusion.

Over recent years, many studies on the kinetics of lipid mixing as a result of the fusion of enveloped viruses with target membranes have been accomplished to elucidate the spatial and temporal structure of the fusion site. Various fluorescence assays are valuable tools for the continuous monitoring of membrane fusion, in particular the octadecylrhodamine B chloride (R 18) fluorescence dequenching (Hoekstra et al. 1984). The plasma membrane of human erythrocytes bearing sialic acid receptors has been used widely as a model target membrane to investigate fusion of R 18-labeled influenza virus. The fusion event can be experimentally controlled by adjusting the pH of the suspension medium (Stegmann et al. 1985; Clague et al. 1991). Several parameters of the dequenching kinetics such as the initial rate and the extent have been employed to characterize the factors affecting fusion. Particular attention has been given to the lag phase between the low

pH-trigger and the onset of fusion of influenza virus with erythrocyte membranes in suspension seen by fluorescence dequenching. This delay is sensitive to pH and temperature. At conditions optimal for the fusion (pH ~ 5.0, 37 °C) the lag time is <1 s and can only be resolved by using "stop-flow" techniques whereas below 30 °C delays in the range of 1–10 s (Clague et al. 1991; Herrmann et al. 1993 a) or even longer up to 10 min (Stegmann et al. 1990) have been observed. Several factors affect the delay time, e.g., the surface density of low pH activated HA (Clague et al. 1991), the lipid composition of the outer leaflet of the target membrane (Clague et al. 1991; Herrmann et al. 1990, 1993 a), and the hydration repulsion forces (Herrmann et al. 1993 b).

However, the lag time measured for a suspension of virus-cell complexes may reflect only the behaviour of a subpopulation of viruses, which undergoes at the earliest time point fusion with the target membrane upon lowering the pH. Since delay turned out to be a valuable characteristic for investigating the mechanism of viral fusion, it remains to be elucidated whether this lag time designated here *lag time* t_{lag} can be used as a parameter which reflects the properties of the whole population of fusing species.

To address this issue we have investigated the kinetics of the low pH-induced fusion of influenza virus A/PR 8/34 with erythrocyte membranes measured by the fluorescence dequenching assay using the probe R 18. We suggest for viral fusion measured in suspension, that the fluorescence dequenching curve reflects the distribution function of individual delay times τ_{lag} provided that the redistribution of the fluorescent probe upon fusion is not rate limiting (Clague et al. 1993). The dequenching curve of virus-cell fusion offers a rather simple way to estimate a representative *mean lag time* μ_{lag}. Even under conditions at which no *lag time* t_{lag} could be resolved (<0.5 s, pH 5.0, 30 °C), it is possible to assess the *mean lag time* μ_{lag} which is still of the order of several seconds (~15 s). However, the *lag time* t_{lag} does not always reflect the fusion behaviour of the whole fusing virus population. Significant differences in the dependence of both lag times on virus inactivation at low pH were established. We will show that such an analysis of delay times offers a new approach to judge the validity of different kinetic models of HA-mediated fusion.

Material and methods

Materials

Octadecylrhodamine B chloride (R 18) was purchased from Molecular Probes (Junction City, OR). Fresh blood from healthy donors was obtained from the Blood Bank, Berlin-Lichtenberg, and was used within 3 days after sampling. Purified influenza virus was kindly provided by Ulrike Gimsa (Federal Health Office, Institute of Veterinary Medicine, Berlin).

Red blood cell and ghost preparation

After removal of buffy coat and plasma red blood cells were washed three times in phosphate-buffered saline (PBS, pH 7.4). Unsealed erythrocyte ghosts were prepared according to Dodge et al. (1963).

Labeling of virus for fusion. 1.25 µl of a 1.75 mM stock solution of R 18 in ethanol were added to 250 µl influenza virus A/PR 8/34 (1 mg protein/ml) followed by rapid vortexing (Herrmann et al. 1993 a, b). After incubation for 30 min at room temperature (in the dark) viruses were washed with ice-cold PBS to remove unbound fluorophore, and resuspended to a concentration of 1 mg virus protein/ml. The final concentration of added probe corresponds to approximately 2 mol% of total viral lipid. The protein concentration of viruses as well as of ghosts was determined according to Lowry.

Inactivation of influenza virus. A/PR 8/34 was inactivated by preincubation at low pH (5.0), 20 °C, for different periods in the absence of the target membrane. Subsequently, the virus suspension was neutralized and kept on ice.

Virus binding to cell membranes. Labeled A/PR 8/34 (0.1 mg protein) was incubated for 45 min on ice with 0.2 ml of erythrocyte ghost suspension (6–7 mg protein/ml). Afterwards, the suspension was washed in 10 to 15 vol. of ice-cold PBS and resuspended by adding PBS to a final concentration of 1 mg virus protein/ml.

Fusion analysis. Fluorescence dequenching of labeled viruses attached to ghost membranes was measured using a SHIMADZU PC5001 spectrofluorometer. 30 µl of virus-erythrocyte ghost suspension was transferred into a standard cuvette containing 1.97 ml of prewarmed sodium acetate buffer, preadjusted to low pH, and stirred continuously.

The fluorescence was monitored at an excitation and emission wavelength 560 nm and 590 nm, respectively (cut-off filter 570 nm, time resolution 0.5 s). For maximum fluorescence $F(\infty)$, TRITON X-100 from Fluka Chemie AG (Buchs, Switzerland) was added to a final concentration of 0.5%. The percentage of fluorescence dequenching FDQ was calculated as described previously (Blumenthal et al. 1987):

$$\%FDQ = 100\% \cdot \frac{F(t) - F(0)}{F(\infty) - F(0)} \quad (1)$$

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.

Data analysis. The first derivative of the fluorescence dequenching curves (d[FDQ]/dt) was obtained by the Savitzky-Golay-algorithm using the software TableCurve of JANDEL Scientific.

Proteinase K digestion of virus. Virus A/PR 8/34 was incubated for different times at pH 5.0, 20 °C, and subsequently neutralized and digested with proteinase K (Boehringer Mannheim) (30 min, 37 °C) and precipitated with trichloroacetic acid. SDS-PAGE was done as described (Doms et al. 1985) under reducing conditions. HA bands

(stained with COOMASIE BLUE) were quantitated by intensity measurements on digitized images of electrophoretic gels by using the software SigmaScan/Image of JANDEL Scientific.

Lag times deduced from fluorescence dequenching kinetics

The formation of the active fusion conformation of HA which eventually results in merging of the virus with the target membrane proceeds via several complex steps the precise nature of which are currently unknown. The scheme of a kinetic pathway reflecting such a multistep process is shown in Fig. 1 A containing essential elements of several models published previously (Bentz et al. 1990; Bentz 1992; Stegmann et al. 1990; Blumenthal et al. 1991; Schoch et al. 1992; Clague et al. 1993).

With respect to its fusogenic properties the HA is originally (neutral pH) in a non-activated state (HA_{NA}). Low pH triggers a conformational transition and an aggregation of HA's (HA_A). Subsequently, a cascade of activation steps before the final fusion event occurs involving intermediate structures. Conformational changes of the HA, occurring while the virus is not appropriately bound to the target membrane, will result in an inactivation of HA (HA_I).

In order to keep the model as simple as possible only one intermediate (I_1) is considered. The intermediate can transform to the fusion pore (FP). Eventually, after stabilization the pore progresses to the fusion site (FS) ensuring a mixing of lipids (LM) between both fused membranes as well as of internal space contents.

As indicated some of the early steps may be reversible which is in line with recent observations. By taking advantage of the slower fusion with HA-expressing cells, Morris et al. (1989) have shown that in the early phase which follows the low pH trigger, fusion can be arrested by switching the pH back and reactivated by reacidification. At longer time intervals fusion was not inhibited by raising the pH indicating that later steps of the activation cascade are independent of an acidic environment.

Only the final step of the scheme – the lipid mixing – can be monitored by the fluorescence dequenching assay, while steps preceding membrane fusion cannot be made directly visible. These steps are reflected by a delay between the low pH trigger ($t=0$) and the onset of fluorescence dequenching. For a given fusion site this time corresponds to τ_{lag}. This *lag time* is determined by the rate constants of the activation steps. However, in suspension (cuvette experiment) the *lag time* t_{lag} of the onset of fluorescence dequenching (shown schematically in Fig. 2 (dashed line)) reflects only those viruses with the shortest delay between low pH trigger and the onset of fusion. The *lag time* t_{lag} does not necessarily provide a parameter typical for the whole fusing species. The kinetics of fluorescence dequenching for virus-cell membrane fusion reflects the cumulative distribution function of the individual *lag times* τ_{lag} of viruses provided the R 18 redistribution is not rate-limiting (see Discussion).

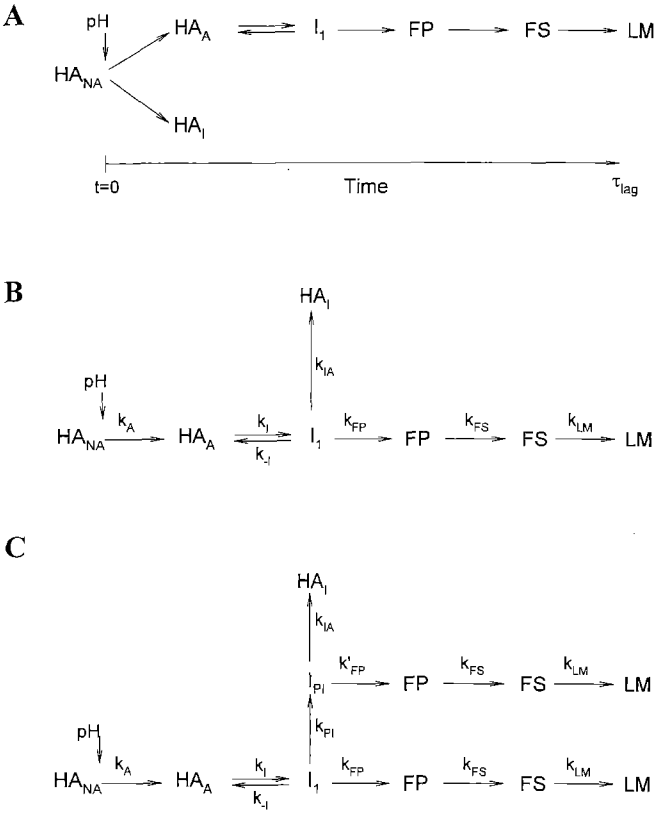

Fig. 1 A–C. Schemes of the low pH triggered activation of a fusion site (FS) of hemagglutinin (HA) of influenza virus. In scheme **A** the process of the inactivation of HA (HA_I) at low pH is different from that leading to the formation of the fusion site whereas in models **B** and **C** the inactivation proceeds via steps of the activation of the fusion junction. The formation of the fusion site is a multistep process: HA_{NA} – cleaved, non-activated HA (neutral pH form); HA_A – HA after triggering the conformational change at low pH; I_I – intermediate, I_{PI} – partial inactivated intermediate; FP – fusion pore; FS – fusion site; LM – lipid mixing. Part of the steps may be reversible. For details see text. The arrow indicates the low pH trigger

The first derivative – $d(FDQ)/dt$ – as a function of time relates the individual lag times to their relative frequency expressed by $d(FDQ)/dt$ (solid line, Fig. 2). From that the *mean lag time* can be obtained (even if the *lag time* t_{lag} is too short to resolve) either numerically or by fitting the experimental curve, e.g. to a kinetic model. We obtained the *mean lag time* numerically from the first derivate $d(FDQ)/dt$.

Results

Temperature dependence of virus membrane fusion

We have measured the temperature dependence of the fusion kinetics of influenza virus prebound to red blood cell membranes between 15 °C and 30 °C at pH 5.0 (Fig. 3). With decreasing temperature (*i*) the final extent of fluorescence dequenching declined and (*ii*) fluorescence dequenching proceeded with a slower rate. A *lag time* t_{lag} between the low pH trigger and the onset of fusion became visible at temperatures <30 °C.

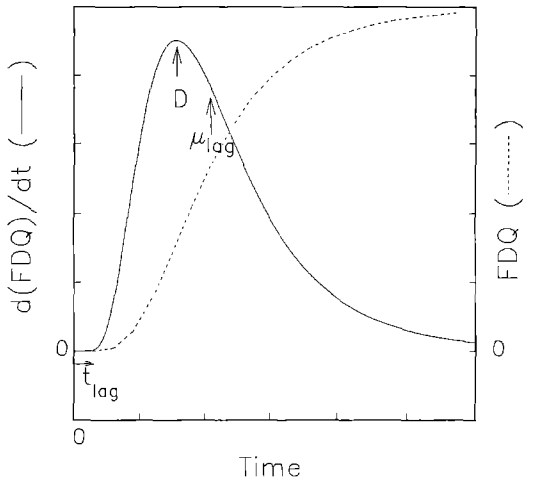

Fig. 2. Typical kinetics of low pH-mediated virus-cell fusion measured in suspension (cuvette experiment) by fluorescence dequenching FDQ (dashed line) of the probe R 18 initially incorporated into the virus membrane. t_{lag} is the lag phase between the low pH-trigger ($t=0$) and the onset of FDQ reflecting the earliest time point of fusion events in the suspension. The first derivative d(FDQ)/dt (solid line) is the distribution function of individual delay times τ_{lag} (dashed line). μ_{lag} is the mean lag time of all fusion events, D corresponds to the mode

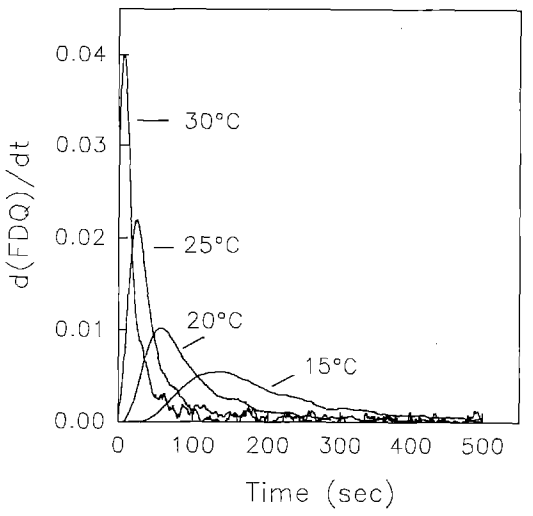

Fig. 4. First derivative d(FDQ)/dt of the fluorescence dequenching (see Fig. 3) of fusion of influenza virus with erythrocyte membranes at pH 5.0 at the various temperatures indicated. Curves were normalized to the same area. The first derivative was obtained by the Savitzky-Golay algorithm

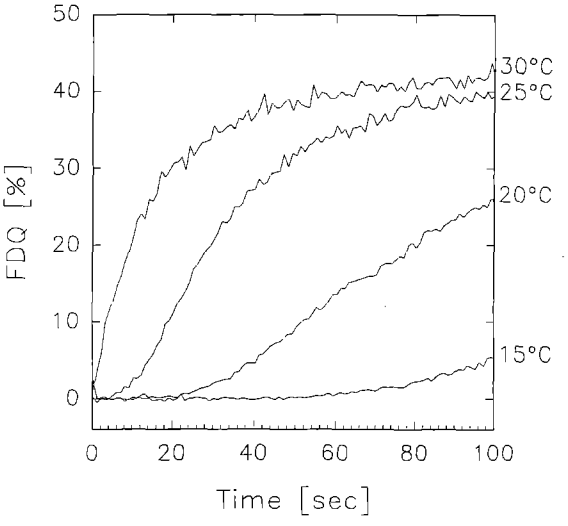

Fig. 3. Temperature dependence of the early phase of low pH-mediated fusion of influenza virus A/PR 8/34 with erythrocyte membranes at pH 5.0 measured by fluorescence dequenching of the R 18 probe. At $t=0$ virus-ghosts complexes were injected into a cuvette containing prewarmed buffer with temperatures as indicated in the figure. Fluorescence was recorded with a time resolution of 0.5 sec. FDQ was calculated according to equation {1}. The lag time t_{lag} (see Fig. 2) can be resolved for temperatures <30 °C

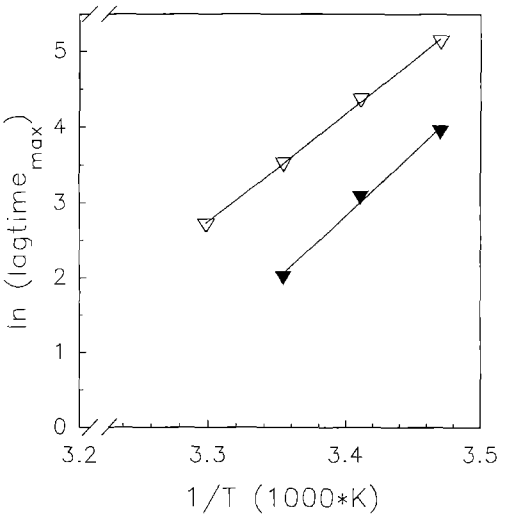

Fig. 5. Arrhenius plots of the mean lag time μ_{lag} (▽) and of the lag time t_{lag} (▼) of fusion of influenza virus with erythrocyte membranes at pH 5.0 at different temperatures. μ_{lag} is the mean value of the first derivative of fluorescence dequenching d(FDQ)/dt. t_{lag} see Fig. 2. For both parameters, data can be fitted by a straight line ($r=0.98$) with a similar slope

In Fig. 4 the first derivative of fluorescence dequenching d(FDQ)/dt normalized to the same area is shown. The curves reflect the relative frequency of the individual lag times τ_{lag} at the temperature indicated. The distribution of τ_{lag} becomes broader and shifts to higher values when lowering the incubation temperature.

The temperature dependence of both the *lag time* t_{lag} and the *mean lag time* μ_{lag} is shown in Fig. 5. No *lag time* t_{lag} could be resolved at 30 °C (as can be seen in Fig. 3). However, the *mean lag time* was about 15 s. Thus, even at elevated temperatures a significant delay between the pH trigger and the onset of fusion (fluorescence dequenching)

occurred on the average. The Arrhenius plot suggests a similar dependence of both parameters on the temperature (see legend to Fig. 5).

One may wonder whether dissociation processes may affect fluorescence dequenching kinetics. In order to assess the stability of virus binding to erythrocyte membranes, we have measured fusion after different periods (up to 10 min) of preincubation of virus-cell complexes at neutral pH. Even after a preincubation at 37 °C, pH 7.4, the kinetics and the extent of FDQ at pH 5.0 were not affected suggesting that the binding is stable.

Inactivation of influenza virus by low pH-preincubation

The rate of the low pH-inactivation depends significantly on the temperature. Inactivation was very rapid at 37 °C, pH 5.0 (data not shown). Therefore, to allow a more gradual inactivation influenza virus A/PR 8/34 was preincubated in the absence of target membranes at pH 5.0 and 20 °C for different intervals (≥2 min). Subsequent to binding to erythrocyte membranes at pH 7.4 fusion was measured at pH 5.0, 20 °C, in order to resolve the *lag time* t_{lag}. As can be seen from the results in Fig. 6 A the fusion activity was impaired by preincubation of the virus at low pH. After prolonged preincubation (90 min) we observed an almost complete inhibition of the fusion activity (FDQ <3%).

In all cases the *lag time* t_{lag} was significantly shorter than the time of the preincubation of virions at low pH. For preincubation periods ≤4 min, we observed only a small increase of the *lag time* t_{lag}, while for longer times of low pH-inactivation a steeper increase of this parameter became evident (Fig. 6 B, 7 A). The absolute length of t_{lag} varied for different preparations of the virus. In particular, for longer periods of low pH preincubation (≥8 min) the determination of the onset of FDQ became more and more inaccurate. To compare different preparations, we have normalized the values of t_{lag} to the control (no preincubation) (Fig. 7 B). In Fig. 7 A we have compared the *lag time* t_{lag} with the *mean lag times* μ_{lag} deduced from the distribution. With increasing time of preincubation the distribution of individual lag times as expressed by the first derivative $d(FDQ)/dt$ became broader upon low pH pretreatment of viruses (not shown). The pronounced contribution of shorter delays disappeared and longer lag times τ_{lag} became significant. It is evident that μ_{lag} is much more sensitive to preincubation at low pH in comparison to t_{lag}. At shorter times of low pH-pretreatment of viruses a small increase of t_{lag} but a pronounced increase of μ_{lag} was observed. Only prolonged inactivation periods (≥6 min) resulted in a higher level of t_{lag}. The value relative to the control (no preincubation) was comparable to that of the *mean lag time* (Fig. 7 B).

To investigate whether HA molecules undergo a conformational change during preincubation at low pH, we have measured the sensitivity of HA to proteinase K. This enzyme digests the acidic, but not the neutral form of HA (Doms et al. 1985). As shown in Fig. 8, a continuous increase of the number of digested HA can be observed during preincubation at pH 5.0, 20 °C.

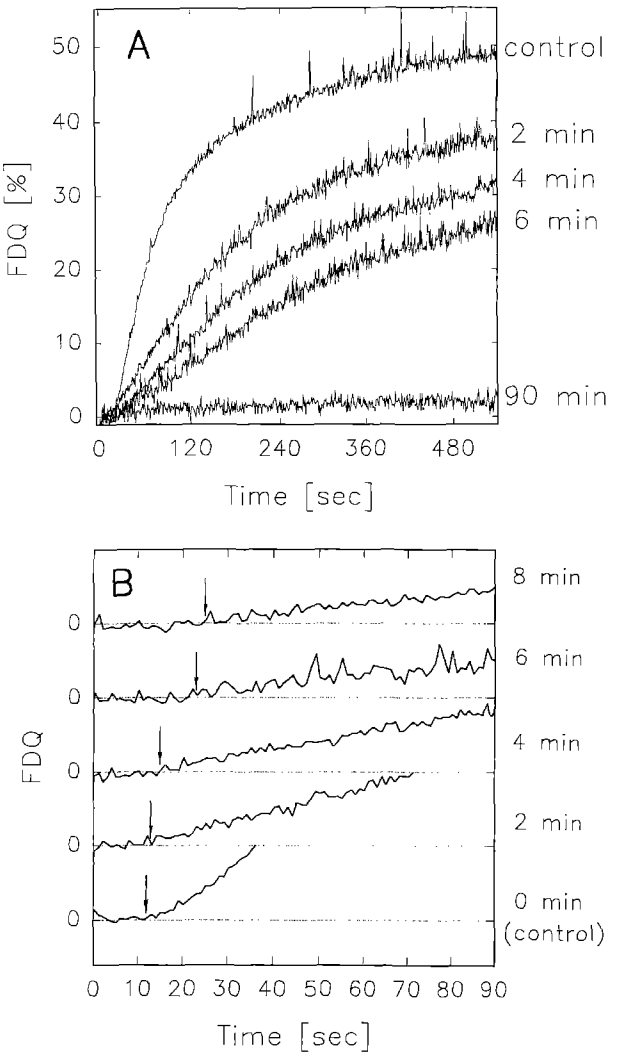

Fig. 6 A, B. Kinetics of low pH-mediated fusion of influenza virus A/PR 8/34 with erythrocyte membranes at 20 °C (pH 5.0) after preincubating the virus at pH 5.0, 20 °C, in the absence of target membranes **A.** Fusion was measured by fluorescence dequenching of R 18 as described in Material and Methods. In **B** the early phase of fluorescence dequenching is shown by expanding the time scale. Arrows indicate the lag time t_{lag} between the low pH trigger ($t=0$) and the onset of fusion as measured by fluorescence dequenching of R 18 (see legend to Fig. 2). Preincubation time is given on the right

Kinetic model of HA-mediated fusion

In order to rationalize the influence of low pH-pretreatment of influenza virus on the kinetics of fusion with erythrocyte membranes we have simulated our experimental data by kinetic models (Fig. 1) which share some similarities with the scheme proposed recently by Bentz (1992). However, an essential point of our modelling was to elucidate whether the inactivation of HA at low pH includes steps of the activation pathway of the fusion sites and affects the kinetics of their formation (Fig. 1, model B and C). Based on the data in literature (see also LAG TIMES DEDUCED FROM FLUORESCENCE DEQUENCHING KINETICS) the following assumptions have been made for the model presented in Fig. 1, B and C.

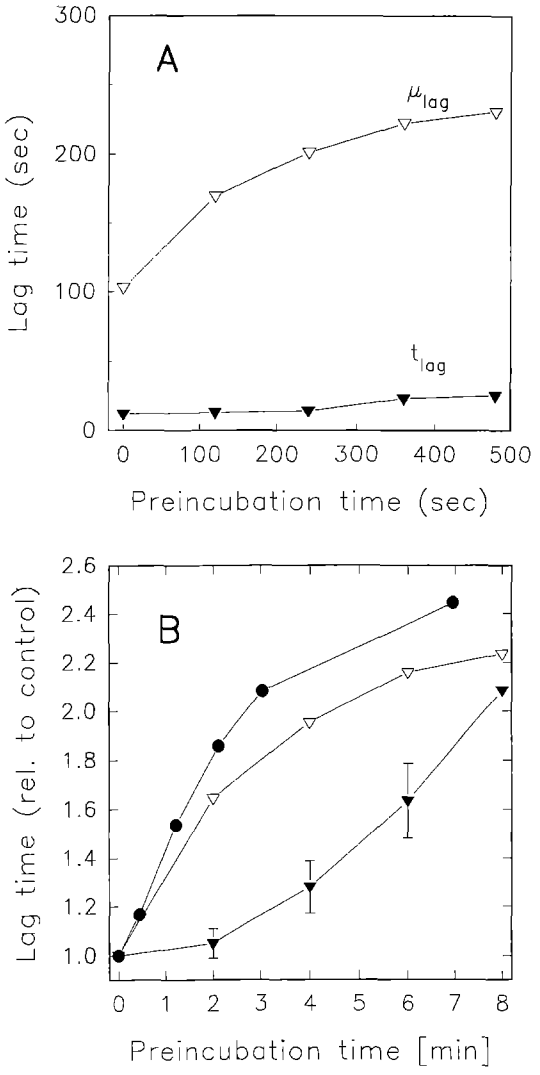

Fig. 7. **A** The mean lag time μ_{lag} (▽) and the lag time t_{lag} (▼) of fusion of influenza virus A/PR 8/34 with erythrocyte membranes at pH 5.0, 20 °C, as a function of time of low pH-treatment of viruses (pH 5.0, 20 °C) in the absence of the target membrane. μ_{lag} is the mean value of the first derivative of fluorescence dequenching d(FDQ)/*dt*. t_{lag} see Fig. 2. Data were taken from Fig. 6 A. **B** Lag times relative to the control (no preincubation): (▽) relative μ_{lag} and (▼) relative t_{lag} (standard error of estimate is given, $n \geq 3$); (●) relative μ_{lag} obtained from kinetics of the model presented in Fig. 1 C (kinetics see Fig. 9 B)

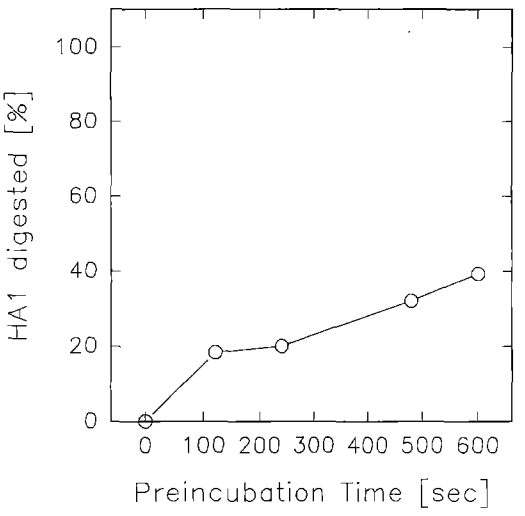

Fig. 8. Conformational change of HA during preincubation of influenza virus A/PR 8/34 at pH 5.0, 20 °C, in the absence of target membranes. Virus was preincubated for different periods as indicated. Subsequently, the virus was preincubated with proteinase K and the amount of digested HA 1 was determined as described in Material and Methods

1. The low pH-triggered conformational change of HA is fast with respect to the lag time of virus-cell fusion, in particular at lower temperature (Stegmann et al. 1990; Korte and Herrmann 1994 and unpublished results). Similarly, the aggregation step of HA trimers necessary to form a fusion pore is considered to be rapid owing to the high surface density of the spikes in the virus membrane (see also Bentz 1992; Stegmann et al. 1990; Clague et al. 1991) and to the fast diffusion of the trimers[1]. Therefore, we have assumed that the rate constant k_A characterizing the step $HA_{NA} \rightarrow HA_A$ is large with respect to the other rate constants ($k_A >> k_{...}$). However, including such a step would not alter any conclusion obtained from this model.

2. The formation of the intermediate I_1 is reversible in a pH dependent manner (see above). At low pH $k_I >> k_{-I}$ whereas at neutral pH $k_I << k_{-I}$ and k_{-I} is regarded to be large. Thus, in terms of the model I_1 is transformed to HA_A immediately upon reneutralization.
3. The formation of the fusion pore (FP) and the fusion site (FS) is characterized by the rate constants k_{FP} and k_{FS}, respectively. Lipid mixing (k_{LM}) monitored by dequenching of R 18 is not rate limiting and needs not to considered in the kinetic model (see Discussion). Thus, the final step in our analysis is the formation of the fusion site. We assume that only one fusion site per virus-cell complex is formed.
4. The inactivation process which eventually yields the inactivated HA-complex HA_I proceeds via steps of the activation of the fusion junction (Ramalho-Santos et al. 1993). In model C we consider a partially inactivated intermediate I_{PI} which can transform to a fusion site (FS) via a fusion pore (FP) but with a slower rate given by the condition $k'_{FP} < k_{FP}$. Alternatively or additionally, one can introduce $k'_{FS} < k_{FS}$. The nature of those partial inactivated intermediates remains open. A reasonable assumption may be that part of the HA-trimers forming a fusion pore are in a conformation nonoptimal for fusion.
5. Complete inactivation (HA_I) occurs also when the virus is bound to the target membrane.
6. There is no need to consider virus binding in the model since the virus is already bound to the target membrane prior to the pH-shift (see Material and Methods).

[1] Gutman et al. (1993) have shown that the lateral diffusion of HA0 and of the fusogenic form of HA of different influenza strains on the surface of CV 1 cells is about $D = 3 \cdot 10^{-10}$ cm^2/s at 22 °C. According to $D = x^2/4t$ (x – distance; t – time), a characteristic time of a diffusion – determined HA-aggregation can be estimated. Setting x equal to ¼ of the virus diameter, $d = 0.1$ µm, an HA-trimer needs only 0.5 s to move this distance

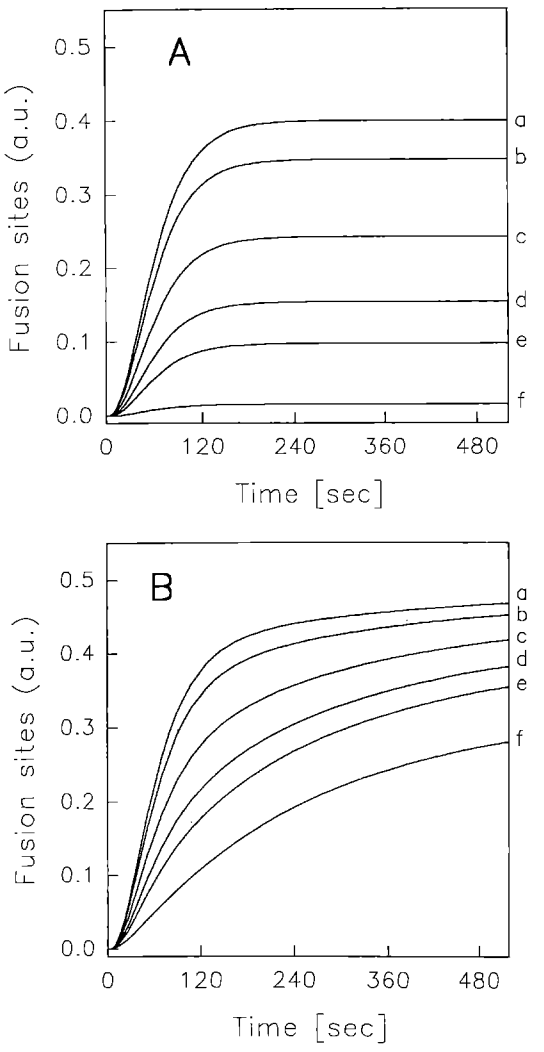

Fig. 9 A, B. Calculated fusion curves on the basis of the models B and C, respectively, presented in Fig. 1. In Fig. 9 B fusion proceeds via two pathways $I_1 \rightarrow$ FP $\rightarrow$ FS and $I_1 \rightarrow I_{PI} \rightarrow$ FP $\rightarrow$ FS (model C) whereas in Fig. 9 A a fusion site can be formed only as a result of the first pathway (model B). **A** and **B**: $k_I = 5$/min; $k_{FP} = k_{FS} = 2$/min; $k_{PI} = 0.5$; $k_{IA} = 0.05$/min; **B** $k'_{FP} = 0.1\ k_{FP}$. Inactivation by low pH-pretreatment was simulated by setting $k_{FP} = 0$ **A** and **B** and $k'_{FP} = 0$ **B** for a given time (indicated at the right): a – control; b–f – preincubation for 27, 73, 127, 182, 418 s respectively

Kinetic schemes B and C in Fig. 1 can be described by a set of differential equations which can be solved by using the software Mathematica 2.2 for Windows. In Fig. 9 A and B the fusion curves $F(t)$ corresponding to the schemes B and C, respectively, in Fig. 1 are shown for several cases: (a) – control, no preincubation ($[HA_{NA}] = 0.5$ at $t = 0$); (b)–(f) preincubation of viruses at low pH for different periods (27, 73, 127, 182, 418 s, respectively). The rate constants were chosen to match the time course of the experiments. Relations between the rate constants were kept as simple as possible:

$k_I = 5$/min, $k_{FP} = k_{FS} = 2$/min; $k_{PI} = 0.5$/min; $k'_{FP} = 0.1\ k_{FP}$ (only model C, Fig. 1); $k_{IA} = 0.05$/min.

Rate constants for the inactivation steps were selected to account for the decrease of FDQ found experimentally after low pH-pretreatment. Low pH-preincubation was modelled by setting $k_{FP} = k'_{FP} = 0$ for given times with $[HA_{NA}] = 0.5$ at $t = 0$.

From the kinetics presented in Fig. 9 B it can be seen that the model gives a reasonable description of the experimental dequenching curves (Fig. 6 A). Not only the number of fusion sites (FS) decreases but also the kinetics of their formation becomes slower. This is obvious from the increase of the *mean lag time* μ_{lag} upon preincubation at low pH deduced from these curves. A reasonable coincidence between experimental and model data was found for μ_{lag} plotted relative to the control (no preincubation) (Fig. 7 B).

In the model shown in Fig. 1 B we have assumed that fusion is only possible via the pathway $I_1 \rightarrow FP \rightarrow FS$ and intermediates with a partial impaired fusion activity (I_{PI}) do not exist. In that case we observed a more rapid decrease of the fusion extent when simulating inactivation by low pH-treatment as described above (Fig. 9 A). However, the important point is that the characteristics of the kinetics were not altered, i.e., μ_{lag} was independent of the inactivation by low pH treatment. It should be emphasized that a similar result would also be obtained when the *inactivation pathway did not share any step of the activation of the fusion site* (model in Fig. 1 A, data not shown).

Discussion

Deducing lag times from the FDQ kinetics

To assess the formation time of the fusion junction between the influenza virus and erythrocyte membranes we have measured fusion in a suspension by the well established R 18 dequenching assay. We have interpreted the fluorescence dequenching kinetics as a cumulative distribution function of individual lag times τ_{lag} of viruses bound to the membrane. For the supposition that the dequenching kinetics reflects the kinetics of the activation of the fusion junction the following requirements have to be fulfilled:

(i) The kinetics of R 18 redistribution in the target membrane upon fusion is rapid and negligible with respect to the characteristic time of the formation of the fusion junction.

Indeed, it has been shown theoretically that for fusion of labeled influenza virus with cell membranes dye redistribution is fast once continuity has been established between both membranes (Rubin and Chen 1990; Chen and Blumenthal 1989). They have estimated the halftime of R 18 redistribution to be in the order of 20 ms which is significantly lower than the *lag times* t_{lag} measured under our conditions. Moreover, for complete removal of quenching R 18 does not need to redistribute over the entire erythrocyte. For a 100 fold dilution of R 18, which is sufficient for complete dequenching (Arbuzova et al. 1994), the dye has to redistribute only over 2% (3 μm^2) of the target membrane surface by taking into account that (a) the surface concentration of R 18 in the viral membrane is about 2 mol%, (b) the membrane area of an influenza virus and an erythrocyte correspond to 0.03 μm^2 and 130 μm^2, respectively. Thus, dye redistribution is not a

rate-limiting step of fluorescence dequenching[2]. However, this conclusion should be carefully checked for each system (Stegmann et al. 1993; Arbuzova et al. 1994).

(ii) Binding of virus to the target membrane does not contribute to the delay times. Our experimental conditions are in agreement with this prerequisite since (a) binding has already been accomplished before triggering the activation of the fusion pore and (b) is stable during the time course of the experiment.

Thus, it is reasonable to assume that the kinetics of fluorescence dequenching for influenza virus fusion are determined by the distribution of the rate of the formation of a single fusion junction but not by dye diffusion.

To elucidate the relation between the *lag time* t_{lag} and the mean (μ_{lag}) of the individual lag times τ_{lag} we have investigated the kinetics of fluorescence dequenching as a function of temperature. Both delay times increased when the temperature was decreased. The Arrhenius plot revealed a linear relationship between delay times and temperature. For both delay times the slope was similar suggesting that they (i) are determined by the same processes and (ii) can be used alternatively to characterize fusion activation. Although beyond our present study, we want to point out that the linear dependence of lag times in the Arrhenius plot does not necessarily mean that a single step is rate-limiting in the complex process of fusion activation (for detailed discussion see Bentz 1992). It should be mentioned, that the correlation between the slopes of both lag times is in accordance with our conclusion that dye redistribution is not a rate-limiting factor for the delay since the onset of FDQ (*lag time* t_{lag}) should not be affected by the redistribution rate.

The relevance of measuring lag times of virus-membrane fusion has been already pointed out. Several advantages of deducing the mean of individual lag times from FDQ curves can be put forward: *(i)* The *mean lag time* μ_{lag} is based on the whole population of bound viruses undergoing fusion with the target membrane. In contrast, the *lag time* t_{lag} reflects only the viruses which form stable fusion pores most rapidly. Although we have found that the frequently used term *lag time* t_{lag} may behave like the *mean lag time* μ_{lag} and, thus, may be used to characterize the whole population of fusing viruses, this observation cannot be generalized. During inactivation we observed a significant difference between both lag times in their sensitivity towards low pH-pretreatment of influenza virus (see below). *(ii)* The *mean lag time* μ_{lag} can be obtained even under conditions where the *lag time* t_{lag} is too short in order to resolve it appropriately. E.g., at 30 °C t_{lag} could not be resolved (<0.5 s) while μ_{lag} was still about 15 s. Using only t_{lag} as a measure would result in an underestimate of the time required to activate the fusion junction.

Often, the initial rate of fluorescence dequenching corresponding to the steepest part of the experimental curve is used to characterize the kinetics. It does not provide a measure for detecting changes in the kinetic pathway of the formation of the fusion junction. For instance, when simulating the inactivation by low pH-treatment on the basis of the model presented in Fig. 1A we did observe a decline of the initial rate without any alterations of the kinetic pathway and of the rate constants.

[2] The situation is different for cell-cell fusion, for which the rate of dequenching is also determined by fluorophore diffusion (Rubin and Chen 1990)

Inactivation of influenza virus

Similar to Ramalho-Santos et al. (1993) we observed a continuous decrease of the fusion extent upon preincubation of A/PR 8/34 at low pH. Moreover, we found an increase of both delay times, t_{lag} and μ_{lag}, respectively. This has not been reported previously. This may be related to technical limitations because the *lag time* t_{lag} upon preincubation cannot be resolved by "conventional" fluorescence spectroscopy under conditions usually selected for measuring influenza virus fusion (pH ~ 5.0, 37 °C).

The increase of the delay time upon low pH-preincubation may be rationalized on the basis of recent observations of Clague et al. (1991). These authors have shown that the kinetics of low pH-induced fusion of erythrocytes bound to cell membranes containing influenza HA depends on the density of pH-activated HA. The lag time decreased with increasing surface concentration of HA. Thus, a reasonable hypothesis is that extended lag times after low pH-inactivation can be explained by depletion of HA's with a fusion competent conformation.

In order to elucidate this notion we have performed mathematical simulation of the fusion kinetics. The development and comparison of kinetic models allow a more detailed interpretation of the data. The main purpose of the simulation of experimental data was a qualitative description reflecting the typical features of the dequenching kinetics. This approach allowed us to keep the number of intermediates low as well as the relation between the rate constants as simple as possible. Here, we have compared various models which differ in the inactivation pathway as well as in the influence of inactivation on the formation of the fusion site. In model A (Fig. 1) the inactivation pathway at low pH was independent of that of the activation of a fusion junction. In the second scheme, B, inactivation shares steps of the activation of the fusion pore, but inactivated HA's are not involved in those structures. However, both reaction schemes did not provide a qualitative description of our experimental curves and parameters. Thus, the inactivation process cannot be described solely by a depletion of fusion-competent HA-trimers which does not affect the formation of the fusion junction (see above).

In model C shown in Fig. 1 we have assumed that (1) the inactivation process involves essential steps of the formation of the fusion site and (2) the existence of intermediates along the inactivation pathway which can still transform to a fusion site ($k'_{FP} \neq 0$) but with a slower rate $k'_{FP} = 0.1\ k_{FP}$. The latter model reasonably reflects the alterations of the experimental kinetics after low pH treatment of influenza viruses. By simulating the inactivation process we established a continuous increase of the relative *mean lag time* μ_{lag} comparable to that obtained from experimental curves of FDQ (Fig. 7B). With increasing preincubation times at low pH the fraction of intermedi-

ate I_{PI} relative to intermediate I_1 rises. Thus, the kinetic contribution of the slower pathway leading to the fusion site ($I_{PI} \rightarrow FP \rightarrow FS$) becomes more prominent and yields an increase of μ_{lag}. After prolonged preincubation with $I_1 = 0$ and $I_{PI} \neq 0$, μ_{lag} reaches a plateau. In principal, the model also allows one to explain the weak sensitivity of t_{lag} at shorter times of preincubation at low pH. As long as $HA_A \neq 0$ and/or $I_I \neq 0$ the formation of the fusion site can proceed via the fast pathway ($I_1 \rightarrow FP \rightarrow FS$). The onset of FDQ should not be significantly affected. The small increase of experimentally determined t_{lag} under these conditions may due to limitations of detecting the onset of FDQ. The depletion of I_1 upon prolonged incubation ($I_I \rightarrow 0$) would result in a steep increase of t_{lag} which is in agreement with our experimental observations.

We have assumed that complete inactivation (HA_I) can also occur when the virus is bound to the target membrane. Therefore, even without preincubation at low pH (control) only a fraction of the viruses attached to the target form a fusion site. This is in accordance with our experimental data because the extent of fused viruses was significantly less than 100%. In the frame of the model the extent of complete inactivation is determined by the relations k_{IP}/k_{FP} and k_{IA}/k'_{FP}. However, the inactivation process of the viruses bound to the target membrane has to be considered carefully. Recently, it has been suggested that the presence of viral receptors on the target may prevent inactivation (Niles and Cohen, 1991; Ramalho-Santos et al. 1993). The limited extent of bound viruses undergoing fusion may also be explained by a complete lack of fusion activity in a fraction of the viruses.

Several conclusions can be drawn from our modelling of the fluorescence dequenching kinetics obtained upon triggering low pH-induced influenza virus fusion: 1. The inactivation pathway of hemagglutinin shares certain stages of the formation of the fusion pore. While this has been already suggested on the basis of experimental data (Ramalho-Santos et al. 1993), comparison of different models allow a better and clear judgement on the characteristic features of the inactivation pathway. 2. The alterations of the fusion kinetics after low pH preincubation of influenza virus cannot simply be explained by a depletion of fusion active HA's. Our data are rather compatible with intermediate structures of HA along the inactivation pathway which can still transform into a fusion site. 3. This could have major implications for the investigation of the fusion pore. On the one side, one has to be aware that the population of those pores is heterogenous with respect to structure and properties. On the other hand, investigations of the low pH structure of HA which are usually done in the absence of the target membrane does not necessarily reflect a complete fusion inactive form of HA. However, before conclusive conclusions can be obtained further studies on this point are warranted.

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