

# A long-lived state for influenza virus–erythrocyte complexes committed to fusion at neutral pH

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The low pH-induced fusion of influenza virus with intact erythrocyte plasma membranes is preceded by a delay time following pH reduction, that is itself pH- and temperature dependent. At 37°C/pH 4.8, lipid mixing between virus and target membranes begins < 2 s after pH reduction, whereas at 4°C/pH 4.8, fusion does not commence until > 10 min after pH reduction. We have found that within this time period at 4°C, a population of virus acquires the capacity to subsequently undergo fusion with high efficiency at elevated temperatures and pH 7.4. Both the kinetics and the extent of this pH 7.4 fusion depend upon the time of pre-incubation at pH 4.8/4°C. Incubation at pH 7.4/4°C, following this pre-incubation does not result in fusion, but the capacity to fuse at pH 7.4/37°C is retained for a time period exceeding 1 h. The longevity of this fusion committed state makes it amenable to biochemical and immunological analysis. We have shown that it is insensitive to dithiothreitol, neuraminidase and trypsin, but is incapacitated by thermolysin or protease K. We conclude that only the HA2 sub-unit of influenza haemagglutinin is a necessary protein component of later stages of the fusion pathway.

Influenza haemagglutinin; Membrane fusion; Octadecyl rhodamine

## 1. INTRODUCTION

Fusion between membranes of influenza virus and target cells is mediated by the influenza haemagglutinin glycoprotein (HA) in a low pH-induced conformation [1,2]. Thus a fusion reaction is conveniently triggered by changing the pH around virus–cell complexes from neutral to 5. Cloning of HA and expression of the gene product on the plasma membrane of cells demonstrated that this protein was sufficient to induce polykaryon formation with a pH dependence similar to that observed for fusion of intact virus with cells [3]. The development of the octadecyl rhodamine (R18) fluorescence dequenching assay [4] allowed direct measurement of the fusion kinetics [5].

By using pre-bound virus and stopped-flow mixing to rapidly reduce the pH, it was shown for vesicular stomatitis virus (VSV) that the onset of fluorescence dequenching (lipid mixing) was preceded by a delay time of 2 s at a pH close to the threshold for fusion, which decreased sharply with decreasing pH values [6]. A similar result has subsequently been shown for influenza–

erythrocyte ghost fusion (delay times < 4 s at 37°C [7,8]). The presence of a delay time indicates a complexity to the fusion reaction one might not otherwise have suspected for such a relatively simple biological system. The fusion kinetics can not be characterized by a single rate-determining step. Recognition of this fact and the manageable time scale of cell–cell fusion or virus–cell fusion at lower temperatures has prompted a dissection of the delay time into component parts. For influenza HA-mediated fusion of virus (X31 strain) the pH- [8] and temperature dependence [9] of the delay time have been characterized. In this paper we introduce the use of temperature-jumps (achieved by dilution), together with the R18 fluorescence dequenching assay to further characterize components of the delay time for low pH-induced fusion of influenza X31 virus with human erythrocytes.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Influenza, X31 strain, was prepared as previously described [10]. <sup>35</sup>S-Labelled virus was produced in MDCK cells according to Stegmann et al. [9]. Fresh blood was obtained from the NIH blood bank. Trypsin, thermolysin, neuraminidase (type V from *Clostridium perfringens*), and dithiothreitol (DTT) were all obtained from Sigma. Protease K was obtained from Boehringer-Mannheim.

### 2.2. Labelling of virus and binding to erythrocytes

2 µl of R18 in ethanol (1 mg/ml) was added to 0.5 ml of virus suspension (1 mg/ml), whilst vortexing. After incubation for 10 min at room temperature, free R18 was removed by elution of the labelled influenza from a Sephadex G25 PD10 column (Pharmacia, Piscata-

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Abbreviations: BHA, bromelain digested haemagglutinin; DTT, dithiothreitol; HA, haemagglutinin; PBS, phosphate-buffered saline; R18, octadecyl rhodamine; VSV, vesicular stomatitis virus.

way, NJ). The labelled virus was then incubated for 20 min with 0.25 ml of washed erythrocytes ( $5 \times 10^9$  cells per ml). After two washes the pellet was resuspended in 0.5 ml of phosphate buffered saline (PBS).

### 2.3. Fluorescence monitoring of fusion kinetics

An SLM 8000 spectrofluorimeter (SLM Instruments Inc., Urbana, IL) was used for all experiments. Excitation and emission wavelengths were 560 and 590 nm, respectively. 10  $\mu$ l of virus-cell suspension was added to a cuvette containing 2 ml of PBS/citrate buffer at a pre-set temperature and pH, which was stirred by a small magnetic bar. When a pre-incubation was incorporated into the protocol, 10  $\mu$ l of virus-cell suspension was first added to 100  $\mu$ l of buffer at the stated temperature to give the final pH indicated. This sample was then added to the cuvette at the specified time. Re-elevation of the pH to pH 7.4/4°C after pH 4.8/4°C incubations, was achieved by washing with 1.5 ml of cold PBS, pH 7.4, and addition of a resuspended pellet to the cuvette at the time indicated. Some experiments involved incubation with the cold PBS for 1 h, before proceeding with the fusion assay; during this time agents such as DTT, neuraminidase and various proteolytic enzymes were included. Re-elevation of the pH whilst monitoring the fluorescence intensity was achieved by injection of a small volume of 0.5 M Tris directly into the cuvette at the specified time. All fusion extents were normalized to a fluorescence value obtained after addition of 0.05% (w/v) Triton X-100, which was arbitrarily assumed to correspond to 100% dequenching. Monitoring of the effects of pre-treatments was carried out by SDS-PAGE analysis of  $^{35}$ S-labelled virus bound to erythrocytes at 1 cpm per 10 erythrocytes and treated in the same way. Gels were scanned using a Pharmacia Ultrascan densitometer.

## 3. RESULTS

Fig. 1 shows a typical time-course of R18 dequenching, resulting from fusion of influenza (X31) with intact erythrocyte membranes at 4°C/pH 4.8. We observe a delay of 10–20 min, which was always constant for a given virus/erythrocyte preparation. We then took virus-cell complexes after various times of incubation at pH 4.8/4°C and added them to an excess of buffer at pH 7.4/37°C. Fusion was observed (as judged by lipid mixing) at neutral pH, with virus-cell complexes for which lipid continuity had not yet been established during the low pH incubation period (Fig. 2, curves a–c).

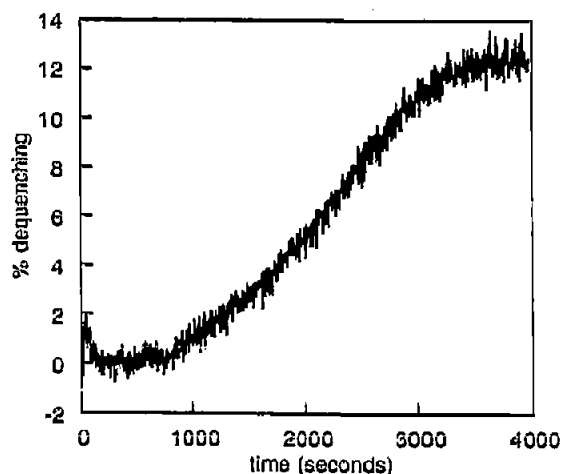


Fig. 1. R18 fluorescence dequenching, resulting from fusion between X31 influenza virus and erythrocytes at pH 4.8/4°C.

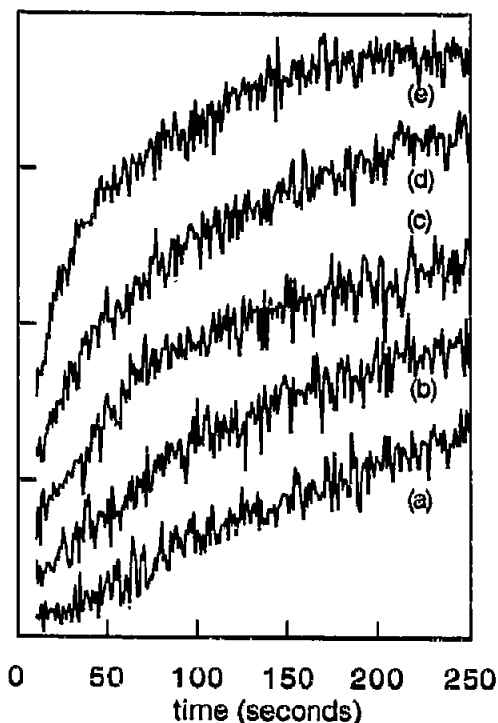


Fig. 2. Influenza X31–erythrocyte fusion. R18-labelled virus-cell complexes were pre-incubated for (a) 1, (b) 2, (c) 10, (d) 30 min in pH 4.8 buffer at 4°C and then added at time = 0 s to a 20  $\times$  volume of pH 7.4 buffer at 37°C. (e) virus-cell complex added from pH 7.4/4°C buffer to pH 4.8/37°C buffer at time = 0 s. Fluorescence dequenching curves have been offset for clarity; y axis increment is 9% fluorescence dequenching.

Furthermore, the kinetics and extent of this neutral fusion were dependent on the time of low pH incubation. Curve a of Fig. 2 is particularly interesting, as a delay time is evident. From Fig. 1 it can be seen that in the case of curve 2d, a small amount of lipid mixing has already taken place by the time temperature and pH are elevated (30 min). No fluorescence dequenching was observed, if after 10 min at 4°C the pH was elevated to 7.4 but the temperature was kept constant, nor at pH 7.4/37°C without pH 4.8/4°C pre-incubation.

A 10 min incubation of virus–erythrocyte complexes at pH 4.8/4°C, efficiently produces a pH 7.4/37°C, fusion-committed population (Fig. 2, curve c). Whilst no fusion is observed at pH 7.4/4°C, the committed state is effectively retained under these conditions, for long time periods (> 1 h). Retention is unaffected by incubation with 20 mM DTT (Fig. 3a), which is expected to dissociate the HA1 subunit of HA in its acid conformation [11]. We next examined sensitivity to trypsin. Like DTT one expects trypsin to dissociate the bulk of the HA1 sub-unit [1], leaving only the 1–27 fragment attached to HA2. We find that pH 7.4/37°C fusion is slightly accelerated by trypsin treatment of the fusion-committed complex (Fig. 3b). The lack of observed inhibition with both treatments is consistent. The enhance-

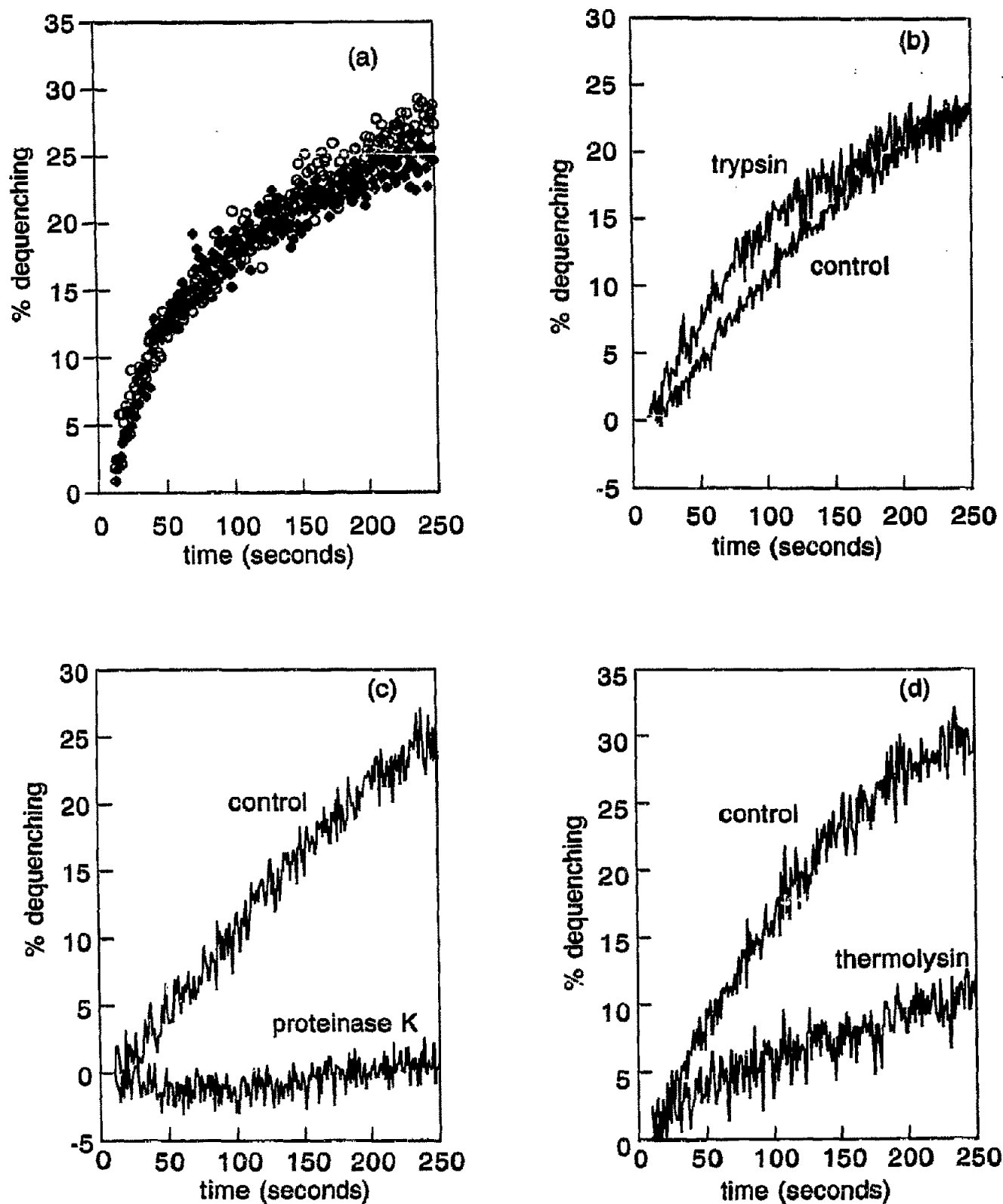


Fig. 3. Sensitivity of fusion commitment to various treatments. Virus-erythrocyte complexes were incubated at pH 4.8/4°C for 9 min. They were then incubated in 1.5 ml of PBS, pH 7.4/4°C for the specified time period. During this incubation the following substances were included (a) 20 mM DTT, 60 min (filled diamonds; controls are represented by open circles) (b) 0.1 mg/ml trypsin, 45 min, (c) 0.1 mg/ml proteinase K, 45 min, (d) 0.05% (w/v) thermolysin, 1 mM CaCl<sub>2</sub>, 75 min. Control curves were incubated for the same times in buffer without active agent. Following this incubation, the complexes were pelleted and resuspended in 100 µl of pH 7.4/37°C buffer, before addition to a stirred cuvette containing PBS, pH 7.4/37°C at time = 0 s.

ment of the fusion rate by trypsin treatment may be due to its effect on the erythrocyte surface, which has been shown to be primarily the degradation of sialoglycoprotein-a [12]. One possibility is that a steric constraint is thereby removed.

Incapacitation of the fusion-committed state can be achieved by treatment with proteinase K or with thermolysin (Fig. 3c and d). This demonstrates that protein components remain essential for the fusion pathway to proceed. Thermolysin digestion did not dissociate the virus-erythrocyte complex, whereas the proteinase K treatment released ~30% of bound virus, as judged by the fluorescence intensity of the virus-cell complexes after washing. It was important to examine the effects of all these treatments under our experimental conditions (i.e. virus bound to erythrocytes). Fig. 4 shows SDS-PAGE analysis of our treatments. At pH 7.4 virus is not susceptible to protease or to DTT. A 10 min pH 4.8/4°C incubation renders it susceptible. Trypsin, DTT, thermolysin and proteinase K all lead to disappearance of the HA1 band, when erythrocytes are pelleted with bound virus. We chose to use thermolysin because it has been shown to cleave BHA2 [13] and might therefore be expected to cleave HA2 under our conditions (in contradistinction to trypsin). The HA2 band co-migrates with the M protein band on SDS-PAGE and a visual impression can be misleading. Densitometric scanning of lanes showed that ~50% of this band disappears after thermolysin treatment (by ratioing against the constant nuclear protein band). This is consistent with at least partial digestion of HA2.

Taken together these results indicate that retention of the fusion state which we have identified and progression of the fusion reaction from this point, is independent of the influenza HA1 sub-unit. We also find that retention of the fusion committed state during pH 7.4/4°C incubation is unaffected by 0.4 mg/ml neuraminidase (data not shown). Without prior low pH incubation this treatment removes all virus capable of fusion from the cell surface within 30 min, by destruction of the receptor to which HA1 binds. This supports our interpretation of the protease and DTT experiments, in particular by using an agent whose effect is not predicated on a low pH-induced conformational change of HA.

#### 4. DISCUSSION

Influenza virus fusion at 4°C was first demonstrated by White et al. [14] and subsequently characterized by Stegmann et al. as a multi-step event [9]. In the latter study, confirmation that R18 dequenching at this temperature corresponds to fusion was provided by electron microscopic studies. In this paper we have taken advantage of the convenient time scale offered at 4°C to accumulate an intermediate of the fusion pathway characterized by its ability to fuse at pH 7.4/37°C and

by its inability to progress along the fusion pathway at pH 7.4/4°C. Under the labelling conditions we have used, no spontaneous R18 exchange from virus to erythrocyte is observed at pH 7.4/37°C without pH 4.8/4°C pre-incubation. We thus have a stable intermediate, the production or destruction of which we can assess by the criterion of kinetics and extent of R18 fluorescence dequenching following a temperature jump to 37°C/pH 7.4.

##### 4.1. Dequenching kinetics

Consider Fig. 2, curves a and e, i.e. fusion at 37°C/pH 7.4 following a 1 min accumulation of the fusion-committed state (curve a) and a conventional fusion at 37°C/pH 4.8 (curve e). The dequenching rate for (curve a) is clearly slower and a delay period of 20–30 s is evident. The presence of this delay time indicates that the completion of fusion from this committed state is a multi-step process. The delay time for curve e is beyond the resolution of this experimental configuration but has previously been shown to be about 1 s [8]. Yet for curve a, the pH-dependent component of the fusion pathway precedes the fusion trigger (temperature) at time = 0 and therefore cannot be a rate-limiting step. Why then should the kinetics of pre-activated fusion at pH 7.4 be slower? And furthermore, why should these kinetics depend on the time of pre-incubation at pH 4.8/4°C? (Fig. 2, curves a–d).

For cells expressing HA (GP4f) it was previously found that the entry into a fusion-committed state (5–10 s) represented a minor component of the delay time at pH 4.8/37°C (by reversing the pH at different points during the delay, [15]). One may expect the kinetics of virus fusion at pH 4.8/37°C to also be determined by steps beyond the initial activation and commitment to fusion. On the basis of the observation that the delay time is sensitive to the composition of the target mem-



Fig. 4. SDS-PAGE analysis of pre-treatments. <sup>35</sup>S-Labelled virus, bound to erythrocytes pre-incubated at pH 4.8 or 7.4 for 10 min at 4°C and then treated at pH 7.4/4°C with agents specified in the legend to Fig. 4 at the same concentrations and for the same time period. (A) Control, (B) trypsin, pH 7.4, (C) trypsin, pH 4.8, (D) DTT, pH 7.4, (E) DTT, pH 4.8, (F) thermolysin, pH 4.8, (G) proteinase K, pH 4.8. NP, nuclear protein, NA, neuraminidase; M, matrix protein.

brane and to the HA surface density, we postulated that the relative hydrophobicity of the two fusing membranes is of crucial importance to the fusion kinetics [8]. We now propose that after 1 min at 4°C/pH 4.8 (Fig. 2a) the effective hydrophobicity of the fusion milieu is low, relative to that produced in less than 1 s at 37°C/pH 4.8 (Fig. 2e) or by longer incubations at 4°C/pH 4.8, and that the rate-limiting steps for fusion are dependent on this property. It may be that these experiments and this interpretation refer to stages of the fusion pathway 'post-commitment', whereas commitment itself may be a more specific process.

#### 4.2. The nature of the intermediate

Susceptibility of the committed state to proteinase K and thermolysin (Fig. 3c and d) demonstrates that protein components are required to complete the fusion reaction and that they are accessible to protease. Our data thus rule out a temperature-sensitive pure lipid intermediate at this stage of the pathway. Proteinase K sensitivity also reinforces the negative data obtained with trypsin or DTT (see below), for which it could otherwise be argued that the relevant proteins at a putative fusion junction (presumably a small sub-population) are inaccessible to macromolecules.

For the soluble bromelain fragment of HA (BHA), susceptibility to DTT, trypsin and protease K is conditional on the same early low pH-induced conformational change [16]. We have used two treatments expected to dissociate the bulk of the HA1 sub-unit, once the HA has adopted the protease sensitive form (which influenza HA (X31) has been shown to adopt at 4°C within 15 s [9]). Resistance of the committed state to (i) 20 mM DTT, and (ii) trypsin, and confirmation that these treatments cause dissociation of HA1 under our experimental conditions (Fig. 4), imply that HA1 is not required for its retention, nor for the fusion pathway to proceed. It is striking that both of the agents (i.e. proteinase K and thermolysin), which we find destroy the commitment to fusion, are known to digest the HA2 sub-unit. In this case we have not formally excluded the influence of erythrocyte or other viral proteins, however, it is difficult to envisage how their proteolysis might abrogate fusion whilst binding is maintained.

Insensitivity to neuraminidase also shows that both

binding of virus and retention of fusion capacity is now independent of the initial HA1-sialic acid interaction. This result is consistent with a study by van Meer et al. [17] which has shown complexes between influenza-infected cells and ganglioside-containing liposomes to acquire neuraminidase resistance before lipid mixing is initiated. In summary, we propose that our results support an early hypothesis that the initial recognition/binding and fusion functions are compartmentalized to the HA1 and HA2 sub-units, respectively.

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