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Biochimica et Biophysica Acta 1330 (1997) 17–28

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Pores formed by influenza hemagglutinin [1]

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Received 2 January 1997; revised 26 May 1997; accepted 29 May 1997

Abstract

Low pH-induced fusion mediated by the hemagglutinin (HA) of influenza virus involves a conformational change in the protein that leads to the insertion of a ''fusion peptide'' of the protein into the target membrane. It has been suggested that this insertion, aided by the formation of a complex of multiple HA trimers, would lead to perturbation of the bilayer structure of the membrane, initiating fusion. Here we present data showing that the interaction of the bromelain released ectodomain of the protein (BHA) with liposomal membranes at low pH leads to pore formation, at least at low temperatures. Strongly temperature-dependent low pH-induced inactivation of BHA resulted in a complete lack of activity of BHA above 10°C. Even at 0°C, only about 5% of the BHA participated in pore formation. Viral HA was less rapidly inactivated and still induced pores at 37°C. BHA-induced pore formation showed a sigmoidal time course. Once BHA had formed a pore in one liposome, it did not form a pore in a further liposome. Quantitative analysis of pore formation indicated that one single BHA trimer sufficed to produce a pore. These data indicate that fusion peptide insertion perturbs the membrane and that the formation of a complex of trimers is not a prerequisite for the perturbation. © 1997 Elsevier Science B.V.

Keywords: Membrane fusion; Pore formation; Hemagglutinin; Influenza virus; Liposome

1. Introduction

Fusion of influenza virus with target membranes is mediated by the trimeric integral membrane protein, hemagglutinin (HA) (for reviews, see [1–3]). Each monomer of the trimer consists of two disulfide-linked subunits [4], the smaller one of which, HA2, is membrane-anchored and has a hydrophobic N-terminus, the so-called ''fusion peptide''. Low pH induces a conformational change in the protein [5], which results in the insertion of the fusion peptide into the target membrane [6,7] and the viral membrane [8]. The expression of HA on the surface of cells causes their fusion with neighbouring cells at low pH [9]. On the basis of experiments with cell

Abbreviations: BHA, Bromelain released ectodomain of influenza hemagglutinin; EDTA, ethylenediaminetetraacetic acid; HA, hemagglutinin; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MES, 2-morpholinoethane-sulfonic acid monohydrate; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl) phosphatidylethanolamine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine

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[1] This study was supported by grant 3100-042953.95/1 (to T.S.) and 31-32188.91 and 31-42045.94 (to G.S) of the Swiss National Science Foundation.

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PII S0005-2736(97)00134-X

lines that expressed varying densities of HA, it was demonstrated that multiple HA trimers are required for fusion [10,11]. Some data indicate that these trimers interact cooperatively [10,11]. It is often assumed that several HA trimers form a higher order oligomer or ''fusion complex''.

Insertion of the fusion peptide could change the structure of the lipid bilayer of the target membrane locally, particularly if the formation of a fusion complex would concentrate these peptides. Fusion of influenza virus with erythrocytes induces hemolysis [12–14] and the leakage of small molecular weight substances from liposomes [12], indicating that during fusion, pores are formed in the membrane of the fusion product. In this paper, the term pore is used to indicate any kind of defect leading to the leakage of substances across a membrane. Shangguan et al. [15] have shown that a rather large pore is generated during the fusion of influenza virus with liposomes, allowing the leakage of dextrans with a molecular weight of 10 000. However, it is not clear if this is the result of fusion with a leaky viral membrane or is caused directly by fusion peptide–membrane interactions. On the one hand, a preparation of membrane-free HA aggregates that are held together by hydrophobic interactions between the membrane anchors (HA rosettes) also had lytic activities at low pH [16–18]. HA rosettes are made by detergent solubilization of viral membranes, followed by purification of the protein and removal of the detergent [16]. On the other hand, the ectodomain of HA, prepared by bromelain digestion of HA (BHA), induced no [16] or very slow [17] hemolysis, although the fusion peptide of BHA is known to insert into the target membrane [19,20]. Therefore, it seems possible that residual detergent at least contributed to the lytic activities of HA rosettes. Alternatively, the lack of activity of BHA could be due to its instability at low pH values [21,22].

It was often suggested that changes in the structure of the bilayer created by fusion peptide insertion could be crucial for fusion (see reviews [1,23]), since, in order to merge the viral and the target membrane, the lipids at the site of fusion have to deviate at least temporarily from a bilayer structure [24]. Thus, structural changes in the membrane induced by HA resulting in pore formation might be related to the formation of lipid intermediate structures that are required for fusion. In this way, the lipid intermediates in the bilayers would lead to the formation of the first aqueous connection between the viral and the target membrane interior, the ''fusion pore''. However, electrophysiological measurements have shown that the fusion pores form before significant lipid mixing takes place, and that the pores may be entirely proteinaceous, resembling an ion channel made of HA trimers [25].

In this paper, we present data showing that BHA is able to interact with liposomal target membranes at low pH values, resulting in pore formation, at least at low temperatures. Strongly temperature-dependent low pH-induced inactivation of BHA was responsible for a complete lack of activity of BHA at temperatures above 10°C. Only a fraction of the BHA participated in pore formation even at 0°C. In contrast, viral HA was less rapidly inactivated, and still induced pores at 37°C. The use of BHA trimers instead of HA rosettes or viral HA allowed us to establish that one single BHA trimer was able to make a pore.

2. Materials and methods

2.1. Liposome preparation

Multilamellar vesicles were produced by resuspension of dry lipid films of egg phosphatidylcholine, egg phosphatidylethanolamine (both from Avanti Polar Lipids, Birmingham, AL, USA), gangliosides (Sigma, St. Louis, MO, USA; type III from bovine brain, estimated molecular weight 1500 g/mol) at a molar ratio of 6:3:1 in buffer containing 145 mM NaCl, 2.5 mM HEPES and 1 mM EDTA, pH 7.4. Subsequently, the suspension was frozen and thawed five times and large unilamellar vesicles were made from the multilamellar vesicles by extrusion (five–ten times) through 0.1 μm defined-pore polycarbonate filters (Nucleopore, Pleasanton, CA, USA) [26]. After extrusion, residual multilamellar liposomes were removed by centrifugation. Phospholipid phosphate was determined according to Böttcher et al. [27]. The size distribution of the liposomes was determined by dynamic laser light scattering at 632 nm using a He–Ne laser, an ALV-125 goniometer and an ALV-5000 correlator.

2.2. Virus

The X-31 recombinant strain of influenza A virus (from plaque C-22 [28]) was grown for us by the Schweizerisches Serum- und Impfstoffinstitut (Bern, Switzerland) in the allantoic cavity of embryonated eggs, and purified, handled and stored essentially as described before [29]. Viral phospholipid was extracted according to Folch et al. [30] and phospholipid phosphate was determined according to Böttcher et al. [27].

2.3. BHA preparation

BHA was prepared as described by Brand and Skehel [31] with minor modifications as specified by Harter et al. [19]. Briefly, virus was pelleted by centrifugation and resuspended in 100 mM Tris–HCl buffer, 57 μM 2-mercaptoethanol, pH 8.0. The protein was then released from the virus by digestion with 2 mg/ml bromelain (Calbiochem, La Jolla, CA, USA) at 37°C for 20 h. Subsequently, virus was removed by centrifugation and BHA was purified from the supernatant by molecular sieve chromatography on Sephadex G-75 in buffer containing 3.5 mM HEPES, 1 mM EDTA and 145 mM NaCl. As assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the protein was more than 95% pure. Protein concentrations were determined according to Bradford [32] using the BioRad protein assay (BioRad Laboratories, Hercules, CA, USA; using bovine serum albumin as the reference).

2.4. Calcein leakage measurements

Leakage of the small molecular weight fluorescent dye, calcein, from liposomes was assessed by monitoring its fluorescence at 515 nm, with excitation at 495 nm, using an SLM 8000 D spectrofluorimeter. At high concentrations, the fluorescence of calcein is self-quenched. Dilution of liposome-entrapped calcein into the aqueous buffer (5 mM HEPES, 10 mM MES, 15 mM sodium citrate, 135 mM NaCl, 1.0 mM EDTA, pH 5.0) surrounding the liposomes therefore leads to an increase in fluorescence intensity. Calcein was entrapped into liposomes by hydrating the lipid film in buffer containing 2.5 mM HEPES, 1 mM EDTA, 100 mM NaCl and 50 mM calcein at pH 7.4 or in buffer containing 5 mM HEPES, 10 mM MES, 15 mM citrate, 85 mM NaCl, 1.0 mM EDTA and 50 mM calcein at pH 5.0, respectively. Non-entrapped dye was then removed by molecular sieve chromatography on Sephadex G-75 with buffer containing 3.5 mM HEPES, 1 mM EDTA and 145 mM NaCl at pH 7.4 or in buffer containing 135 mM NaCl, 15 mM sodium citrate, 10 mM MES, 5 mM HEPES and 1 mM EDTA at pH 5.0, respectively. For evaluation of the kinetic measurements, the fluorescence data were normalized. The fluorescence intensity, F_∞, of completely dequenched calcein was obtained after lysis of the liposomes with the detergents Triton X-100 (7.6 mM) or octaethyleneglycol mono *n*-dodecyl ether (C12E8, Nikko Chemicals, Tokyo, Japan; 5.0 mM). The efflux of dye function, E(t), was calculated from the relative fluorescence intensities, F(t), according to Eq. (1), where F_0 represents the initial fluorescence

$$E(t) = (F_\infty - F(t))/(F\infty - F_0) \quad (1)$$

For easier comparison with the fusion data, $1 - E(t)$ is plotted in some of the figures, rather than E(t). This function has an initial value of zero and reaches a value of 1 when all liposomes are lysed. We observed an increase in fluorescence intensity, probably due to spontaneous leakage, when liposomes were kept at low concentrations, especially at 37°C or when the solution was stirred, even if no protein or virus was added. The increase in fluorescence intensity after 24 hours was about 15 to 35% of the difference between F_0 and F_∞. For that reason, long time experiments were done without further stirring, after mixing the components for several seconds. In this case, the fluorescence intensity F_s of a liposome solution, which was incubated for the same time under the same conditions, but in the absence of BHA, was taken as the initial level of fluorescence. The efflux E(24 h) was then calculated according to:

$$E(24h) = [F_\infty - F(24h)]/(F_\infty - F_s) \quad (2)$$

The lag time before the onset of leakage was defined as the time between the addition of BHA or virus and the intercept of the tangent to the inflection point of the leakage curve with the time axis [33], as first proposed by Bentz [34].

2.5. Fusion measurements

Fusion between virus and labeled liposomes was measured with a resonance energy transfer assay [35].

Labeled liposomes contained 0.6 mol.% each of *N*-(lissamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (*N*-Rh-PE) and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE). Fluorescence was recorded at excitation and emission wavelengths of 465 and 530 nm, respectively, with a 515-nm long-pass filter placed between the cuvette and the emission monochromator [36] on a SLM 8000 D spectrofluorimeter with continuous stirring in a thermostated cuvette holder. All measurements were carried out in buffer containing 135 mM NaCl, 15 mM sodium citrate, 10 mM MES, 5 mM HEPES and 1 mM EDTA at pH 5.0 or 7.4. For calibration of the fluorescence scale, the initial residual fluorescence intensity, F_0, was taken as the zero level and the intensity at infinite probe solution, F_∞, as the maximum. The latter value was obtained by lysis of the liposomes with 5.0 mM octaethyleneglycol mono *n*-dodecyl ether (C12E8, Nikko Chemicals) with correction for dilution. Hence, the calibrated fluorescence, F_n, was obtained by:

$$F_n = [F(t) - F_0]/(F_\infty - F_0) \quad (3)$$

In some experiments, dextran (MW 19 600 g/mol), at a concentration of 4.1 mM, was incorporated into liposomes containing *N*-NBD-PE and *N*-Rh-PE. A much higher pressure was needed to extrude the liposomes in this case, but a similar extrusion protocol as described above was followed.

3. Results

3.1. BHA induces pore formation in liposomes at 0°C

Influenza virus and HA rosettes have hemolytic activity and induce the leakage of small molecular weight markers from liposomes at low pH [12–17]. However, BHA, which, in contrast to HA rosettes, is not prepared in the presence of detergent, had little or no lytic activity [16,17]. Therefore, the activity of HA rosettes could be due to residual detergent and, in this case virus-induced lysis, could be a consequence of fusion, in line with the results obtained by Shangguan et al. [15], rather than resulting from the interaction of HA with target membranes. On the other hand, BHA is not very stable at low pH values and, thus, the lack of activity could be due to its inactivation [21].

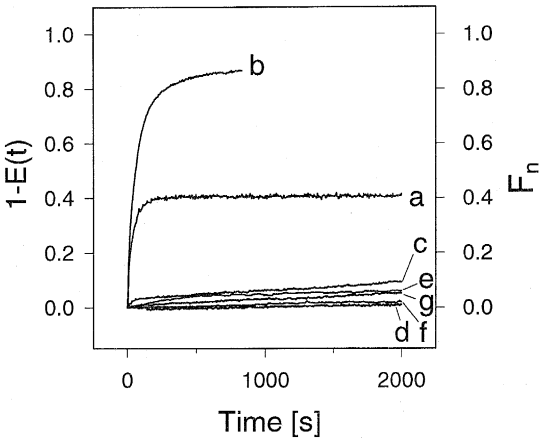

Fig. 1. Comparison of fusion and leakage at 37°C. At time 0, virus (a, b, d, e) or BHA (c, f) was injected into a cuvette containing liposomes at pH 5.0 (a, b, c, g) or pH 7.4 (d, e, f). The liposomes either contained calcein and leakage of this substance from liposomes was measured (b, c, e, f, g) as described in Section 2, or fusion was measured (a, d). In the latter case, liposomes contained the fluorescent phospholipid analogues *N*-NBD-PE and *N*-Rh-PE and fusion was measured by a resonance energy transfer assay, as described in Section 2. F_n: normalized NBD fluorescence. The leakage data are expressed as $1-E(t)$ (see Section 2) for ease of comparison with the fusion data. The Eq. (1) $-E(t)$ represents the amount of calcein that has leaked from the liposomes. All experiments were done at 37°C, at 5 μM liposomal phospholipid and 25 nM BHA or 5 μM viral phospholipid (a, b, d, e). (g) Leakage from liposomes under the same conditions as in c but in the absence of BHA.

To investigate these issues, we first compared the fusion of virus with large unilamellar liposomes (composed of phosphatidylcholine, phosphatidylethanolamine and gangliosides in a 6:3:1 ratio) with virus-induced leakage at pH 5.0, 37°C (Fig. 1). Fusion, measured using the resonance energy transfer assay of Struck et al. [35], leveled off at a fluorescence increase of 41% at pH 5.0, corresponding to 82% fusion [37,38]. Virus-induced leakage of the small molecular weight substance, calcein, from liposomes, measured by the relief of self-quenching of the dye upon its dilution in aqueous buffer, reached a level of 86%. At this pH and temperature, BHA induced less than 5% leakage (Fig. 1). At neutral pH, there was neither leakage nor fusion. BHA does not induce fusion [17].

To investigate if the lack of activity of BHA at this

temperature was due to the instability of BHA at 37°C, fusion and leakage were also investigated at 0°C (Fig. 2). In the absence of target membranes, the fusion activity of viral HA is rapidly affected by low pH incubation at 37°C, but not at 0°C [39]. At 0°C, virus-induced leakage reached 91% after 30 min, but fluorescence dequenching measurements of fusion had reached only 8% at this time point, corresponding to 16% fusion. Therefore, in contrast to the data published by Shangguan et al. [15], these data indicate that leakage was also from liposomes that did not fuse with the virus, suggesting that it could be caused by interactions of HA with liposomes, rather than being a secondary consequence of fusion. Significantly, at this temperature, BHA was found to induce 69% leakage within 30 min at pH 5.0. Both fusion and leakage showed a sigmoidal time course. No leakage or fusion took place at neutral pH (Fig. 2). The detailed pH-dependence of BHA-induced pore formation confirmed that a conformational change in the protein, which exposes the fusion peptide, was involved (Fig. 3).

Thus, it seemed likely that the lack of activity of BHA at 37°C was due to strongly temperature-dependent inactivation. To investigate this in more detail, the activity of BHA was measured at a range of temperatures (Fig. 4, panel A). We found that the rate of BHA-induced leakage decreased with increasing

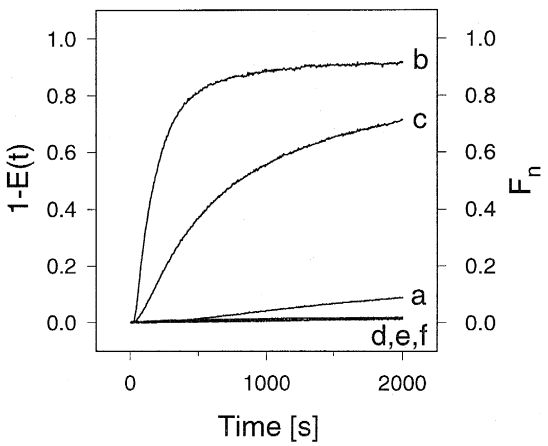

Fig. 2. Comparison of fusion and leakage at 0°C. At time 0, virus (a, b, d, e) or BHA (c, f) was injected into a cuvette containing liposomes at pH 5.0 (a, b, c) or pH 7.4 (d, e, f) at 0°C. Leakage (b, c, e, f) and fusion (a, d) were measured as in Fig. 1. All other conditions were as in Fig. 1.

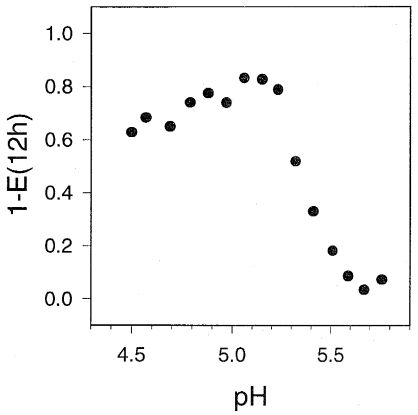

Fig. 3. Dependence of leakage on pH. BHA (1.15 nM) was incubated with calcein-containing liposomes (2.5 mM phospholipid) at 0°C and at various pH values. The extent of leakage was measured after 24 h, as described in Section 2. $1-E(12\ h)=$ amount of calcein that has leaked from the liposomes after 24 h.

temperature. Moreover, when BHA was incubated at low pH for 10 min at 0°C before the addition of liposomes, the rate of leakage was much reduced (Fig. 4, panel B). These data indicate that BHA is more rapidly inactivated at low pH than is viral HA, and that this inactivation is much faster at higher temperatures. Doms and Helenius [21] have shown that BHA trimers dissociate at low pH and at room temperature. In accordance with their data, we found that, as measured by high-performance liquid chromatography (HPLC) gel filtration, about 50% of BHA (1.0 μM) dissociated after a treatment at 25°C and pH 5 for 10 min, followed by reneutralization (data not shown). At lower concentrations, there should be at least as much dissociation.

The leakage of calcein across the membrane was the result of a membrane defect induced by HA, and not the result of complete destruction of the liposomes, as evidenced by the capacity of the liposomes to undergo fusion (Fig. 1). Also, negative stain electron microscopy showed intact liposomes after incubation with BHA at pH 5 (results not shown). Moreover, if liposomes containing dextran (MW 19 600) were prepared as described in Section 2, they could, in contrast to liposomes containing buffer, be pelleted by centrifugation in an Eppendorf centrifuge. After incubation with various concentrations of BHA at pH 5, 0°C, under conditions where calcein would have leaked completely (16 μM liposomal lipid, 3 nM

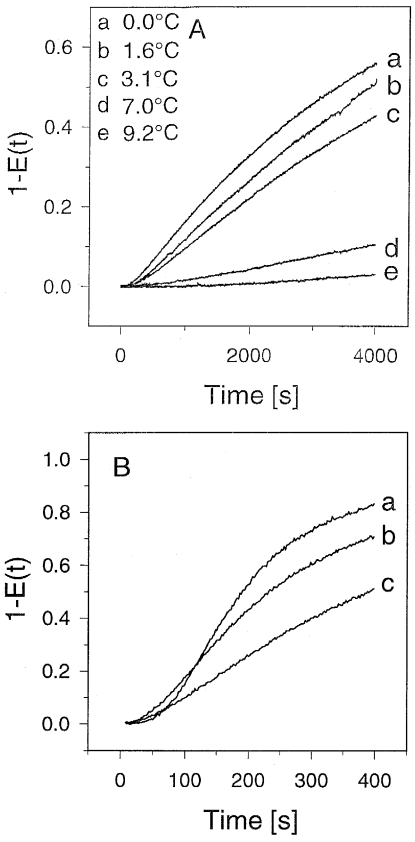

Fig. 4. Inactivation of BHA. Panel A: 10 nM BHA, pH 7.4, was added to a solution of calcein-loaded liposomes (10 μM phospholipid) in acidic buffer to a final pH of 5.0 at various temperatures. 1−E(24 h): as in Fig. 3. Panel B: BHA (29 nM) was incubated at pH 5.1 and 0°C for 0 s (a), 200 s (b) or 600 s (c) prior to the addition of calcein-loaded liposomes (2.5 μM phospholipid) at time 0, and leakage was measured.

BHA, 30 min), 92% of the liposomes could still be pelleted, indicating that high molecular weight molecules were retained in their interior.

BHA-induced leakage apparently reached a final level after incubation for several hours. To investigate if BHA caused leakage of only a fraction of liposomes or lysed all liposomes eventually, various amounts of BHA were incubated with liposomes at 0°C and pH 5.0 for 2, 24, 48 and 170 h. As shown in Fig. 5, leakage reached a constant level after 24 h. Further incubation did not increase the extent of leakage. If calcein-loaded liposomes were added to a sample of BHA and liposomes that had already reached a final level of leakage, no further BHA-induced leakage was observed (data not shown). Therefore, the interaction of BHA with liposomes was most likely irreversible; once a BHA molecule has bound to a liposome and formed a pore, it is not able to form a pore in a further liposome. To investigate at which point pore formation became irreversible, empty liposomes were incubated with BHA at low pH and, after various periods of time, calcein-loaded liposomes were added to the mixture. As shown in Fig. 6, after preincubation of BHA with liposomes for 10 min, less than 10% of the subsequently added

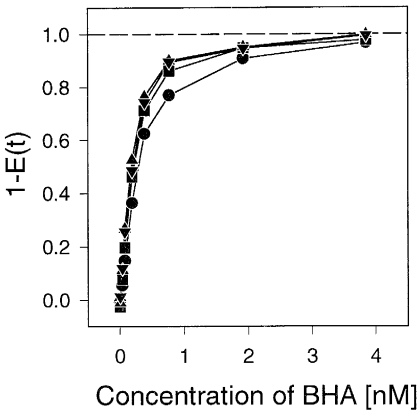

Fig. 5. Leakage from liposomes versus BHA concentration. BHA was added to calcein-containing liposomes (2.5 μM phospholipid) at pH 5.1, 0°C. The extent of leakage was measured after 2 h (circles), 24 h (squares), 46 h (triangles) and 170 h (inverted triangles).

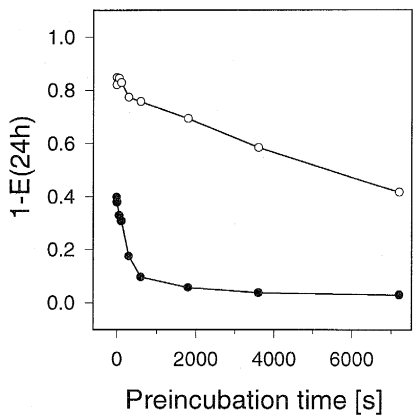

Fig. 6. Preincubation of BHA with liposomes at pH 5.0, 0°C. BHA (1.15 nM) was incubated with unlabelled liposomes (2.5 μM phospholipid) without calcein (dots) or without liposomes (circles) at pH 5.0, 0°C for a certain period of time. Then, calcein-loaded vesicles were added (2.5 μM). Leakage was measured after 24 h.

calcein-loaded liposomes (which would have been lysed if BHA was incubated at low pH in the absence of liposomes) were lysed under these circumstances.

3.2. Determination of the minimum number of BHA trimers required to form a pore

To determine the minimum number of BHA-trimers per liposome required to form a pore, we assumed that the binding of the protein to liposomes is non-cooperative, i.e., that the trimers bind to liposomes independently. Furthermore, it was taken into account that one BHA trimer induced leakage in only one liposome, as shown above. The fraction of emptied liposomes can then be calculated as a function of the protein/liposome ratio, assuming that the liposomes are of equal size. Dynamic laser light scattering measurements, as described in Section 2, showed that the size distribution of the liposomes, which were made by repeated extrusion through defined-pore filters, was very narrow, as described previously [26], around a mean diameter of 103 nm. In that case, the fraction $P_\mu(m)$ of liposomes with exactly m proteins are Poisson-distributed according to:

$$P_\mu(m) = e^{-\mu}\mu^m/m! \quad (4)$$

where μ is the average number of BHA trimers per liposome. The fraction of liposomes with one or more pores can be calculated from the efflux function, E(t), provided that the mode of leakage (''all or none'' or more gradual) is known. To determine the mode of leakage, we first measured the self-quenching of calcein incorporated in liposomes at a range of concentrations (not shown). Liposomes containing 50 mM calcein were then incubated at pH 5, 0°C with various concentrations of BHA for 24 h, resulting in a variety of leakage levels (Fig. 7). The liposomes were separated from the free dye by gel filtration chromatography, and the quenching of the calcein remaining in the liposomes was determined [40]. It was found that, even under conditions where more than 80% of the calcein had leaked out of the liposomes, the quenching in those that still contained the dye was still close to the quenching of 50 mM calcein (Fig. 7), clearly indicating an ''all or none'' mode of leakage for BHA. In this case, the fraction of emptied liposomes equals $[1 - E(t)]$. Assuming that m_p proteins are required to form a pore, then the fraction of liposomes that do not lose their content equals the sum of all fractions of liposomes with less than m_p proteins:

$$E(\mu, m_p) = SP_\mu(i),\ i = 0 \ldots m_p - 1 \quad (5)$$

and the fraction of emptied liposomes becomes:

$$1 - E(\mu, m_p) = 1 - SP_\mu(i),\ i = 0 \ldots m_p - 1 \quad (6)$$

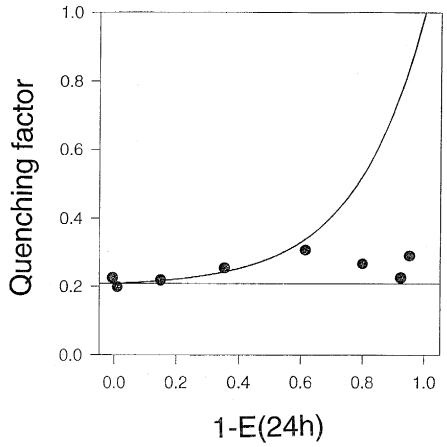

Fig. 7. ''All or none'' mode of leakage. Liposomes containing calcein (50 mM) were incubated with various concentrations of BHA at pH 5, 0°C and, after 24 h, the liposomes were separated from the leaked dye, and the quenching of calcein remaining in the liposomes was determined as described in the text (dots). The quenching expected in the case of an ''all or none'' mode of leakage is represented by a straight line and the curve shows the quenching expected for a gradual release of dye.

The final extent of leakage at several different concentrations of liposomes and BHA was calculated according to Eq. (6) (Fig. 8, panel A) and measured (Fig. 8, panel B). The measured data points were fitted using Eq. (6). Best fits were obtained with $m_p = 1$, yielding an exponential function, with a mean square residue of 0.009. For $m_p = 2$, the mean square residue was 0.015 and for $m_p = 3$, it was 0.021. These data indicate that the minimum number of BHA trimers required to form a pore was one. However, the concentrations of BHA at which lysis occurred were twenty-fold higher than expected if every BHA trimer would be active, as shown graphically in Fig. 8, panel B. Therefore, these data could either mean that only 5% of the BHA had pore-forming activity, or that the formation of very large complexes of BHA trimers (of the order of twenty trimers) in the solution was required prior to binding

to the liposomes in order to produce a pore. Considering that BHA is rapidly inactivated at low pH (Fig. 4) and that fragments of BHA containing the fusion peptide were found to bind to liposomes singly [41], the former possibility is more likely.

3.3. The kinetics of leakage

As can be seen most clearly in Fig. 4, panel A, BHA-induced leakage showed a sigmoidal time course. The occurrence of a ''lag phase'' before the onset of leakage indicated that there were at least two rate-limiting steps in the reaction leading to leakage. These steps could involve the conformational change in BHA, binding of the protein to the liposomes, insertion of the fusion peptide into the target membrane, and the perturbation of the target bilayer, which leads to pore formation. Preincubation of BHA with liposomes at pH 7.4 did not significantly shorten the lag, indicating that BHA-receptor binding was not rate-limiting. As preincubation of BHA at low pH in the absence of liposomes seemed to shorten the lag phase (cf. Fig. 4, panel A), we investigated whether or not the conformational change in BHA was rate-limiting. To this end, we preincubated BHA at 0°C, pH 5.0 for various times, in the absence of liposomes, and determined the lag, as described in Section 2 (Fig. 9). It was found that preincubation for up to 200 s shortened the lag, but longer preincubations did not shorten it further. Therefore, the conformational change contributes to the measured lag, but is not solely responsible for it. These data indicate that there were more than two steps leading to leakage.

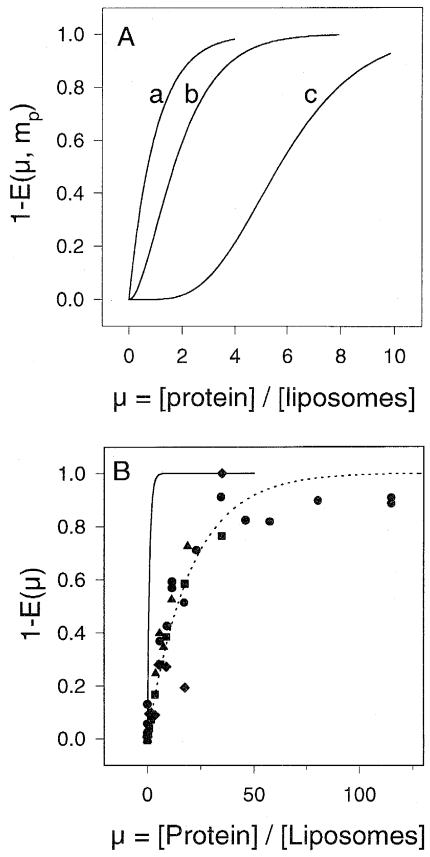

Fig. 8. Leakage from liposomes at different BHA trimer concentrations. Panel A: The fraction of emptied liposomes $[1-E(\mu, m_p)]$ was calculated using Eq. (6) (see text) for $m_p = 1$ (a), 2 (b) and 6 (c) and plotted versus the protein/liposome ratio, μ. Panel B: BHA was added to calcein-containing liposomes at pH 5.1, 0°C. The extent of leakage $[1-E(\mu)]$ was measured after 24 h as described in Section 2. The final phospholipid concentrations were 2.5 μM (circles), 20 μM (squares), 25 μM (triangles) and 50 μM (rhombi). Solid line: theoretical curve for $m_p = 1$ was calculated as described for panel A; dotted line: theoretical curve for $m_p = 1$ and interaction of one twentieth of the protein.

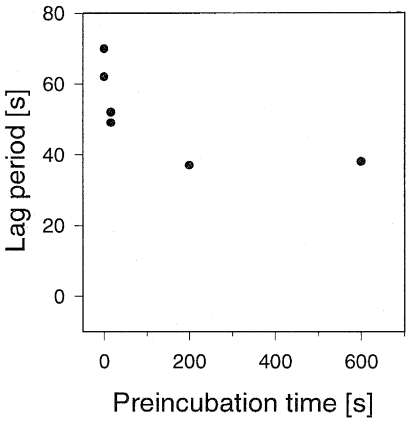

Fig. 9. Lag versus preincubation time of BHA at a low pH value. BHA (29 nM) was incubated at 0°C and pH 5.1 for the time indicated. Then, liposomes (2.5 μM phospholipid) were added and calcein leakage was monitored. The lag time was determined graphically, from the injection of BHA to the intercept of a tangent to the leakage curve at its inflection point, as described in Section 2.

4. Discussion

In this paper, we have shown that a single trimer of BHA, the soluble ectodomain of HA, is able to

form a pore in a liposome at the pH of HA-induced fusion. BHA was rapidly inactivated at low pH in a strongly temperature-dependent fashion and, therefore, significant pore formation at pH 5 was seen at temperatures below 10°C only. BHA-induced pore formation was preceded by a lag phase. Receptor binding of the molecule to liposomes did not appreciably contribute to the lag phase, and there still was a (shortened) lag after the low pH-induced conformational change in BHA, indicating that there were more than two rate-limiting steps in the reaction.

Temperature-dependent low pH-induced inactivation is probably the reason why previous experiments with BHA at 37°C showed no [16] or only very slow [17] hemolysis. The fusion and hemolytic activities of viral HA and HA rosettes are also inactivated at low pH values in a similar temperature-dependent fashion [16,39] but much more slowly than BHA, indicating that the transmembrane C-terminal part of HA is important for the stability of the molecule [21]. Even at 0°C, we found that only 5% of BHA participated in pore formation. Most likely, the remainder of the BHA was inactivated before it could interact with the membrane. For membrane-anchored HA, inactivation probably involves insertion of the fusion peptide in the membrane containing HA, rather than the target membrane [8], and also aggregation of HA trimers [42], most likely through hydrophobic interactions between their fusion peptides. Two mechanisms are likely to contribute to the inactivation of BHA. At low pH values, BHA molecules were shown to aggregate into rosettes, which are held together by hydrophobic interactions between the fusion peptides in the center of the rosette [43]. Since inactivation was irreversible and BHA trimers were shown to interact singly with the target membrane [41], BHA molecules aggregated in this fashion are most likely to no longer be capable of interactions with the membrane. Also, dissociation of the trimers of the low pH form of BHA contributed to the inactivation [21]. Alternatively, it is possible that some BHA trimers did bind to liposomes and insert their fusion peptides, but without forming a pore. However, binding experiments (R. Jiricek, unpublished) seem to indicate that the fraction of BHA that binds to liposomes is not very much larger than the fraction that causes pore formation.

The kinetic distinction between virus-induced leakage and fusion at 0°C (Fig. 2) and the pore-forming activity of BHA indicate that the leakage, which was shown to occur during influenza virus–liposome fusion [15], is caused by the interaction of HA with the target membrane. It remains a possibility that lipid mixing during fusion is also a leaky process, as suggested by Shangguan et al. [15], and that there is leakage after fusion, because the viral membrane has defects, as suggested by Young et al. [44]. It should be noted that Shangguan et al. [15] used a strain of virus whose HA has considerable sequence differences from the one used here.

HA-induced fusion may require the cooperative interaction of multiple HA trimers [10,11]. Cooperativity was first demonstrated using two cell lines expressing 1.9-fold different densities of the protein, which showed a 4.4-fold difference in the extent of fusion with liposomes [11]. Moreover, on the basis of the duration of the lag phase that preceded the fusion of nine cell lines expressing different densities of HA with erythrocytes, it was concluded that fusion involved the cooperative action of three to four trimers [10]. Originally, it was suggested that multiple HA trimers have to form a higher order oligomer or ''fusion complex'' in order to initiate fusion. In that case, most of the lag was thought to arise from the formation of the fusion complex. However, the data presented by Danieli et al. [10] and the results of other experiments with cell-surface-expressed HA [45] suggested that the cooperativity was not at this stage of the reaction, but at a later stage. In agreement with these observations, BHA-induced pore formation was also preceded by a lag phase, and the pores were formed by single trimers, as we have shown here. If the events that take place during the lag preceding BHA-induced lysis are comparable to those that take place during the lag preceding fusion, then it is most likely that fusion peptide–target membrane lipid interactions are rate-limiting and that these could play a key role in fusion.

In the case of BHA, these interactions may involve the formation of a certain arrangement of two or three fusion peptides in the membrane at the site of fusion. It is not clear how insertion of the fusion peptide would lead to pore formation. Pores are induced by synthetic fusion peptides, but, in most cases, small unilamellar vesicles were used as targets [46–49] and, because of the curvature strain on these

membranes, they are easily lysed. On using large unilamellar vesicles as targets, a clear correlation was found between the ability of synthetic influenza fusion peptides with different sequences to produce pores and the fusion activity of the corresponding wild-type or mutant HAs [50]. Typical lytic amphipathic helical peptides, which differ from the fusion peptide because they have positive charges, have been proposed to induce leakage either by forming pores according to a barrel–stave mechanism, involving clusters of transmembrane helices [51], or by increasing the negative curvature strain on membranes [52]. Fusion peptides probably do not form pores of the barrel–stave type. Studies with photoactivatable lipids have indicated that the fusion peptide of BHA is inserted as an α-helix [20]. The orientation of the inserted peptide has been found to be parallel to the plane of the membrane [53], or oblique [54,55]. The tryptophan residue of the molecule is close to the hydrocarbon/polar interface [56]. Even though there may be differences between BHA and viral HA, in the sense that viral HA might penetrate deeper into the target membrane [6], there are no indications that fusion peptides would span the membrane, like they should in a barrel–stave model. Moreover, other viruses that have fusion proteins with internal, rather than N-terminal, fusion peptides, also cause hemolysis and induce pore formation in membranes [13,57] and it is hard to imagine how these would span the membrane.

Alternatively, fusion peptides could have lytic properties because they increase the negative curvature strain on membranes, a mechanism proposed for lytic amphipathic peptides [58]. Synthetic influenza fusion peptides were shown to have effects that are compatible with this proposal, at low pH values, and peptides corresponding to mutants that lack fusion activity did not have these [59]. Although this seems to provide a plausible link between fusion and lytic activities of fusion peptides, considering that the formation of structures with negative curvature is thought to be required for fusion [1,23], it was recently shown that changes in the composition of the membrane that would affect the negative curvature strain have little effect on fusion or leakage [15]. Whether BHA is actually capable of altering membrane curvature or not remains to be determined.

The pore-forming activities of other peptides that do not span the membrane or resemble amphipathic lytic peptides, like magainin 2 [60] or melittin [61,62], cannot be easily understood. Most of these peptides presumably induce pore formation only as multimers, indicating the formation of a larger, defined structure. Considering the orientation of the influenza fusion peptide in the membrane, and the fact that a single trimer of BHA has pore-forming activity, the mechanism of pore formation by these types of peptides might give the most relevant information on BHA-induced pore formation.

Although influenza HA is the most extensively studied membrane fusion protein, and many details of the conformational change in the protein at the pH of fusion are known, how the protein succeeds in mixing the lipids of the viral and target membrane is not understood. Insertion of the fusion peptide into the target membrane seems to play a key role in the process. Studies with synthetic fusion peptides modeled after wild-type- or mutant fusion peptides almost invariably show a positive correlation between the pore-forming ability of the synthetic peptide and the fusion activity of the corresponding HA [48–50,59]. Therefore, it is likely that the effect of these peptides on the membrane, which gives rise to pore formation, is comparable to that which gives rise to lipid mixing. Such studies can now be performed with BHA, providing a more sophisticated model for observing the changes in the lipid membrane and obtaining information on the lipid intermediates that are involved in fusion.

Acknowledgements

We thank Dr. R. Glück and R. Mischler of the Swiss Institute for Serum and Vaccines for the preparation of influenza virus, A. Arbuzova for helpful discussions and Dr. M. Winterhalter for critical review of the paper.

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