Biochemistry

 Volume 34, Number 17 May 2, 1995

Accelerated Publications

Calorimetric Detection of Influenza Virus Induced Membrane Fusion†

Simon Nebel, Ingrid Bartoldus, and Toon Stegmann*

Department of Biophysical Chemistry, Biozentrum of the University of Basel, Klingelbergstrasse 70, CH 4056 Basel, Switzerland

Received January 13, 1995; Revised Manuscript Received March 10, 1995®

ABSTRACT: Membrane fusion induced by the hemagglutinin glycoprotein of influenza virus has been extensively characterized, but the mechanism whereby the protein achieves the merger of the viral and target membrane lipids remains enigmatic. Various lipid intermediate structures have been proposed, and the energies required for their formation predicted. Here, we have analyzed the enthalpies of fusion of influenza with liposomes by titration calorimetry. If a small sample of virus in a weak neutral pH buffer was added to an excess of liposomes at low pH, a two-component reaction was seen, composed of an exothermic reaction and a slower endothermic reaction. The exothermic reaction was the result of acid−base reactions between the neutral pH virus sample and low pH buffer and low-pH-induced changes in the virus. The endothermic reaction was not observed in the absence of liposomes and much reduced if acid-inactivated virus, which had lost its fusion but not its binding activity, was added to liposomes. The endothermic reaction was more temperature dependent than the exothermic reaction; its pH dependence corresponded with that of fusion and its enthalpy was higher if fusion was more extensive. These data indicate that most of the endothermic reaction was due to membrane fusion. The experimentally determined enthalpy of fusion, 0.6−0.7 kcal per mol of viral phospholipids, is much higher than expected on the basis of current theories about the formation of lipid intermediates during membrane fusion.

Low-pH-induced fusion mediated by the hemagglutinin (HA)[1] glycoprotein of influenza virus is the most extensively characterized membrane fusion event [for reviews, see Wiley & Skehel (1987), White (1992), and Bentz (1993)]. At the pH of fusion, a conformational change in the protein leads to the exposure of a "fusion peptide" (Skehel et al., 1982; Doms & Helenius, 1986; White & Wilson, 1987; Stegmann et al., 1990; Bullough et al., 1994), which is then inserted into the target membrane for fusion (Stegmann et al., 1991; Tsurudome et al., 1992). As a result, HA becomes an integral membrane protein in both the viral and the target membrane simultaneously. Despite the extensive knowledge about the structure of HA and the conformational change, little is known about the mechanism whereby HA induces the merger of the lipids of the two membranes.

It is clear that for membrane fusion the lipids of the two bilayers involved in fusion must, at least transiently and locally, form a nonbilayer structure (Wilschut & Hoekstra, 1986). Several structures have been proposed for these lipid intermediates in the case of influenza virus, such as inverted micellar intermediates (Burger et al., 1990; Bentz et al., 1990, 1991; Ellens et al., 1990) and stalks (Siegel, 1993a,b). Direct experimental verification of the existence of any intermediate lipid structures is difficult for a lack of techniques that can detect rare, local, and short-lived structures. Since fusion of influenza with liposomes can take place under conditions where inverted micelles do not form (Stegmann, 1993) and

† This study was supported in part by the Swiss National Science Foundation (Grant 3-33512.92).

* Corresponding author. Telephone: +41 61 267 2194. Fax: +41 61 267 2189. E-mail: stegmann@urz.unibas.ch.

® Abstract published in *Advance ACS Abstracts,* April 1, 1995.

[1] Abbreviations: HA, Hemagglutinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(4-morpholino)ethanesulfonic acid; PC, egg phosphatidylcholine; PE, egg phosphatidylethanolamine.

0006-2960/95/0434-5705$09.00/0

since fusion is not enhanced under conditions where they do form (Alford et al., 1994), inverted micelles are most likely not involved in influenza-induced membrane fusion. Stalks are hour-glass shaped (toroidal) structures composed of two fused outer membrane leaflets and the unfused inner leaflets of the fusion partners. Recent experiments showing that glycosylphosphatidylinositol-anchored HA induces the fusion of the outer membrane leaflets of the two bilayers participating in fusion, but not of the inner membrane leaflets (hemifusion), suggest that intermediate structures which have some of the properties expected of stalks may form during fusion (Kemble et al., 1994; Stegmann, 1994). Siegel (Siegel, 1993a,b) has calculated the free energies of the lipid intermediates involved in fusion via a stalk mechanism and compared these with the free energies of intermediates in inverted micelle formation. This theoretical analysis has yielded new insights into the mechanism of membrane fusion. For example, assuming that the formation of structures with the least energy would be favored, stalks are more likely to be involved in membrane fusion than inverted micellar intermediates, even under conditions where the latter could in principle form (Siegel, 1993a,b). However, such hypotheses remain untested, as the energies involved in membrane fusion have not yet been measured.

Here we present the first analysis of the enthalpies involved in influenza-induced fusion with liposomes by isothermal calorimetry. Upon addition of virus to liposomes at low pH, a two-component reaction was seen. It is shown below that this reaction was the result of an exothermic reaction, largely due to acid−base reactions between the buffered virus suspension and the low pH buffer and an endothermic reaction which was largely associated with membrane fusion. The enthalpy of this reaction was much larger than the calculated free energy difference between bilayers and stalk intermediates.

MATERIALS AND METHODS

Liposomes and Virus. Influenza virus, strain X-31, was grown for us by the Schweizerische Serum und Impfstoffinstitut (Bern, Switzerland) in embryonated eggs, purified by equilibrium density gradient centrifugation, and stored frozen at −70 °C in 0.3 M sucrose and 2.5 mM HEPES, pH 7.4. For calorimetry, aliquots were quickly thawed, pooled, and diluted with at least 18 volumes of 145 mM NaCl, 0.1 mM EDTA, and 2.5 mM HEPES, pH 7.4. The virus was then pelleted by centrifugation in an SW.28 rotor for 1 h at 25 000 rpm and resuspended in a small volume of the same buffer by repeated passage through the 25 Gauge needle of a hypodermic syringe. For the quantitation of viral phospholipid contents, lipids were extracted according to Folch (1957), and phosphate was assayed according to Böttcher (1961). Acid-inactivated virus was produced by incubating aliquots of freshly thawed virus at pH 4.8 for 15 min at 37 °C, after which the sample was neutralized, pelleted, and resuspended as indicated above. The fusion activity of virus and acid-inactivated virus was checked regularly by previously published methods (Stegmann et al., 1985).

Large unilamellar vesicles were produced from multilamellar vesicles by extrusion through 0.1 μm, or where indicated through 0.2 μm, defined-pore polycarbonate filters (Nucleopore, Pleasanton, CA) (Mayer et al., 1986). After extrusion, remaining multilamellar liposomes were removed by centrifugation. The multilamellar vesicles were produced by resuspension of dry lipid films in 145 mM NaCl and 2.5 mM HEPES, pH 7.4. Subsequently, the suspensions were frozen and thawed at least five times before extrusion. Small unilamellar vesicles were produced from large unilamellar vesicles by sonication. All liposomes were stored under argon at 4 °C. Phospholipid phosphate was assayed according to Böttcher (1961). Phospholipids were from Avanti Polar Lipids (Birmingham, AL), bovine brain gangliosides (type III, estimated molecular weight 1500) from Sigma (St. Louis, MO). Liposomes contained the phospholipids indicated in the main text, and in addition, trace amounts of *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine to enable the measurement of viral fusion activity by a resonance energy transfer assay as previously published (Stegmann et al., 1985).

Calorimetric Measurements. The instrument we used was a Microcal OMEGA MC-2 titration calorimeter (Microcal, Northampton, MA) as described by Wiseman et al. (1989). Ten or 15 μL of sample was injected slowly, over a period of 15 or 20 s, respectively, into the stirred reaction cell, containing 1.2778 mL of buffer at 35 °C. The solution in the syringe was maintained at the same temperature. The buffer was composed of 135 mM NaCl, 15 mM sodium citrate, 10 mM MES, and 5 mM HEPES, set to various pH with HCl or NaOH, and sometimes contained liposomes (final concentration 0.5 mM phospholipids), which came with a small volume of the buffer in which they were produced (145 mM NaCl, 2.5 mM HEPES, 0.1 mM EDTA, pH 7.4). An equivalent amount of the same buffer was included in control measurements without liposomes. The reference cell was filled with a corresponding buffer. All buffers and samples were degassed thoroughly before use. The calorimeter was calibrated electrically. Data acquisition by computer was done using software developed by Microcal, using the "Origin" program.

RESULTS

Membrane Fusion can be Detected by Calorimetry. Ten microliters of a concentrated influenza virus suspension in a weak neutral pH buffer was injected slowly with stirring into a reaction cell containing an excess of large unilamellar vesicles. The vesicles were composed of phosphatidylcholine, phosphatidylethanolamine, and gangliosides (6:3:1 ratio) at pH 5.1, 35 °C. As shown in the calorimetric tracing (Figure 1A), a two-component reaction was seen. Immediately after the onset of the injection (arrows), the reaction was apparently exothermic, reaching a minimum within 8 s after the end of the 15 s injection. A slow, broader, less intense endothermic reaction ensued, after which the signal returned to baseline. Three to four additional injections of virus gave identical results. To evaluate the results quantitatively, the enthalpy of the reaction was calculated by integration from the start of the injection until the final return to baseline. Since exothermic reactions by convention have a negative, whereas endothermic reactions have a positive sign, there was both a positive and a negative contribution to the integral. The result was a net exothermic reaction of −38.2 μcal per injection of 22 nmol of virus with a standard deviation of 2.7 μcal for four injections (Table 1). These data were highly reproducible between samples

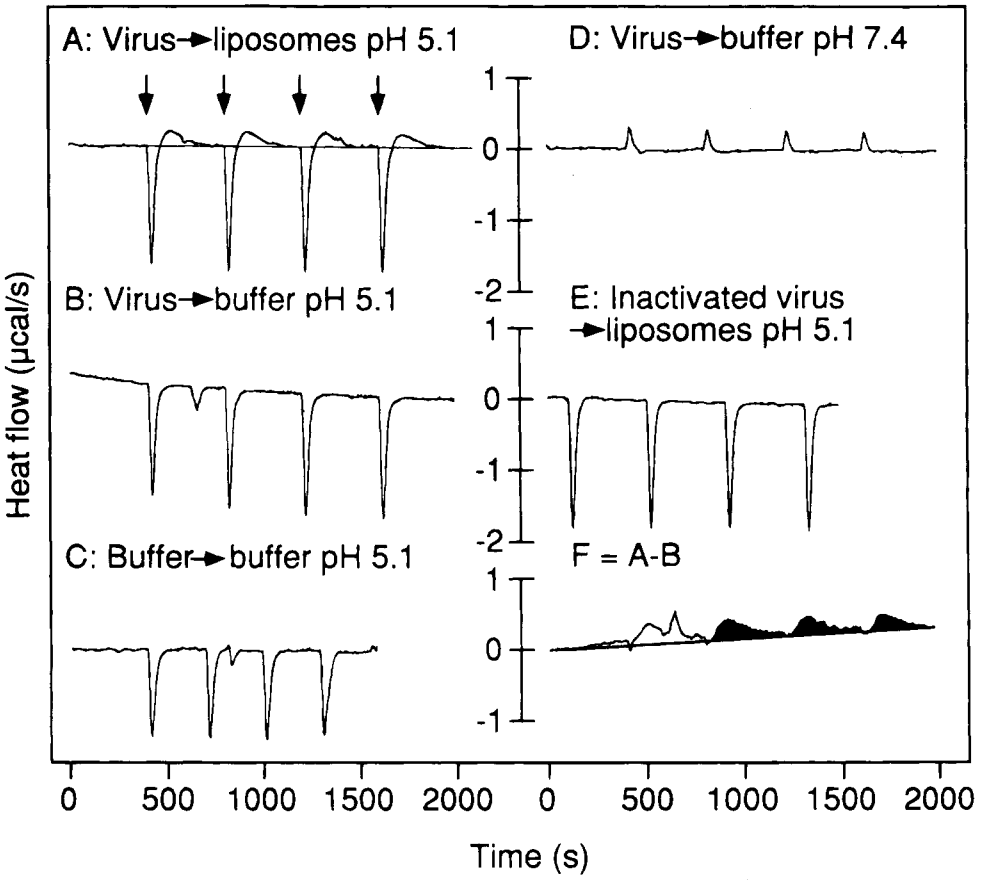

FIGURE 1: Calorimetric measurements of influenza virus−liposome interactions. Four injections each, starting at the arrows (as shown in A) of 10 μL of virus (A, B, D), acid-inactivated virus (E) or buffer (C) into a stirred cell containing pH 5.1 buffer only (B, C), liposomes in pH 5.1 buffer (A, E) or pH 7.4 buffer (D). For clarity, baselines are shown only in A and F. Injections took 15 s each. The cell was maintained at 35 °C. Final concentration of the liposomes was 0.5 mM phospholipid. Each injection of virus corresponded to about 20 nmol of viral phospholipid (see Table 1 for precise concentrations). (F) Result of a subtraction of the data set of panel B from that of panel A. The peaks between the first and the second injection of panel B and between the second and the third injection of panel C are electronic disturbances. For this reason, we have not further used the data from the first injection shown in A for F. Liposomes were composed of phosphatidylcholine (PC)/phosphatidylethanolamine (PE)/gangliosides in a 6:3:1 ratio and trace amounts of fluorescent probes (see Materials and Methods).

Table 1: Summary of Raw Calorimetric Results[a]

cell contained		syringe contained	
liposomes	pH	virus	buffer
PC/PE/gang		virus[b]	
+	7.4	4.9 ± 0.6	
−	7.4	2.4 ± 0.4	
+	5.1	−38.2 ± 2.7	−43.8 ± 1.7
−	5.1	−60.5 ± 1.2	−41.1 ± 1.4
PC/PE/gang		inactivated virus[c]	
+	5.1	−53.1 ± 2.0	
−	5.1	−60.9 ± 0.7	
phosphatidylserine		virus[d]	
+	5.1	27.7 ± 9.0	
−	5.1	−51.6 ± 2.5	−41.5 ± 2

[a] Shown are the enthalpies per injection ± SD for 4−5 injections of virus or buffer into cells with or without liposomes. [b] 22.0 nmol viral phospholipid per injection. [c] 20.0 nmol. [d] 21.4 nmol. PC/PE/gang stands for phosphatidylcholine/ phosphatidylethanolamine/gangliosides 6:3:1.

of the same virus preparation and between different virus preparations.

If virus was injected into a cell containing pH 5.1 buffer only, without target liposomes, an exothermic reaction (−60.5 ± 1.2 μcal per injection; Table 1) was observed (Figure 1B). This reaction was most likely the result of acid−base reactions between the components of the buffers, between virus and buffer, and other pH-induced changes in the virus. As has been demonstrated previously (Stegmann et al., 1987), under the conditions of this experiment, the fusion peptide becomes exposed as a result of the conformational change induced in HA by low pH, and at this temperature the fusion activity of the virus is subsequently destroyed. Clearly, no endothermic reaction resulted from the conformational change and inactivation.

To measure the contribution of acid−base reactions between the buffers, the weak neutral virus buffer was injected by itself into the reaction cell at pH 5.1. Only an exothermic reaction of around −40 μcal per injection was observed in this case, independent of the absence or presence of liposomes in the cell (Figure 1C, Table 1). Comparison of Figure 1B with 1C indicates that the interactions of virus with low pH buffer resulted in a more extensive exothermic reaction than the interactions of the buffers alone, indicating that the net contribution of conformational and other changes in the virus is exothermic. Taken together, the above data indicate that the addition of virus and/or neutral pH buffer to low pH buffer results in a purely exothermic reaction. Therefore, the endothermic component of the reaction observed after injection of virus into a liposome-containing cell at pH 5.1 must be the result of virus−liposome interactions.

Table 2: Times at Which the Thermograms Reached a Minimum and Returned to Baseline at 35 °C

injected	cell contents	minimum	baseline reached or crossed	return to baseline
buffer, pH 7.4	buffer, pH 5.1	24 ± 1	105 ± 9	
virus	buffer, pH 5.1	23 ± 1	141 ± 14	
virus	liposomes, pH 5.1	24 ± 2	78 ± 4	292 + 34

The thermograms shown in Figure 1A–C all reached a minimum 8 s after the end of the 15 s injection. If buffer was injected into buffer (Figure 1C), it took 105 s for the signal to reach baseline again (90 s after injection), indicating that there no longer was a temperature difference between the sample and the reference cell of the calorimeter (Table 2). If virus was injected into buffer, it took 141 s, reflecting the more extensive exothermic reaction seen in this case. In contrast, if virus interacted with liposomes (Figure 1A), the curve crossed the baseline after 78 s. These data indicate that the endothermic component of the two-component reaction of Figure 1A is due to a reaction which starts after the exothermic reaction but is already taking place when the overall signal is still below baseline. Most likely, acid–base reactions mediated by the diffusion of protons are much faster than virus–liposome interactions such as binding and fusion and due to the geometry of the cell (lollipop-shaped), full mixing of the injected sample of virus with liposomes is slow, explaining why the exothermic reaction precedes the endothermic one.

Interactions between virus and liposomes at low pH involve (i) the binding of HA to the liposomal gangliosides, (ii) insertion of the fusion peptide from the HA2 subunit of the protein into the target membrane, and (iii) membrane fusion. To investigate the relative contributions of receptor binding and these two latter steps to the endothermic component of the reaction, several experiments were performed. For the first series of experiments, we exploited the fact that HA1–ganglioside interactions also take place at neutral pH. If virus was allowed to prebind to liposomes at neutral pH on ice for several hours and then the mixture was injected into a cell containing low pH buffer, a two-component reaction, qualitatively similar to the one shown in Figure 1A, was seen (not shown), indicating that prebinding does not eliminate the endothermic component. Second, virus was injected into a cell containing liposomes at neutral pH. A slight endothermic reaction was observed (Figure 1D and Table 1), which could be due to virus–liposome binding at neutral pH. If buffer alone was injected into neutral pH buffer, no reaction was seen, and if virus was injected into buffer at neutral pH, a barely significant endothermic reaction was seen (Table 1). Therefore, at neutral pH there is an endothermic, albeit limited (around 2.5 μcal per injection), contribution of receptor binding.

For the second series of experiments, a preparation of virus was used which had been acid-inactivated by subjecting the virus to low pH in the absence of target membranes. The preparation was then neutralized and concentrated again in the weak neutral pH buffer used for calorimetry. Acid-inactivated virus retains the capacity to bind to ganglioside-containing liposomes but can no longer insert fusion peptides into the target membrane and consequently has no fusion activity (Stegmann et al., 1987, 1990). Importantly, if acid-inactivated virus was injected into a cell containing liposomes at low pH, only an exothermic reaction was seen (Figure 1E). However, quantitative evaluation of the data showed the enthalpy observed upon injection of acid-inactivated virus into low pH buffer containing liposomes to be higher by roughly 7 μcal per injection[2] than that observed if the buffer did not contain liposomes. Since there is no fusion, or insertion of fusion peptides into the target membrane under these circumstances, this indicates that the endothermic contribution of binding at pH 5.1 might be larger than that at pH 7.4 (Table 1; see also under "quantitative analysis" below).

Together, these data indicate that most of the endothermic component of the reaction is only seen under conditions where there is (1) insertion of the fusion peptide into the target membrane, and (2) membrane fusion. To distinguish between these two reactions, we made use of the differences in temperature dependence between them. Whereas fusion has a steep temperature dependence, the rate of fusion at 0 °C being 40 times lower than at 37 °C (Stegmann et al., 1990), insertion of the fusion peptide into the target membrane takes place within about 10 s even at 0 °C (Stegmann et al., 1991; Tsurudome et al., 1992). Thus we injected virus into a cell containing liposomes at 30 °C. Using this batch of virus and liposomes, we found that fusion at 30 °C was 1.5 times slower than at 35 °C, as measured by a fluorescence resonance energy transfer assay. However, the rates determined in the fluorimeter are not precisely comparable to the kinetics of reactions during calorimetry, because the latter are also affected by the slow mixing of the samples and the rate of heat dissipation across the cell. It was found that the endothermic, but not the exothermic, component of the reaction was slower at 30 °C (Figure 3). Unfortunately, we could not measure at much lower temperatures, because the endothermic component would then drown in the instrument noise. Taken together, the above data suggest that the endothermic component of the reaction is due to membrane fusion.

Two additional experiments confirmed this proposal. First, fusion of influenza virus with liposomes composed of pure phosphatidylserine is almost twice as extensive as that with the ganglioside-containing zwitterionic liposomes that were used for the above experiments (Stegmann et al., 1989). The excess fusion with negatively charged liposomes is at least partially due to a nonphysiological fusion reaction, as we have shown previously (Stegmann et al., 1986, 1989). If phosphatidylserine liposomes were used as fusion targets, a two-component reaction was seen, similar to that in Figure 1A (not shown). However, the endothermic component of the reaction was much larger than with ganglioside-containing zwitterionic liposomes, such that the overall reaction was endothermic (Table 1). Thus, if fusion is more extensive, the endothermic component of the reaction is enhanced.

Second, if the endothermic component resulted largely from membrane fusion, then the enthalpy of fusion can be calculated (ignoring, for the moment, the contribution of binding, see below) from the difference between the enthalpy of the reaction of virus with liposomes at low pH (Figure 1A) and the enthalpy resulting from injection of the virus alone into a low pH buffer (Figure 1B). To demonstrate

[2] Please note that these results are per injection of 10 μL. The concentrations of the different virus preparations vary sligthly (see Table 1 for concentrations).

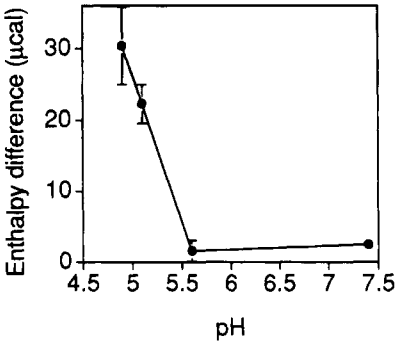

FIGURE 2: pH dependence of the endothermic component. Shown is the difference between the enthalpy of the reaction upon injection of virus into a cell containing liposomes minus that observed upon injection of virus into buffer at several pH values. The results are expressed per injection of 22 nmol of virus. Error bars are one SD.

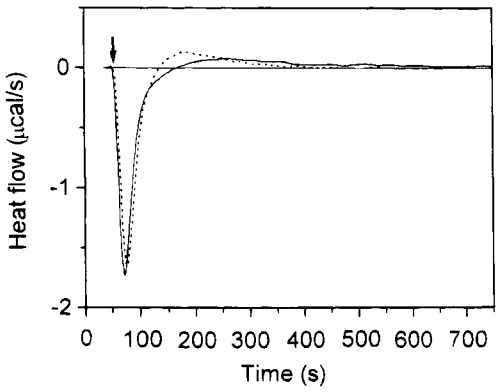

FIGURE 3: Fusion at 30 °C vs 35 °C. Single injection, starting at the arrow, of 17.7 nmol of virus into the cell of a calorimeter containing liposomes (as in Figure 1) at pH 5.1, 35 °C (dashed) or 30 °C (solid). In contrast to the other experiments in the paper, this experiment was performed with the help of a slightly improved Microcal Omega calorimeter, which had a more sensitive nanovolt preamplifier.

this graphically, we have subtracted the data points of Figure 1B from those of Figure 1A and plotted the results in Figure 1F. The darkened areas show the resultant endothermic component. In line with the results shown in Table 2, the resultant rose above baseline only after a noticeable delay of several seconds with respect to the onset of the exothermic reaction. If the resultant endothermic component was determined at several different pH values, a significant enthalpy was detected at pH 4.8 and 5.1, but not at pH 5.6 or above (Figure 2). Fusion of influenza with these receptor-containing zwitterionic liposomes has a very strict low pH dependence, with a threshold at pH 5.6 and an optimum at pH 4.8−5.2 (Stegmann et al., 1986). Therefore, the pH dependence of the endothermic component closely corresponded to that of fusion. Taken together, the above data indicate that the endothermic component was the result of membrane fusion.

Contents Mixing. Fusion of influenza virus with target membranes involves the merger of the lipids of the virus and liposomes and the coalescence of the contents of virus (core proteins, RNA) and liposomes (buffer). We attempted to measure the enthalpy associated with the coalescence of the internal contents of the virus and liposomes by reconstituting viral membranes without RNA and core proteins and performing calorimetric measurements of their interactions with liposomes. However, these attempts were unsuccessful because, after each successive injection of reconstituted membranes into a cell, the signal did not return to the original baseline but, instead, to a new, much lower baseline. Thus, we could not interpret the results. An indirect estimate of the contribution could be obtained from fusion of virus with liposomes of different diameter. Upon injection of 15.4 nmol of virus into pH 5.1 buffer at 35 °C, containing liposomes with a diameter of 0.2 μm, we measured −25.5 ± 2.1 μcal per injection, and for a diameter of 0.1 μm, −21.7 ± 2.1 μcal (four injections). Therefore, dilution of the viral contents into an eight times larger liposomal interior did not significantly change the enthalpy associated with fusion. Thus, the contribution of mixing the contents of virus and liposomes is negligible.

Quantitative Analysis of the Results. The enthalpy difference between the reactions shown in Figure 1A and 1B (darkened area in Figure 1F) is about 22 μcal per injection or 1013 ± 134 cal per mol of viral phospholipid at pH 5.1 (Table 1). If the enthalpy difference between the injection of acid-inactivated virus into low pH buffer with or without liposomes, 390 ± 105 cal/mol of viral phospholipid, is taken as an estimate of the endothermic contribution of binding at pH 5.1, then the fusion-associated enthalpy is around 623 ± 170 cal/mol of viral phospholipid. Error propagation was performed as recommended by Taylor (1988). Alternatively, we can calculate the fusion-associated enthalpy from the difference between the injection of virus to liposomes at pH 5.1 (as in Figure 1A) and the injection of acid-inactivated virus to liposomes at pH 5.1 (as in Figure 1E). In this case, because the amounts of virus injected were unequal, we first subtracted the contribution of acid−base reactions from the buffers from the data and then normalized for the amount of virus. The difference is 719 ± 195 cal per mol of viral phospholipid and thus very similar to the former estimate. Likewise, the contribution from all irreversible changes in the virus induced by low pH, including the conformational change in HA, can be calculated from the difference between the injection of virus (Figure 1B) and acid-inactivated virus (Figure 1C) into pH 5.1 buffer. This difference, 108 ± 115 cal/mol of viral phospholipid is most likely the sum of several different changes that might have an exothermic or endothermic contribution.

DISCUSSION

In this paper, we have measured the enthalpy of reactions taking place during fusion of influenza virus with liposomes by isothermal calorimetry. Upon fusion, an exothermic and an endothermic reaction were observed. The exothermic reaction resulted from acid−base reactions between the neutral pH buffer of the virus stock solution and the low pH buffer in the calorimeter cell as well as acid-induced changes in the virus. The slower and more temperature dependent endothermic reaction was not observed in the absence of target membranes, much reduced if using acid-inactivated virus that lacked fusion, but not binding activity, and was low pH dependent. As discussed below, we think that low-pH-induced conformational changes in viral proteins or viral inactivation did not contribute significantly to an endothermic reaction. Therefore, we conclude that around 0.6−0.7 kcal/mol of viral phospholipids of the endothermic reaction was due to membrane fusion.

The free energy differences between lipid bilayers and two types of intermediate lipid structures which are thought to

play a role in membrane fusion, stalks, and inverted micelles have been calculated by Siegel (1984, 1986a,b, 1993a,b). Although estimation of these differences does not provide direct information on the energy required for fusion, he reasoned that they are lower bound estimates of the activation energy required for formation of the intermediate structures (Siegel, 1993a,b). Therefore, comparison of the free energy differences for can provide a relative estimate of the likelihood that one or the other intermediate is involved in fusion. Using this elegant approach, it could be demonstrated that inverted micelles would require 4−5 times more energy than stalks in most circumstances, and therefore stalks are more likely to be involved in fusion (Siegel, 1993a). The fusion enthalpy that we have measured here is very large in comparison with those estimates of the energy required for the formation of lipid intermediates. The free energy of one stalk intermediate of the size expected for influenza fusion (Siegel, 1993b) would be around 100−200 kT (Siegel, 1993a; and Siegel, personal communication) above that of the corresponding bilayers, where k is Boltzman's constant and T the absolute temperature in Kelvin. Assuming influenza virus to be about 0.1 μm in diameter, there are about 100 000 phospholipid molecules per virion. Thus, assuming that one stalk forms per fusion event, one would expect free energy differences of 0.6−1.2 cal per mol of viral phospholipid, which is three orders of magnitude less than the fusion enthalpy that we have measured.

Under the conditions of the calorimetric experiments, 100% of the influenza virions that we added to liposomes fused, as measured by a resonance energy transfer assay (not shown). We have found previously that fusion of influenza with liposomes remains essentially limited to one round, i.e., virions fuse only once with a liposome, and there is very little fusion of virions with fusion products (Nir et al., 1986; Stegmann et al., 1989). Binding experiments, carried out as described elsewhere (Stegmann et al., 1995), indicated that approximately 7−10 liposomes bound to one virus during the course of the calorimetric measurements (not shown). It is not known how many stalks could form between one virion and one liposome. Considering that virions and liposomes are spheres contacting each other, it is hard to imagine how more than a few stalks of the dimensions expected in influenza fusion (Siegel, 1993b) could form. Also, as the free energy of a fused bilayer is mostly lower than that of a stalk intermediate, most stalks would proceed to fuse, and therefore the total number of stalks formed per virion is probably low. But even assuming that all virions form 2 stalks with each of 10 liposomes, one would expect the free energy difference to be no more than 12−24 cal/mol of viral phospholipid.

This discrepancy between the calculated lower bound activation energy for fusion and the fusion enthalpy that we have measured may indicate that the energy barrier to membrane fusion is much larger then the free energy difference between stalks and bilayers would suggest. One explanation for this could be that the dimensions of stalks were underestimated; the free energy of stalks increases monotonically with their diameter. However, large stalks would be very unstable (Siegel, 1993a). Alternatively, lipid intermediates other than stalks or inverted micelles may be involved in fusion. On the other hand, there is experimental evidence that the formation of structures resembling stalks, which do not proceed to fuse, can be induced by mutant HA molecules (Kemble et al., 1994; Stegmann, 1994). Therefore, if fusion does involve stalks, it would be most logical to propose that a step preceding stalk formation, e.g., the formation of a fusion complex from multiple HA molecules would be energy-intensive.

There has been one earlier attempt to measure membrane fusion by titration calorimetry. An exothermic reaction was seen during fusion of small unilamellar vesicles, composed of pure phosphatidylserine, induced by Ca^{2+} (Rehfeld et al., 1981). However, the interpretation of these results was confounded by the enthalpy of Ca^{2+} binding to the vesicles, the contribution of the release of curvature strain by the fusion of these vesicles, and that of the aggregation of the vesicles by Ca^{2+}. Thus, we cannot compare our data with those.

We do not think the endothermic reaction, which was observed upon influenza fusion, was due to aspects of the conformational change in HA or the low-pH-induced destruction of the viral fusion activity for three reasons. First, the endothermic reaction was dependent on the phospholipid composition of the liposomes. Using phosphatidylserine liposomes as targets, one would expect a similar conformational change, but more extensive fusion (Stegmann et al., 1989), and in this case a more endothermic reaction was observed (Table 1). Second, all of the changes that involve HA molecules which are not in contact with liposomes, such as the conformational change and inactivation, take place when virus is exposed to low pH at 35 °C, as in Figure 1B, which results in an overall exothermic reaction. Third, no statistically significant (105 ± 118 cal/mol) enthalpy contribution of irreversible low-pH-induced changes, such as the conformational change in HA, was found (compare Figure 1B and 1E).

Theoretically, it is possible that there are differences in enthalpy between HA molecules bound to liposomes and those which are not bound to liposomes, because it has been found that the fusion capacity of membrane bound HAs is not (Sato et al., 1983; Stegmann et al., 1987) or more slowly (Ramalho-Santos et al., 1993) inactivated. Inactivation involves the fusion peptides of HA. Some of these peptides enter the target membrane (Stegmann et al., 1991; Tsurudome et al., 1992), and most likely HA molecules that have their fusion peptides inserted are involved in fusion. Inactivation may occur either because fusion peptides enter the viral rather than the target membrane (Weber et al., 1994) or because of lateral aggregation of the HA molecules (Gutman et al., 1993), which could be mediated by hydrophobic interactions between the fusion peptides. Since only a very small percentage of viral fusion peptides are inserted (Tsurudome et al., 1992), the specific contribution of the HA molecules with inserted fusion peptides to the measured enthalpy is most likely limited even if their conformation is different from the bulk of the HA molecules. The latter will be inactivated; as shown, inactivation gave rise only to an exothermic reaction. For the strain of virus used here, inactivation occurs after the conformational change in HA (Gutman et al., 1993; Stegmann et al., 1987); for another strain the rate-limiting step to the conformational change and inactivation appear the same (Ramalho-Santos et al., 1993). Complete inactivation of all viral HAs occurs during the reaction shown in Figure 1B and results in an overall exothermic reaction which is faster than the endothermic reaction. For these reasons, we think that the endothermic

reaction is not due to an endothermic contribution of aspects of the conformational change in HA, or to inactivation, but is due to membrane fusion.

It is possible that the fusion enthalpy that we have measured to some extent directly reflects an increase in entropy upon lipid mixing, but, in the absence of any data on the entropy or enthalpy of lipid mixing, this is difficult to determine.

It has been speculated that the energy released upon the conversion of HA from its neutral to its acid form would drive the fusion reaction (Siegel, 1993b), because several lines of evidence suggest that the neutral pH conformation of HA could be metastable relative to the low pH structure (Skehel et al., 1982; Ruigrok et al., 1986; Carr & Kim, 1993; Bullough et al., 1994). There are no direct measurements of the energies involved in the conformational change. However, by differential scanning calorimetry of HA it could be shown that the unfolding of the neutral pH form of HA gave rise to a single endotherm at 66.5 °C, with an enthalpy of 980 kcal/mol of HA (Krumbiegel et al., 1993); for low pH treated HA the transition temperature was lower, with an enthalpy of 100 kcal per mol (Remeta et al., 1992). If virus is heated to 62 °C at neutral pH, it becomes fusogenic even at neutral pH (Ruigrok et al., 1986). Thus, if the heat-induced changes in HA and the low-pH-induced changes are at all comparable, one interpretation of these data might be that the neutral to low pH conversion of HA is endothermic, contradicting the notion that the neutral pH structure is metastable, and involves an enthalpy equivalent to the difference between between the neutral and low-pH-transition of 880 kcal/mol HA. Assuming 500 HA trimers per virion, this would imply 13.3 kcal/mol of viral phospholipid for the low pH induced conformational change, or 266 μcal per injection of 20 nmol. Clearly, we have not observed such a large, endothermic reaction (cf. Figure 1B). Therefore, unless there are even larger compensatory exothermic contributions from other processes that are absent in acid-inactivated virions (cf. Figure 1E), our data do not support this interpretation. Perhaps the definitive answer to this question could come from direct measurements of the conformational change in HA by titration calorimetry.

ACKNOWLEDGMENT

We thank dr. D. Siegel for his extensive critical advise, Dr. J. Engel and Mrs. N. Schönbrunner for their criticism of the paper, Dr. J. Seelig for his advise and for the use of his calorimeters, P. Ganz for expert maintenance of the calorimeters, Dr. R. Blumenthal for making the result of his experiments available to us, and Dr. R Glück and R. Misschler of the Swiss Institute for Virus and Vaccines for their generous gift of influenza virus.

REFERENCES

Alford, D., Ellens, H., & Bentz, J. (1994) *Biochemistry 33,* 1977−1987.

Bentz, J. (1991) in *Advances in Membrane Fluidity* (Aloia, R. C., Curtain, C. C., & Gordon, L. M., Eds.) pp 259−287, Vol. 5, Wiley-Liss, Inc., New York.

Bentz, J., Ellens, H., & Alford, D. (1990) *FEBS Lett. 276,* 1−5.

Böttcher, C. J. F., Van Gent, C. M., & Fries, C. (1961) *Anal. Chim. Acta 24,* 203−204.

Bullough, P. A., Hughson, F. M., Skehel, J. J., & Wiley, D. C. (1994) *Nature 371,* 37−43.

Burger, K. N. J., Knoll, G., Frederik, P. M., & Verkleij, A. J. (1990) in *Dynamics and Biogenesis of Membranes* (Op den Kamp, J. A. F., Ed.) pp 185−196, Springer-Verlag, Berlin.

Carr, C. M., & Kim, P. S. (1993) *Cell 73,* 823−832.

Doms, R. W., & Helenius, A. (1986) *J. Virol. 60,* 833−39.

Ellens, H., Bentz, J., Mason, D., Zhang, F., & White, J. (1990) *Biochemistry 29,* 9697−9707.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226,* 497−509.

Gutman, D., Danieli, T., White, J. M., & Henis, Y. I. (1993) *Biochemistry 32,* 101−106.

Kemble, G. W., Danieli, T., & White, J. M. (1994) *Cell 76,* 383−391.

Krumbiegel, M., Blumenthal, R., Ginsburg, A., & Remeta, D. P. (1993) *Biophys. J. 64,* A171.

Mayer, L. D., Hope, M. J., & Cullis, P. R. (1986) *Biochim. Biophys. Acta 858,* 161−168.

Nir, S., Stegmann, T., & Wilschut, J. (1986) *Biochemistry 25,* 257−66.

Ramalho-Santos, J., Nir, S., Düzgünes, N., Pato de Carvalho, A., & da Conceição Pedroso de Lima, M. (1993) *Biochemistry 32,* 2771−2779.

Rehfeld, S. J., Düzgünes, N., Newton, C., Papahadjoupolos, D., & Eatough, D. J. (1981) *FEBS Lett. 123,* 249−251.

Remeta, D. P., Krumbiegel, M., Blumenthal, R., & Ginsburg, A. (1992) *Biophys. J. 62,* A177.

Ruigrok, R. W., Martin, S. R., Wharton, S. A., Skehel, J. J., Bayley, P. M., & Wiley, D. C. (1986) *Virology 155,* 484−97.

Sato, S. B., Kawasaki, K., & Ohnishi, S. I. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80,* 3153−3157.

Siegel, D. P. (1984) *Biophys. J. 45,* 399−420.

Siegel, D. P. (1986a) *Chem. Phys. Lipids 42,* 279−301.

Siegel, D. P. (1986b) *Biophys. J. 49,* 1171−1183.

Siegel, D. P. (1993a) *Biophys. J. 65,* 2124−2140.

Siegel, D. P. (1993b) in *Viral Fusion Mechanisms* (Bentz, J., Eds.) pp 475−512, CRC Press, Boca Raton, FL.

Skehel, J. J., Bayley, P. M., Brown, E. B., Martin, S. R., Waterfield, M. D., White, J. M., Wilson, I. A., & Wiley, D. C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79,* 968−72.

Stegmann, T. (1993) *J. Biol. Chem. 268,* 1716−1722.

Stegmann, T. (1994) *Curr. Biol. 4,* 551−554.

Stegmann, T., Hoekstra, D., Scherphof, G., & Wilschut, J. (1985) *Biochemistry 24,* 3107−3113.

Stegmann, T., Hoekstra, D., Scherphof, G., & Wilschut, J. (1986) *J. Biol. Chem. 261,* 10966−10969.

Stegmann, T., Booy, F. P., & Wilschut, J. (1987) *J. Biol. Chem. 262,* 17744−17749.

Stegmann, T., Nir, S., & Wilschut, J. (1989) *Biochemistry 28,* 1698−1704.

Stegmann, T., White, J. M., & Helenius, A. (1990) *EMBO J 9,* 4231−4241.

Stegmann, T., Delfino, J. M., Richards, F. M., & Helenius, A. (1991) *J. Biol. Chem. 266,* 18404−18410.

Stegmann, T., Bartoldus, I., & Zumbrunn, J. (1995) *Biochemistry* (in press).

Taylor, J. R. (1988) *Fehleranalyse: Eine Einführung in die Untersuchung von Unsicherheiten in physikalischen Messungen,* VCH, Weinheim.

Tsurudome, M., Glück, R., Graf, R., Falchetto, R., Schaller, U., & Brunner, J. (1992) *J. Biol. Chem. 267,* 20225−20232.

Weber, T., Paesold, G., Galli, C., Mischler, R., Semenza, G., & Brunner, J. (1994) *J. Biol. Chem. 18535*−18538,

White, J. M. (1992) *Science 258,* 917−924.

White, J., & Wilson, I. A. (1987) *J. Cell Biol. 105,* 2887−2896.

Wiley, D. C., & Skehel, J. J. (1987) *Annu. Rev. Biochem. 56,* 365−394.

Wilschut, J., & Hoekstra, D. (1986) *Chem. Phys. Lipids 40,* 145−66.

Wiseman, T., Willington, S., Brandts, J. F., & Lung-Nau, L. (1989) *Anal. Biochem. 179,* 131−137.

BI950093A