Irreversible Unfolding of the Neutral pH Form of Influenza Hemagglutinin Demonstrates That It Is Not in a Metastable State†

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ABSTRACT: The thermal denaturation of the proteins of influenza virus has been measured by differential scanning calorimetry in the presence and absence of lipids as a function of scan rate. We have applied theories of irreversible thermodynamics to obtain the activation energy. In the presence of liposomes of dioleoylphosphatidylcholine with the ganglioside, GD_{1a}, the denaturation temperature of the hemagglutinin protein is lowered. This lowering of thermal stability is also reflected in the temperature dependence of the circular dichroism spectra. Quasi-elastic light scattering confirms that liposomes containing GD_{1a} interact with the virus and inhibit the growth in the size of the particle as a function of temperature. Although the virus can fuse with the liposomes at higher temperatures, the enthalpy change for this process is not detectable. Our results also demonstrate that the compact folded structure of the influenza hemagglutinin protein is not a kinetically trapped metastable high-energy form.

Influenza virus has become one of the most studied and best understood viruses (*1*). The fusion of this enveloped virus to a target membrane is accelerated at acidic pH. It is also known from crystallographic studies that the fusion protein of influenza virus, the hemagglutinin protein (HA),[1] undergoes a marked conformational change as a function of pH. At neutral pH the ectodomain of the protein is in a more folded, compact state (*2*), while at acidic pH a segment of the ectodomain forms an extended coiled-coil structure (*3*). This has led to the development of the "spring-loaded trap" model (*4*) in which the conformational change in the fusion protein has been suggested to provide energy for the fusion process. More specifically, it has been suggested that the neutral pH form of HA is in a high-energy state because of conformational constraints imposed on the HA2 subunit of this protein by the surrounding HA1 subunits (*5*). The unfolding of a protein that is in a high-energy metastable state will result in the liberation of energy that can be detected as an exothermic process by differential scanning calorimetry (DSC). This is independent of whether the process is entropy or enthalpy driven. Any equilibrium reaction that favors product formation more at higher temperatures than at lower temperatures, by thermodynamic arguments, must have a positive enthalpy of reaction. The only case in which increased temperature can produce an exothermic reaction is when the process is not in equilibrium but is kinetically limited; i.e., the process does not proceed very rapidly until higher temperatures are reached. This represents the current concept of the HA2 subunit: that it is in a kinetically trapped high-energy state until it can form the lower energy, extended coiled-coil structure only at higher temperatures or lower pH. However, neither the isolated HA (*6*) nor the HA in the intact influenza virus (*7*) exhibits an exothermic process. The finding that there was no exothermic process associated with the unfolding of HA suggested that the more compact folded state of the protein at neutral pH did not represent a high-energy metastable state (*7*). We have further analyzed the unfolding process of HA as a function of scan rate. This results in the transition temperature for unfolding being shifted so that if there was a small exothermic process that was not readily apparent, it would be revealed at some of the scan rates used. In addition, we studied the unfolding process both in the presence and in the absence of large liposomes to determine if there was an exothermic process associated with the interaction of the virus with target membranes.

The unfolding transition was analyzed in terms of an irreversible transition. This can be done by analysis of the shape of the DSC transition (*8*), as well as from the scan rate dependence of the DSC (*9*, *10*). We also compare the thermal behavior of the virus in the presence and absence of target liposomes containing the viral receptor, the ganglioside GD_{1a} (*11*). In addition, the conformational state of the protein was monitored using circular dichroism (CD), and interaction of the protein with liposomes as a function of temperature was monitored using quasi-elastic light scattering.

EXPERIMENTAL PROCEDURES

Materials. Influenza virus X-31, A/Aichi/68 (H3N2), was obtained from Charles River Laboratories (North Franklin, CT) as a suspension with a protein concentration of 2 mg/

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[1] Abbreviations: HA, influenza hemagglutinin protein; LUV, large unilamellar vesicle; DSC, differential scanning calorimetry; T_m, transition temperature; ΔH_{cal}, calorimetric enthalpy; E_a, energy of activation; ΔH^*, activation enthalpy; CD, circular dichroism; DOPC, dioleoylphosphatidylcholine; QUELS, quasi-elastic light scattering.

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mL. The virus was purified by centrifugation, and the hemagglutinin titer per 50 μL of suspension was 2097152. Dioleoylphosphatidylcholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL), and the ganglioside GD_{1a} was purchased from Dr. I. Mikhalyov (Moscow, Russia).

Preparation of Liposomes. A solution of DOPC containing 5 mol % of the ganglioside GD_{1a} was made in chloroform/methanol (2:1 v/v). The lipids were deposited on the wall of a glass test tube by solvent evaporation under a stream of nitrogen. Last traces of solvent were removed under vacuum for 2 h. The film was hydrated with 5 mM Hepes, 5 mM Mes, 5 mM citric acid, 0.15 M NaCl, and 1 mM EDTA, pH 7.4 (Hepes−Mes−citrate) to give a final lipid suspension of 15 mg/mL. The lipid suspensions were further processed with five cycles of freezing and thawing, followed by 10 passes through two stacked 0.1 μm polycarbonate filters (Nucleopore Filtration Products, Pleasanton, CA) in a barrel extruder (Lipex Biomembranes, Vancouver, BC) at room temperature. LUVs were kept in ice and used within a few hours of preparation.

DSC. Stock suspensions of intact virus in Hepes/saline were diluted with one-half volume of 5 mM Mes, 5 mM Hepes, 5 mM citrate, 150 mM NaCl, and 1 mM EDTA with or without the presence of 5 mg/mL LUVs. The buffer or liposome suspension was preadjusted to the final desired pH prior to mixing with the virus. The protein concentration of the resulting suspension of intact virus was 1.33 mg/mL. The final pH of the suspension was verified. The viral suspension and corresponding buffer were degassed and loaded into the sample and reference cell, respectively, of an N-DSCII instrument from Calorimetry Sciences Corp. (American Forks, UT). After the first heating scan, no additional thermal transitions were observed on cooling or on reheating, even when the first heating was stopped at a temperature just past that of the denaturation temperature at pH 6.

Data Analysis of DSC. The enthalpy was calculated on the basis of the HA protein comprising 29.6% of the total protein of influenza virus (*12*). The reported enthalpy values for the denaturation of HA agree well at neutral pH with that previously reported for the isolated protein (*6*). The enthalpy values for the other proteins are presented as their values relative to that of HA. All scans were analyzed with the program Origin v5.0, to obtain the transition temperature (T_m) and the calorimetric enthalpy (ΔH_{cal}). To obtain the energy of activation E_a, the data were analyzed with equations for irreversible transitions (*9*, *10*), i.e., assuming that the initial unfolding is followed by an irreversible step such as aggregation and given by a first-order kinetic constant which changes with temperature according to the Arrhenius equation: $k = Ae^{-E_a/RT}$, where A is the Arrhenius constant, R is the universal gas constant, and T is the temperature in Kelvin. From the relationship $\nu/T_m^2 = (AR/E_a)e^{-E_a/RT_m}$, one can obtain E_a from the linear regression of the plot of $ln\ \nu/T_m^2$ versus $1/T_m$, where ν is the scan rate and T_m is the transition temperature. E_a was also calculated from the equation $E_a = eRT_m^2C_p^m/\Delta H_{cal}$, where $e = 2.7183$ and C_p^m is the experimental heat capacity obtained at the maximum of the calorimetric curve. We also measured ΔC_p at the temperature of the maximum of the endotherm as the difference in C_p, at this temperature, of baselines constructed from the region below and above the transition.

CD Spectroscopy. The CD spectra were recorded using an AVIV Model 215 CD instrument (AVIV Associates, Lakewood, NJ). A virus suspension containing 0.067 mg/mL virus in 10 mM phosphate buffer, 1.0 mM EDTA, and 140 mM NaCl, pH 7.4, was prepared. For pH 6 runs, a small aliquot of 1 M citrate was added prior to use. When lipid was present, it was added to the virus, after the LUVs were adjusted to the desired pH, to give a final concentration of 0.25 mg/mL. Control spectra with lipid alone were used as a baseline. The sample was contained in a 10 or 1 mm path length quartz cell that was placed in a thermostated cell holder. The temperature was maintained at a specific value during spectral measurements, but the overall rate of temperature increase was approximately 2 deg/min over the period of the experiment to correspond with the DSC measurements. The heating rate was programmable and was controlled by the instrument. The CD data are expressed as the relative ellipticity, which is a measure of the temperature dependence of the conformational transition.

Quasi-Elastic Light Scattering (QUELS). The size distributions of the LUVs, a suspension of the virus, and a mixture of virus and LUVs were determined as a function of temperature with quasi-elastic light scattering using a Brookhaven Model B1 9000AT digital correlator equipped with a BI-200sm goniometer, version 2.0, and a BI-900AT digital correlator system. A suspension of 0.67 mg/mL influenza X-31 at pH 6, 5 mM Mes, 5 mM Hepes, 5 mM citrate, 150 mM NaCl, and 1 mM EDTA was used in the absence or in the presence of 2.5 mg/mL LUVs of DOPC with 5 mol % GD_{1a}. A control was done with 2.5 mg/mL LUVs. The temperature of the sample compartment was regulated with fluid circulating from a constant temperature bath. The temperature was monitored at the sample using a thermister probe. The sample was prepared in an fashion identical to that described for the DSC. The temperature was raised in steps, and the scattering from the sample was measured at 90° at the indicated temperatures over a period of 3−5 min. Size distribution was calculated with a nonnegatively constrained least-squares algorithm, with software provided by the instrument manufacturer.

RESULTS

DSC. The thermal transitions of suspensions of influenza virus were measured at both pH 7.4 and pH 6.0, both in the absence and in the presence of liposomes composed of DOPC with 5 mol % GD1a (Figure 1). The lipid composition was kept simple with a single phospholipid component, DOPC, that makes stable liposomes and with the addition of GD1a to serve as a receptor for virus binding to the liposomes. The measurements were also made at several different scan rates. All of the observed transitions were found to be irreversible since no transitions were observed on cooling or on subsequent reheating. The curves were deconvoluted as a function of pH and scan rate in the absence as well as in the presence of lipid, and values for ΔH_{cal} were obtained (Table 1). The data at pH 7.4 in the absence of lipid agree very well with that previously reported (*7*), even though the experiments were carried out in another instrument using a different batch of virus. However, the transition temperature for the peak corresponding to HA, in the absence of added liposomes, at pH 6.0 is lower than that previously reported (*7*). It should be noted, however, that the hemag-

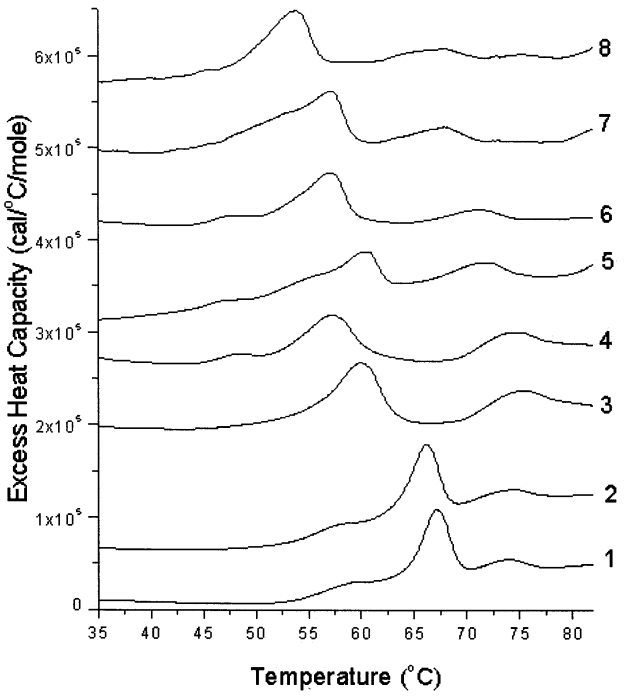

FIGURE 1: DSC heating curves of influenza X-31 at pH 7.4 and 6.0 in the presence and absence of lipid. The viral protein concentration was 1.33 mg/mL. The molar heat capacity was calculated per mole of HA monomer. When present, the lipid concentration was 5 mg/mL LUVs of DOPC with 5 mol % GD1a. Curves: 1, scan rate 2 K/min, pH 7.4, no lipid; 2, scan rate 2 K/min, pH 7.4, with lipid; 3, scan rate 2 K/min, pH 6.0, no lipid;4, scan rate 2 K/min, pH 6.0, with lipid; 5, scan rate 0.5 K/min, pH 6.0, no lipid; 6, scan rate 0.5 K/min, pH 6.0, with lipid; 7, scan rate 0.125 K/min, pH 6.0, no lipid; 8, scan rate 0.125 K/min, pH 6.0, with lipid.

Table 1: Thermodynamic Parameters of the Major DSC Transitions of Influenza X-31

			peak 1		peak 2[a]		peak 3	
pH	presence of lipid	scan rate (K/min)	T_m (°C)	ΔH^b	T_m (°C)	ΔH (kJ/mol of monomer)	T_m (°C)	ΔH^b
7.4	−	2	61	335	67.0	1172	74	293
7.4	+	2	59	293	66.9	1340	74	439
6.0	−	2	54	335	60.0	1423	76	1046
6.0	+	2	48	167	57.2	1464	75	962
6.0	−	0.5	55	502	60.3	920	71	670
6.0	+	0.5	48	167	57.0	1255	71	251
6.0	−	0.125	51	795	57.1	1172	67	377
6.0	+	0.125	46	167	53.7	1550	66	544

[a] Corresponds to the hemagglutinin peak. [b] Value relative to that of the hemagglutinin peak.

glutinin titer of the present lot of X-31 virus is considerably higher than the one previously used. It is possible that this lot of virus begins to become activated at a slightly higher pH. In the region between pH 5 and pH 6 activation of the virus is very sensitive to changes in pH (*6*). The denaturation temperature we determine at pH 6.0 in the present study is in very good agreement with that reported by Remeta et al. for the isolated HA (*6*). The present paper only uses data from this single new lot of virus. At pH 6 there is a systematic decrease of about 3 deg in the transition temperature for peak 2 (HA denaturation) upon the addition of liposomes containing a receptor for HA, the ganglioside GD1a. Since this study was done with intact native virus in

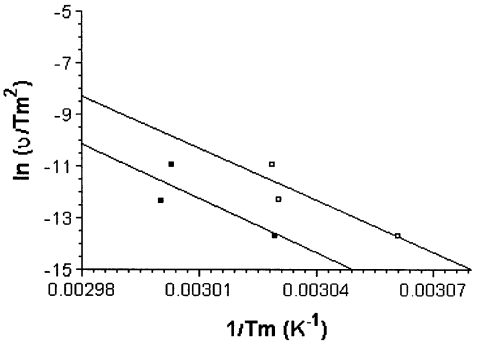

FIGURE 2: Plot of $\ln(\nu/T_m^2)$ vs $1/T_m$ used to obtain the activation energy. Points correspond to data obtained in the presence (□) and in the absence (■) of LUVs of DOPC and 5 mol % GD_{1a} at pH 6.

which the HA was in its native membrane orientation and environment, it also meant that other influenza virus proteins were also present. In comparison to HA, the transition temperature for peak 3 does not change significantly with the addition of lipid, indicating that the shift observed with HA denaturation upon addition of lipid is specific for certain proteins. As expected, controls performed with LUVs of the lipid alone in the absence of virus did not exhibit any detectable phase transition because of the low phase transition temperature of DOPC. This would not be the case if other lipids had been used. The scan rate dependence was determined at pH 6.0. Interestingly, peak 3, showing no change in transition temperature with pH or with lipid, has the largest dependency of the transition temperature on scan rate (Table 1). This indicates that there is no experimental or instrumental factor introducing a systematic error.

The data at pH 6 using different scan rates were analyzed according to the formula $\nu/T_m^2 = (AR/E_a)e^{-E_a/RT_m}$ (Figure 2). The E_a obtained were 576 ± 40 kJ/mol in the absence of lipid and 556 ± 29 kJ/mol with lipid. These values are the same, within the experimental error. Using the relationship, $E_a = eRT_m^2C_p^m/\Delta H_{cal}$, we calculate similar values of E_a at pH 6 of 576 ± 94 and 550 ± 60 kJ/mol, without and with lipid, respectively. At pH 7.4, somewhat higher E_a values of 786 and 994 kJ/mol in the absence and presence of lipid, respectively, were found. We estimate ΔC_p at pH 6 to be 55 ± 10 kJ K^{-1} mol^{-1} in the absence of lipid and 99 ± 7 kJ K^{-1} mol^{-1} in the presence of the liposomes.

CD. We have measured the temperature dependence of the CD signal at 222 nm at pH 7.4 (Figure 3) and at pH 6.0 (Figure 4). If the data for the intact virus are calculated on the basis of the HA content of the virus, the magnitudes of the CD spectra are about 20% lower than those for the isolated HA. This suggests that the other proteins of influenza virus have a lower helical content. CD runs were made with the virus suspension at a concentration of 0.067 mg/mL in 10 mM phosphate buffer, 1.0 mM EDTA, and 140 mM NaCl adjusted to pH 7.4 or 6.0. The CD spectra both at 25 and at 85 °C are similar to those previously reported (*7*) and are independent of the presence of lipid. In the absence of lipid, the CD spectra at pH 7.4 exhibit the same marked change in ellipticity at about 60 °C as had been reported by Ruigrok et al. (*13*) using the ectodomain of HA as well as the results of Remeta et al. (*6*) using the intact hemagglutinin and our results using the intact virus (*7*). To compare the thermal dependence in the presence and absence of lipid, we have presented the data as the relative change in ellipticity at 222

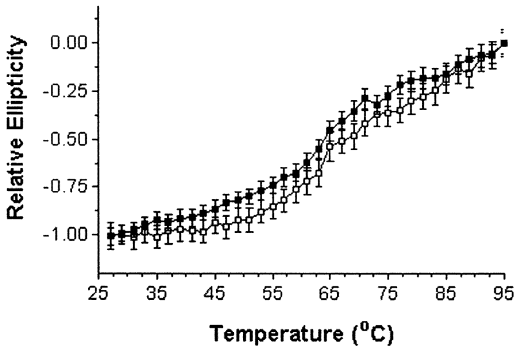

FIGURE 3: Fractional change in the observed ellipticity at 222 nm of influenza X-31 as a function of temperature at pH 7.4. The concentration of viral protein was 0.067 mg/mL in 10 mM phosphate buffer adjusted to pH 7.4 (■) or with the addition of 0.25 mg/mL LUVs of DOPC with 5 mol % GD_{1a} (□).

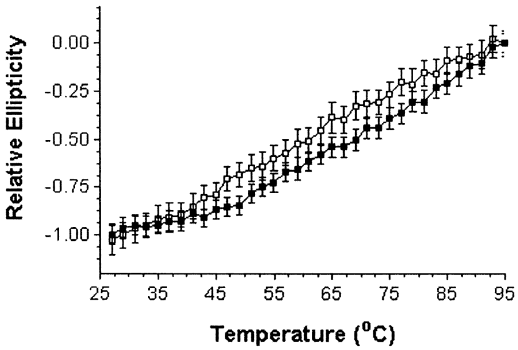

FIGURE 4: Fractional change in the observed ellipticity at 222 nm of influenza X-31 as a function of temperature at pH 6.0. The concentration of viral protein was 0.067 mg/mL in 10 mM phosphate buffer adjusted to pH 6.0 (■) or with the addition of 0.25 mg/mL LUVs of DOPC with 5 mol % GD_{1a} (□).

nm. The conformational change observed at both pH 7.4 (Figure 3) and pH 6 (Figure 4) with the intact virus is irreversible upon recooling.

QUELS. The virus alone, the virus mixed with LUVs, and the LUVs alone all exhibited a rather narrow distribution of particle sizes, even at higher temperatures (Figure 5). The average size of the virus increases with increasing temperature both in the presence and in the absence of the LUV, while the LUVs alone do not change in size when heated. The calculated mean diameters of the samples as a function of temperature are given in Table 2. The analysis of the LUV alone (not in Table 2) is 115 nm and is close to what is expected of lipid extruded through 100 nm pore membranes. The size of the liposomes does not change with temperature. However, the size of the virus is less than other estimates in the literature of about 120 nm diameter (*14*, *15*). The mixture of virus and LUV is smaller than that of the LUV alone. It is possible that the virus particle is partially broken up by the higher temperature and the lower pH. Our main focus is to have independent evidence that there is interaction between the virus and the LUV. This is clearly shown by the suppression of the increase in particle size of the virus caused by the presence of the LUV. The increase in size can result from both the aggregation of particles and membrane fusion. It is known that influenza virus fuses at higher pH with increased temperature (*5*, *6*, *15*). The temperature and pH dependence of this process has been studied extensively (*6*). At pH 6 the half-maximal rate of fusion occurs at about 60

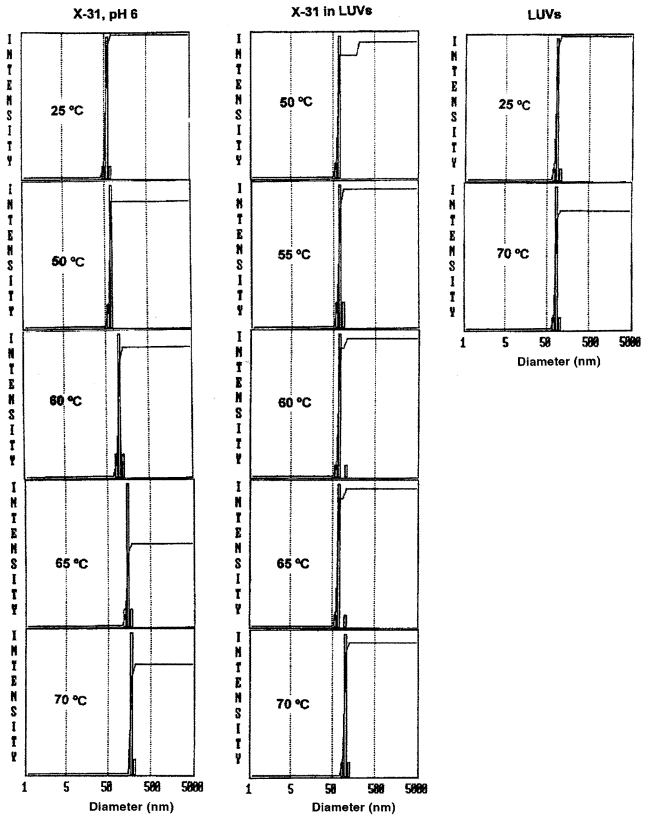

FIGURE 5: Temperature dependence of particle size. The calculated distribution of particle sizes as a function of temperature is shown for a suspension of 0.67 mg/mL influenza X-31 at pH 6 in the absence (left column) or in the presence (middle column) of 2.5 mg/mL LUVs of DOPC with 5 mol % GD_{1a}. A control with 2.5 mg/mL LUVs alone is shown in the right-hand column.

Table 2: QUELS Analysis of Size

	mean diameter (nm)	
temp (°C)	virus	virus + liposomes
50	87	86
60	134	95
65	182	95
70	236	124

°C, the same temperature as the denaturation of hemagglutinin. Therefore, some of the increase in size with temperature of the mixture of virus and LUV is almost certainly due to fusion. A single virus particle can fuse with more than one liposome, but a single liposome cannot fuse with several virus particles. At pH 6.0, the rate constant for aggregation is the same as at pH 5.0, but the rate constant of fusion was about 5-fold lower (*16*). Even though there is more membrane material in the sample with virus plus LUV, the binding of the virus to the liposome appears to stabilize it from growing into a much larger particle.

DISCUSSION

At pH 6 the activation energy for the unfolding of HA is not significantly affected by addition of lipid. The only effect that lipid has on the calorimetric transition of HA is to lower the temperature at which this protein unfolds. Since the kinetics of the process is not greatly altered, it suggests that the lipid is affecting the thermal stability of the protein. The effect is greater at pH 6.0 than at pH 7.4. This occurs without

a change in the enthalpy of denaturation, indicating that the ΔC_p of the reaction is small. The thermal stability of the protein is decreased either by lowering the pH or by increasing the temperature. The loss in stability as a result of decreased pH (*17*, *18*) or an increase in temperature (*6*, *13*, *18*) is largely a consequence of a loss of tertiary structure and not secondary structure. This suggests that the interaction of the virus with liposomes weakens the interactions that stabilize the tertiary structure. This would then facilitate membrane fusion. It has been suggested that the low-pH fusogenic state of HA is a more dissociated structure (*19*), and this is in accord with the finding that SDS can dissociate the HA trimer in nonreducing electrophoresis gels at pH 5 but not at pH 7.4 (*7*). It has also been suggested that the binding of viral fusion proteins to membranes induces their unfolding, which is a critical step in the fusion process (*20*, *21*).

Nevertheless, there is some loss of helical structure with increased temperature (*6*, *7*, *13*). Addition of the lipid had no effect on the CD spectrum at either 25 or 95 °C. However, at pH 6 (Figure 4), particularly in comparison to the data at pH 7.4 (Figure 3), the lipid shifts the temperature required for partial unfolding to lower values. This indicates that the lipid also has a small effect in destabilizing the secondary structure of HA, in addition to or possibly coupled with its effect on the tertiary structure.

The enthalpy of unfolding of the HA protein is about 4.5 cal/g. This value is intermediate between that observed for a comparable weight of a membrane protein and a soluble globular protein (see ref *7*). In addition, ΔC_p, in terms of weight, for the unfolding of HA at pH 6 is 0.21 ± 0.07 cal K^{-1} g^{-1} for the virus alone and 0.38 ± 0.05 cal K^{-1} g^{-1} in the presence of the liposomes. These values are indicative of a highly hydrophobic protein that exposes a number of hydrophobic groups to water upon unfolding (*22*). A positive value of ΔC_p upon protein denaturation has been associated with the exposure of hydrophobic groups (*23*−*25*). The somewhat larger value of ΔC_p in the presence of lipid suggests that not only does the lipid loosen the structure of the protein at lower temperature, but it also facilitates a greater exposure of hydrophobic groups upon denaturation.

It is known that the rate of membrane fusion between influenza virus and target membranes is more rapid at lower pH and at higher temperatures (*5*, *6*, *26*). Remeta et al. have done a more extensive study of these dependencies (*6*). At pH 5.5 or below, there is a significant rate of fusion below 40 °C. We therefore chose to study the properties of the virus at pH 6.0 so that there would not be extensive fusion prior to heating the virus and liposome mixture. The presence of the LUVs prevents some process that leads to large size particles, independently demonstrating that the virus is affected by the presence of lipid.

How much energy is involved in membrane fusion and can a source of energy be identified that could be coupled with reducing the activation energy for fusion? An attempt was made to measure the enthalpy of fusion between influenza virus and liposomes triggered by the addition of an acidic solution using isothermal titration calorimetry (*27*). This study yielded an estimate of a large endothermic enthalpy of fusion of 0.6−0.7 kcal/mol of viral phospholipids. However, the procedure used to obtain this estimate required a large correction for the enthalpy of protonation of the virus. Our DSC data indicate that either the enthalpy of fusion is small or it occurs over a wide range of temperatures. The most convincing argument indicating that the enthalpy of fusion is small comes from the data at pH 7.4. The conformational change in HA is rapid compared with the time scale of the DSC measurements (*18*). There is considerable evidence that this conformational change is required for rapid fusion. The observed conformational thermal transition in HA at pH 7.4, using fluorescence, occurs between about 60 and 65 °C (*6*), yet the transition observed by DSC occurs at 67 °C at a scan rate of 2 K/min (*7*). Thus the enthalpy associated with the triggering of membrane fusion is not observable by DSC. It is possible that an additional transition due to fusion is "buried" under the observed DSC transition, but if this is so, its enthalpy must be small. At pH 6, the unfolding of HA is observed between about 55 and 60 °C (*6*), in the same temperature range as the DSC transition, but the enthalpy of the DSC transition does not change with scan rate, as the transition is shifted through this temperature region, nor is any other transition easily identified. This again indicates that the enthalpy of membrane fusion and of the conformational change in HA required for fusion must be small, at least for the conditions studied here. The results suggest that above this temperature range, when the free energy change for both protein unfolding and for membrane fusion is highly favorable, the process is entropy driven.

In the present work we demonstrate that there is no exothermic process that can be observed concomitant with the unfolding of HA. The conformational change leading to the activation of HA likely occurs within seconds at high temperature, since it occurs within minutes at lower temperatures (*18*). At the range of scan rates used there would not be much difference in the temperature at which this conformational change occurs with the different scan rates that we used. However, the temperature of the calorimetric transition can be shifted at pH 6 from 60 to 54 °C by slowing the scan rate and adding lipid. Yet in none of the scans is there even the hint of an exothermic process observed. This means that there is no kinetically trapped high-energy folded state of the protein at pH 6 at low temperature. Nevertheless, it does not negate the important finding that the HA protein has to undergo a conformational change to expose the fusion peptide in order to become fusogenic (*5*). Our conclusion that there is no metastable high-energy intermediate is independent of the question of the reversibility of the transition and whether the DSC transitions we observe are equilibrium processes. The fact that the process is irreversible could not explain why no exothermic process is observed. Where then does the energy for fusion come from? It should be remembered that the energy of the membrane before and after fusion is not very different. The role of the fusion protein is to lower the activation energy of the process and thereby accelerate it. Does an enzyme have any pent up energy it can use to break a covalent bond in a substrate? This is not very likely. Nevertheless, there may be some conformational rearrangements in HA that occur upon acidification that are coupled to the fusion process. This would include processes that are driven by favorable entropy changes. Certainly, immediately upon acidification the HA molecule will be in a state that will spontaneously rearrange. However, at higher pH this protein would not be in a

metastable state as a consequence of the HA1 subunits constraining the HA2 from adopting the extended coiled-coil structure.

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