# Thermal denaturation of influenza virus and its relationship to membrane fusion

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The X-31 strain of influenza virus was studied by differential scanning calorimetry (DSC), CD and SDS/PAGE analysis as a function of both temperature and pH. A bromelain-treated virus was also studied by these methods. The major transition observed in the intact virus was a result of the denaturation of the haemagglutinin (HA) protein. At pH 7.4, this transition was similar in the intact virus and the isolated HA, but was absent in the bromelain-treated virus. However, at pH 5 the denaturation temperature and enthalpy were both higher for HA in the virus than in the isolated protein, indicating that HA interacts with other molecular components in the intact virus. The transition observed by DSC occurs at a higher temperature than does the thermal transition observed by CD. The temperature of the CD transition coincides with the temperature at which the fusogenicity of the virus increases, and probably corresponds to the formation of an extended coiled-coil conformation. Analysis by SDS/PAGE at neutral pH under non-reducing conditions demonstrates a selective loss of the HA protein trimer, resulting in the formation of aggregates in the range of temperatures of 55 to 70 °C. In contrast, at acidic pH, the HA protein is largely in the monomeric form at 25 °C, and there is little change with temperature. There is thus a weakening of the quaternary structure of HA at acidic pH prior to heating. At the temperature at which the virus exhibits an increased fusogenicity at neutral pH, there is a loss of secondary structure and a beginning of a destabilization of the trimeric form of HA. This temperature is lower than that required for the major endothermic peak observed in DSC experiments. The results demonstrate that there is no kinetically trapped high-energy form of HA at neutral pH.

Key words: fusion energy, membrane protein, protein denaturation, viral fusion.

## INTRODUCTION

Influenza virus has become one of the most studied and best understood viruses. In particular, the mechanism by which the haemagglutinin (HA) protein of the virus facilitates membrane fusion has been studied extensively. The HA protein is composed of two disulphide-linked polypeptide chains. One of these chains, HA1, is composed of 328 amino acids. It forms part of the ectodomain of the protein, and contains a globular moiety that functions as a sialic-acid-receptor-binding site. The other polypeptide component of HA, HA2, has 221 amino acids and is linked to HA1 by a disulphide bond. HA2 has a transmembrane segment, and its ectodomain is thought to be responsible for the fusogenic activity of the protein. The intact HA protein self-assembles into a trimer.

Influenza virus requires acidic pH for maximal fusion activity. Much attention has been drawn to the large conformational change that occurs as a consequence of acidification. The most detailed structure of the neutral-pH form comes from the crystallographic study of the ectodomain of the HA protein [1] that shows the viral fusion peptide sequestered within a folded structure of the HA2 subunit. At acidic pH, there is a major conformational change in the HA2 subunit, resulting in an extended coiled-coil structure, terminating in the region of the N-terminal fusion peptide [2,3]. These observations have led to the development of the 'spring-loaded trap' model [4]. In this model, it is suggested that the non-fusogenic, neutral-pH conformation of HA is in a kinetically trapped high-energy state, and that energy is released as a consequence of a conformational change in the protein. In agreement with this spring-loaded trap model, it has been shown that the metastable neutral-pH conformation can be transformed to the fusogenic form by 'loosening' the structure of HA with either heat or urea [5]. There have been several studies elucidating the nature of the conformational changes of HA or its fragments with temperature. Wiley and co-workers [6] have shown that the ectodomain of HA undergoes a conformational change at 63 °C that is marked by a partial loss of secondary structure, a decrease in tryptophan fluorescence emission wavelength and an increased susceptibility to tryptic hydrolysis. This is close to the same temperature at which Kim and co-workers [5] have shown that the HA protein in the intact virus undergoes increased susceptibility to cleavage by proteinase K. In addition, preventing this conformational change by introducing additional disulphide cross-links abolishes fusogenic activity [7]. Recently, a differential scanning calorimetry (DSC) study of the isolated HA protein in the form of rosettes has been reported [8]. That study showed a denaturation transition at a temperature of 66 °C at neutral pH, which was somewhat higher than the temperature of 63 °C identified using spectroscopic methods [6].

Fewer studies have been performed on the thermal denaturation of the proteins in the intact virus. One would not necessarily anticipate a protein embedded in the viral envelope at a high concentration to behave in the same way as that of the isolated protein. The state of the protein in the virus is related more directly to the biological properties of the virus.

Abbreviations used: DSC, differential scanning calorimetry; HA, haemagglutinin; M1/2, matrix proteins 1 and 2 respectively; NA, neuraminidase; NP, nucleocapsid protein.

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Of course, such a study also has the added complication of having several proteins present in the membrane. As far as the protein composition is concerned, the viral envelope of the X-31 strain of influenza contains HA, matrix protein 1 (M1) and the nucleocapsid protein (NP) in the proportions of 9:10:13 [9]. The NP and neuraminidase (NA) have similar molecular masses in SDS and, generally, are not well resolved [10]. The proportions of several influenza envelope proteins have been found to be similar for various strains of the virus [11,12]. We demonstrate that no exothermic process occurs on heating, as would be anticipated if the protein was in a metastable high-energy conformation at neutral pH. In addition, the properties of HA in the virus at acidic pH are altered compared with the isolated protein.

## MATERIALS AND METHODS

### Materials

Two strains of influenza virus were used: X:31-A/Aichi/68 (H3N2) and A/PR/8/34 (H1N1). These viruses will be referred to as X-31 and PR-8 respectively. They were purified by centrifugation and obtained from Charles River Laboratories (North Franklin, CT, U.S.A.) as a suspension, with a protein concentration of 2 mg/ml. The HA titre per 50 $\mu$l of suspension was 131072 for X-31 and 16777216 for PR-8. The X-31 strain of influenza virus was treated with bromelain, as described by Compans et al. [13]. Briefly, the virus was suspended at a concentration of 2 mg/ml in a solution containing 0.1 M Tris/HCl, pH 7.2, 1 mM EDTA and 50 mM 2-mercaptoethanol. An amount of bromelain was added that was equal to the concentration (in mg/ml) of viral protein. The virus and enzyme were then incubated at 37 °C for 16 h. The fluid was clarified by low-speed centrifugation, and then the viral cores were pelleted and resuspended in 0.1 M Tris/HCl, pH 7.2/1 mM EDTA at a protein concentration of 1.1 mg/ml.

### DSC studies

Stock suspensions of intact virus in Hepes/saline or of bromelain-treated virus in Tris/HCl buffer were diluted with a one-half volume of 5 mM Mes, 5 mM Hepes, 5 mM citrate, 150 mM NaCl and 1 mM EDTA. The buffer was pre-adjusted to either pH 7.4 or pH 5 before mixing with the virus. The protein concentration of the resulting suspension of intact virus was 1.33 mg/ml; that of bromelain-treated virus was 0.75 mg/ml. The final pH of the suspension was verified and then the viral suspension and corresponding buffer were degassed and loaded into the sample and reference cell, respectively, of a Nanocal instrument from Calorimetry Sciences Corporation (American Forks, UT, U.S.A.). A heating scan rate of 2 °C/min was used for all heating scans presented in the present study. No thermal transitions were observed on cooling, or on reheating. However, in cooling scans very small exotherms were occasionally seen. As is often observed, particularly with membrane proteins, the transition was essentially irreversible. Irreversible transitions may be analysed in terms of an activation energy from the shape of the transition curve or from the scan-rate dependency. However, because of the presence of several proteins in the intact virus and the limited amount of virus sample available, such an analysis was not attempted. The data were analysed using equations for reversible transitions, i.e. assuming that the initial unfolding is reversible, but that it is followed by an irreversible step, such as aggregation. The program Origin v5.0 was used to obtain the transition temperature ($T_m$), the calorimetric enthalpy ($\Delta H_{cal}$) and the van't Hoff enthalpy ($\Delta H_{vH}$).

### CD spectroscopy

The CD spectra were recorded using an AVIV model 61 DS CD instrument (AVIV Associates, Lakewood, NJ, U.S.A.). The sample was contained in a 0.5- or 1-mm path-length quartz cell, which was maintained at a defined temperature in a thermostatically controlled cell holder. The temperature was monitored in the cell and maintained at a specific value during spectral measurements, but the temperature was increased at a rate of approx. 2 °C/min over the period of the entire experiment to correspond to the DSC measurements. The CD data were expressed as the observed ellipticities. For comparative purposes, the spectra of both the intact virus and the bromelain-treated virus were normalized to the same protein concentration and path-length. When the data obtained for the intact virus are calculated on the basis of the HA content of the virus, the magnitudes of the CD spectra are approx. 20% lower than those of the CD spectra of the isolated HA, probably because of the presence of other proteins that have a lower $\alpha$-helical content. CD runs were performed with the virus suspension at a concentration of 0.67 mg/ml in 10 mM phosphate buffer containing 1.0 mM EDTA and 140 mM NaCl, adjusted to either pH 7.4 or pH 5.0.

### Thermal gel analysis

The protein content of the X-31 virus was analysed by SDS/PAGE under non-reducing conditions. The changes in the state of association of the protein were analysed as a function of both the pH and the temperature to which the sample was heated. Aliquots of 15 $\mu$l of influenza virus suspension (containing approx. 20 $\mu$g of viral protein) at either pH 7.4 or pH 5.0 were placed into a series of Eppendorf tubes. The virus samples were then placed in a water bath and heated at a rate of approx. 2 °C/min, as for the DSC experiments. One sample at each of the two pH values was removed at each of the desired temperatures. Samples were cooled to 23 °C, and then each was diluted 1:1 with 15 $\mu$l of SDS/PAGE sample buffer [65 mM Tris/HCl, pH 6.8, containing 10% (v/v) glycerol, 2.1% (w/v) SDS and 0.05% (w/v) Bromophenol Blue] lacking reducing agents. Each sample (20 $\mu$l) was loaded on to 12% (w/v) polyacrylamide BioRad Ready Gels. Lanes containing protein standards (BioRad Precision Protein Standards) were also loaded on the gels. The running buffer used was 3 g/l Tris base at pH 8.3, containing 14.4 g/l glycine and 1 g/l SDS. Gels were run at 200 V for 45 min, and were then stained with BioRad Biosafe Coomassie stain for 2 h and dried overnight. A similar experiment was performed with the bromelain-treated virus, but in this case the heating was not carried out over a series of temperatures. The analysis was performed using the bromelain-treated virus that had been used for the DSC measurements and which had been heated twice to 105 °C, as well as with fresh samples.

Estimates of the molecular mass were calculated by generating a standard curve of $R_F$ against the log of the molecular mass for the set of protein standards, and using a line of best fit to calculate the molecular mass of all distinct bands from their $R_F$ values. The regression coefficient ($r$) was 0.96. Calculations of relative percentages of protein were made from scans of the gels using the programme UN-SCAN-IT (Automatic Digitizing System, version 5.1; from Silk Scientific Ltd, Orem, UT, U.S.A.).

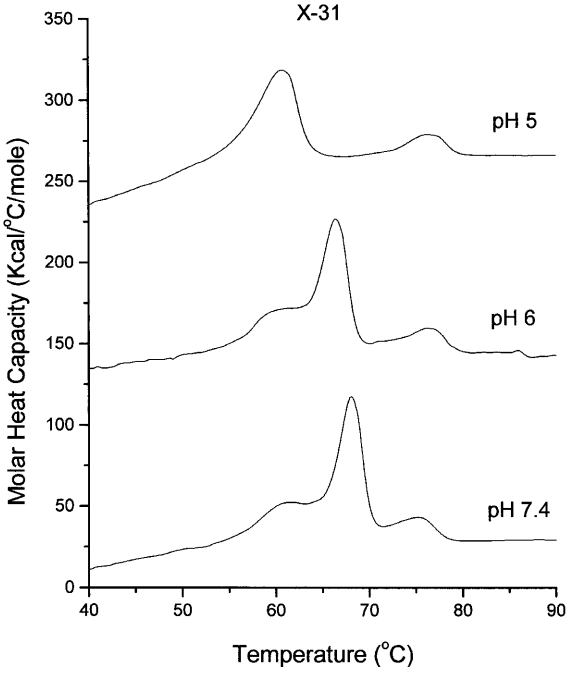


**Figure 1 DSC heating curves of influenza X-31 at three different pH values**

The scan rate was 2 °C/min, and viral protein concentration was 1.33 mg/ml. The molar heat capacity was calculated per mol of HA monomer (1 kcal ≡ 4.184 kJ).

**Table 1 Thermodynamic parameters of the major DSC transition of influenza X-31**

$T_m$, the transition temperature; $\Delta H_{cal}$, the calorimetric enthalpy; $\Delta H_{vH}$, the van't Hoff enthalpy.

| pH | $T_m$ (°C) | $\Delta H_{cal}$ (kJ/mol of monomer) | $\Delta H_{vH}$ (kJ/mol) | Co-operativity | $\Delta H$/g (kJ/g) |
|---|---|---|---|---|---|
| 7.4 | 67.9 | 1222 | 1130 | 0.93 | 19.2 |
| 6 | 66.2 | 1188 | 1075 | 0.90 | 18.8 |
| 5 | 60.1 | 1418 | 636 | 0.45 | 22.6 |

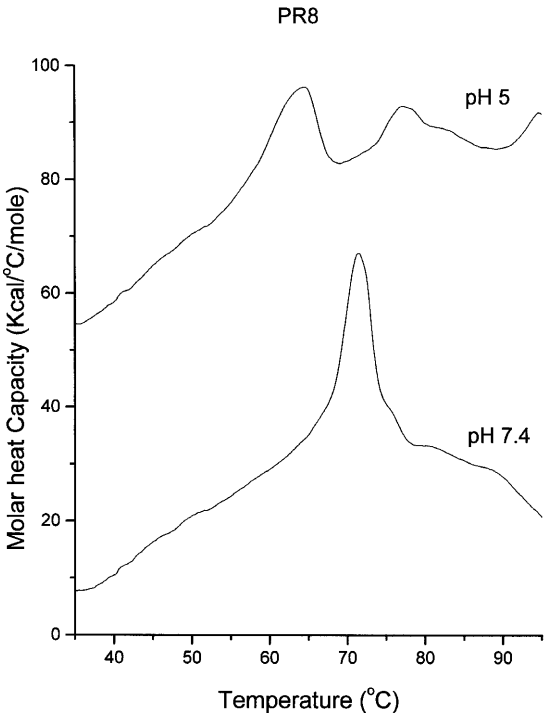


**Figure 2 DSC heating curves of influenza PR8 at two different pH values**

The scan rate was 2 °C/min, and viral protein concentration was 1.33 mg/ml. The molar heat capacity was calculated per mol of HA monomer (1 kcal ≡ 4.184 kJ).

## RESULTS

### DSC experiments

DSC heating scans of a suspension of influenza X-31 were measured at three different pH values (Figure 1). In each of the scans there is one major transition, along with two transitions of lower enthalpy. The thermodynamic parameters of the large transition are summarized in Table 1. For comparison, the DSC of PR8 was determined at pH 5 and 7.4 (Figure 2). Although there are some differences between the two strains, the major transition is similar for the two cases.

The thermotropic properties of the X-31 strain were then studied in greater detail. In addition to the major peak, the parameters of which are summarized in Table 1, there are also smaller peaks at approx. 75 °C and at 62 °C at pH 7.4, and at 75 °C and 52 °C at pH 5 (Table 2). The peak at 52 °C of the pH 5 sample overlaps with the major transition at 60.1 °C. The two peaks were separated by curve deconvolution, and the enthalpy of the minor peaks was also calculated/mol of HA for comparative purposes, since the proteins responsible for these transitions have not been determined.

The DSC curve of the bromelain-cleaved X-31 virus was also measured (Figure 3). Transitions at the temperatures corresponding to the major transition observed in the intact virus are absent. However, two transitions were still observed. The temperatures at which these transitions occur correspond well to the minor transitions observed with the intact virus (Table 2). For comparative purposes, the transition enthalpy of the bromelain-cleaved virus was calculated using the same factors as for the intact virus. However, since there is less protein per virus particle after treatment with the proteolytic enzyme, comparing the bromelain-cleaved virus with the intact virus leads to an overestimation of the enthalpy for the transitions of the remaining proteins. Since these transitions are present after the removal of the ectodomain of HA, they are probably a result of the thermal denaturation of other proteins, or possibly of the remaining core of the HA molecule.

### CD analysis

The CD spectra of X-31 were determined as a function of temperature at pH 7.4 and pH 5 (Figure 4). The CD spectra at pH 7.4 exhibit the same marked change in ellipticity at approx. 60 °C, as had been reported by Ruigrok et al. [6] using the ectodomain of HA, as well as the results of Remeta et al. [8] using the intact HA. At pH 5, however, a gradual change in ellipticity was observed in the present study over a wide temperature range, whereas no change was observed with the ectodomain of HA as a function of temperature [6]. Moreover, with the isolated intact protein, the decrease in the magnitude of the ellipticity was observed only at higher temperatures

**Table 2 Thermodynamic parameters of other DSC transitions of influenza X-31**

Each of the eight data rows correspond to a different transition. Shown are two transitions for each sample, of which there are four: intact virus at pH 7.4 and pH 5.0, and bromelain-cleaved virus at these pH values. $T_m$, the transition temperature; $\Delta H_{cal}$, the calorimetric enthalpy; $\Delta H_{vH}$, the van't Hoff enthalpy.

| Virus particle | pH | $T_m$ (°C) | $\Delta H_{cal}$ (kJ/mol monomer) | $\Delta H_{vH}$ (kJ/mol) |
|---|---|---|---|---|
| Intact | 7.4 | 75.2 | 180 | 1038 |
| Bromelain-cleaved | 7.3 | 74.5 | 264 | 556 |
| Intact | 7.4 | 61.7 | 427 | 569 |
| Bromelain-cleaved | 7.3 | 61.6 | 485 | 385 |
| Intact | 5.0 | 76.2 | 230 | 896 |
| Bromelain-cleaved | 5.0 | 74.0 | 389 | 782 |
| Intact | 5.0 | 53.9 | 251 | 699 |
| Bromelain-cleaved | 5.0 | 52.0 | 389 | 782 |

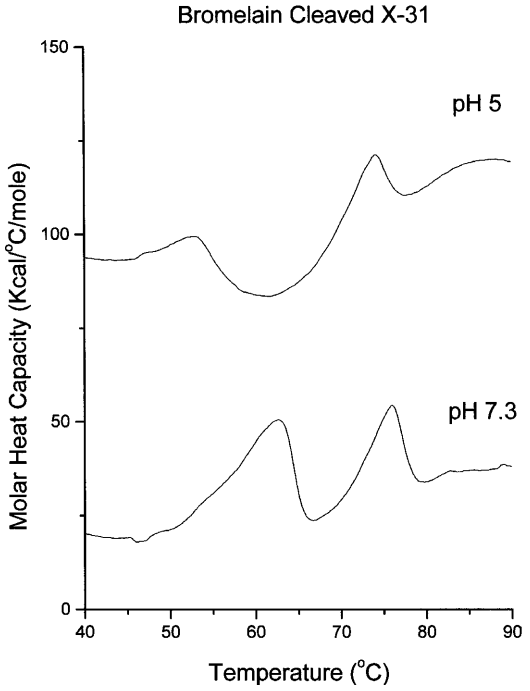


**Figure 3 DSC heating curves of bromelain cleaved X-31 virus at two different pH values**

The scan rate was 2 °C/min, and viral protein concentration was 0.75 mg/ml. The molar heat capacity was calculated per mol of HA monomer for comparative purposes (1 kcal ≡ 4.184 kJ).

and over a narrower range [8]. In the case of the bromelain-treated viral cores, there was also a gradual decrease in magnitude of the CD signal with increasing temperature, and the abrupt change observed at pH 7.4 and at approx. 60 °C is not seen with the cleaved virus (Figure 5). The conformational changes observed at both pH 7.4 (Figure 6) and pH 5 (Figure 7) with the intact virus are irreversible upon cooling. A similar irreversibility was observed with the bromelain-treated virus (not shown).

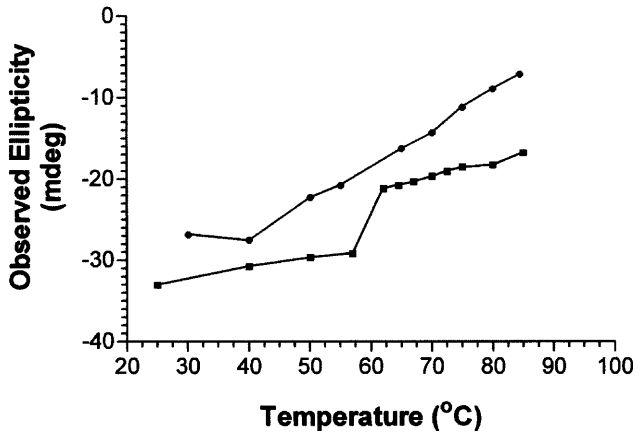


**Figure 4 Dependence of the observed ellipticity at 222 nm of influenza X-31 on temperature**

The concentration of viral protein was 0.67 mg/ml in 10 mM phosphate buffer adjusted to the desired pH. pH values 7.4 (■) and 5.0 (●).

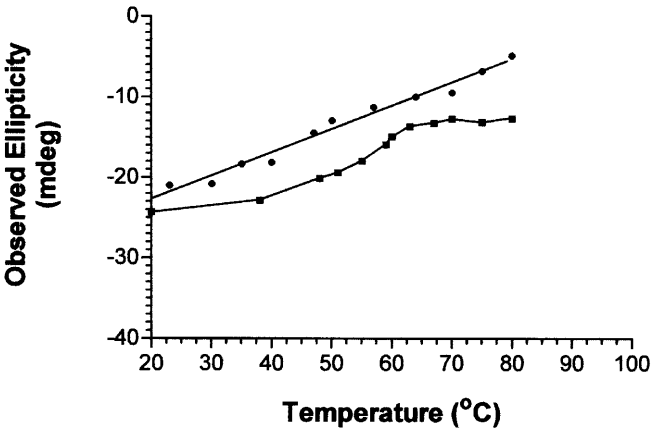


**Figure 5 Dependence of the observed ellipticity at 222 nm of the bromelain-treated influenza X-31 on temperature**

Conditions were the same as those given in the legend for Figure 4. pH values 7.4 (■) and 5.0 (●).

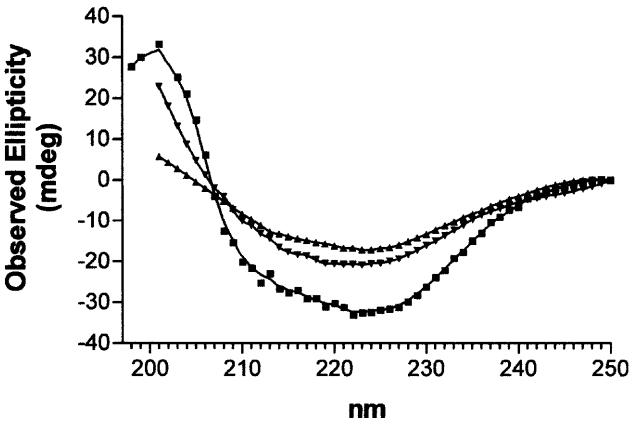


**Figure 6 CD spectra of influenza X-31 at pH 7.4**

Conditions were the same as those given in the legend for Figure 4. ■, 25 °C; ▲, 85 °C; ▼, re-cooled to 25 °C after heating.

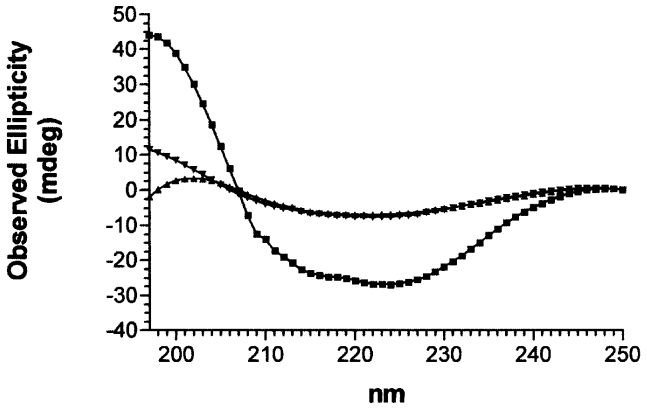


**Figure 7 CD spectra of influenza X-31 at pH 5.0**

Conditions were the same as those given in the legend for Figure 4. ■, 30 °C; ▲, 85 °C; ▼, re-cooled to 25 °C after heating.

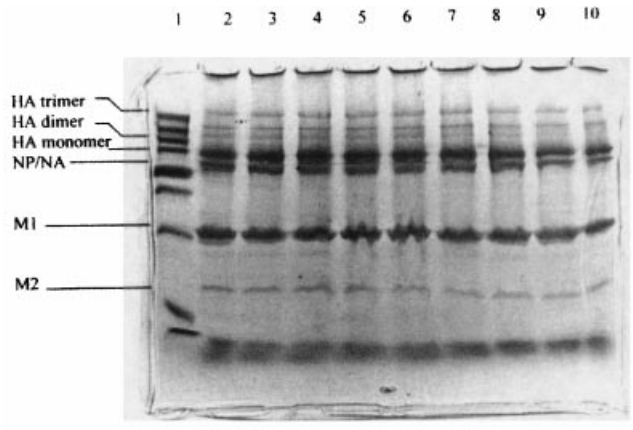


**Figure 8 SDS/PAGE of influenza X-31 at pH 5.0 heated to various temperatures**

Lanes were loaded as follows: lane 1, molecular-mass markers; lane 2, 23 °C; lanes 3–10, 50, 55, 60, 65, 70, 75, 80 and 90 °C respectively.

## SDS/PAGE

SDS/PAGE analysis in non-reducing conditions has been used to analyse the thermal denaturation of proteins in biological membranes. Upon thermal denaturation, some proteins of erythrocyte membranes undergo disulphide exchange, leading to the formation of intermolecular disulphide bonds. This would result in some proteins appearing as higher-molecular-mass aggregates in SDS/PAGE. This phenomenon was used to assign endotherms observed in DSC to the denaturation of particular proteins [14]. There were six different bands shown to arise from non-aggregated forms of the proteins of influenza virus, which were: HA (trimeric), 190 kDa; HA (dimeric), 126 kDa; HA (monomeric), 63 kDa; NP/NA, 56 kDa/51 kDa respectively; M1 28 kDa; and M2, 11 kDa. In the present study, six distinct bands were discernible on the pH 5 gel, with approximate molecular masses of 166, 133, 88, 76, 30 and 15 kDa (Figure 8). Seven bands were present on the pH 7 gel (Figure 9). Molecular masses of these bands were estimated to be 162, 135, 116, 90, 78, 31 and 16 kDa. Most of the bands appear at positions anticipated for the proteins of influenza virus, with the exception of

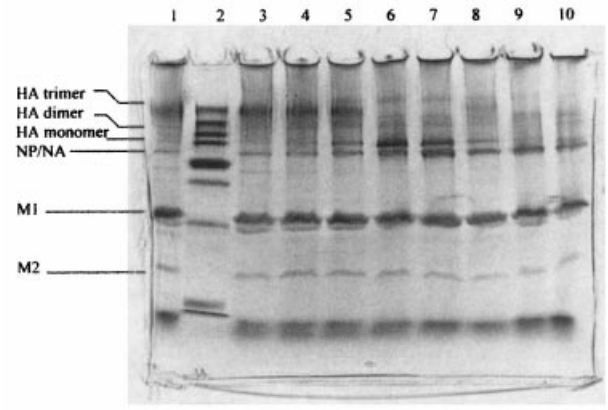


**Figure 9 SDS/PAGE of influenza X-31 at pH 7.4 heated to various temperatures**

Lanes were loaded as follows: lane 1, 23 °C; lane 2, molecular-mass markers; lanes 3–10, 50, 55, 60, 65, 70, 75, 80 and 90 °C respectively.

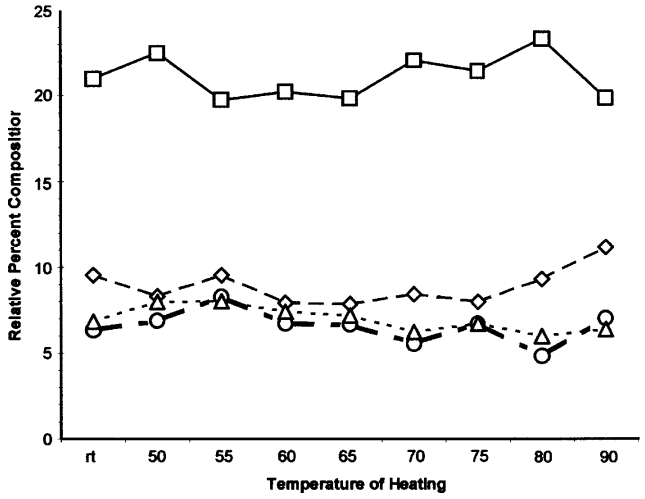


**Figure 10 Intensity of various bands of influenza X-31 proteins analysed by SDS/PAGE under non-reducing conditions after being heated to various temperatures at pH 5**

The results of experiments with HA trimer (□, continuous line), material not entering the gel (◇, broken line), HA monomer (○, emboldened, line formed from long/short dashes) and HA dimer (△, line formed from short dashes). The results shown are the average of two independent determinations that gave qualitatively similar results. The percentage deviation of an individual determination (not shown) was ±20%.

the band at 116 kDa appearing in the pH 7 sample. At pH 5, the relative amounts of each of the proteins remain unchanged as the temperature is increased (Figure 10). In contrast, there is a marked decrease in the amount of HA trimer at pH 7 and between 55 and 65 °C (Figure 11). There is also a corresponding increase in the content of proteins in the wells. The relative percentage of HA monomer present increases in response to the decrease in HA trimer between 55 and 65 °C, but this falls off again at higher temperatures.

The results of the SDS/PAGE analysis confirm that the bromelain-treated virus is devoid of HA protein (Figure 12). There are no bands present corresponding to the HA protein.

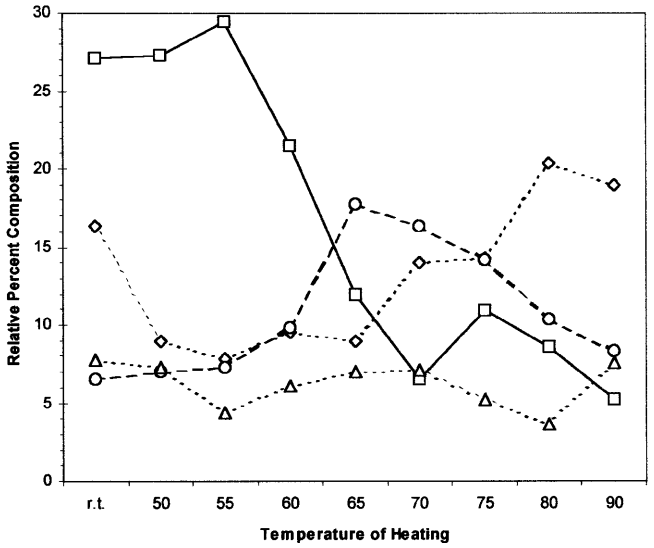


**Figure 11 Intensity of various bands of influenza X-31 proteins analysed by SDS/PAGE under non-reducing conditions, after being heated to various temperatures at pH 7.4**

The results of experiments with HA monomer (□, continuous line), material not entering the gel (◇, line formed from short dashes), HA dimer (△, line formed from short dashes) and HA trimer (○, broken line). Results shown are the average of two independent determinations that gave qualitatively similar results. The percentage deviation of an individual determination (not shown) was ±20%.

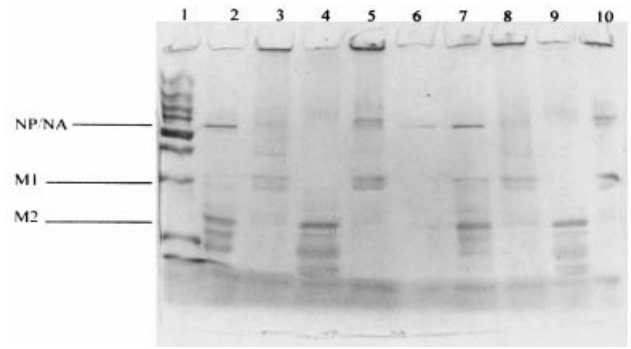


**Figure 12 SDS/PAGE of freshly prepared bromelain-cleaved influenza X-31, and samples that were heated previously to 100 °C at pH 5.0 and at pH 7.3**

Lanes were loaded as follows: lane 1, molecular-mass markers; lane 2, pH 5, not heated; lane 3, pH 5, heated; lane 4, pH 7, not heated; lane 5, pH 7, heated; lane 6, blank; lane 7, pH 5, not heated; lane 8, pH 5, heated; lane 9, pH 7, not heated; lane 10, pH 7, heated.

There should be a fragment of HA2 remaining in the bromelain-treated virus of 3.9 kDa corresponding to the monomer, or of 11.7 kDa corresponding to the trimer. These bands would not be easy to identify, since the monomer probably runs off the gel. There may be some dimer or trimer present, since there are several bands below the band for the M2 protein, as well as some trimer that is not well resolved from the band pertaining to M2. Bands corresponding to the M1, M2 and NP/NA proteins are still present in the gels from the fresh virus, indicating that the viral envelope was still sufficiently intact to prevent influx of the bromelain. After heating at either pH, there is a considerable increase in aggregated material that appears in the well at the top of the gel, and some loss of the NP/NA and M2 proteins.

## DISCUSSION

The HA protein comprises only 29.6% of the total protein of influenza virus [11]. Nevertheless, there are conformational transitions observed by DSC and by CD with the intact virus that can be assigned to changes occurring with HA. In the case of DSC, the properties of the major transition of both the intact virus (Figure 1 and Table 1) and of the isolated HA protein [8] at neutral pH are quite similar. This transition is not shown in the DSC of the bromelain-cleaved virus (Figure 3). The DSC of the intact virus has two additional transitions appearing at 75 and 62 °C (Table 2). These peaks also appear in the DSC scan of the bromelain-cleaved virus, indicating that they arise from the unfolding of other proteins, and not HA. It is known, for example, that the NA protein undergoes thermal inactivation at 50 °C [15], although this does not necessarily correspond to the temperature of thermal denaturation that would be observed by DSC.

There is a change in the behaviour of the HA protein at pH 5.0. These changes occur with the intact virus, the isolated HA protein and the ectodomain of HA, but the observed changes, although similar, are not quantitatively identical. For both the isolated HA [8] and the intact virus (Figure 1), there is a decrease in the transition temperature. However, the extent of the decrease in the transition temperature is much greater for the isolated protein found at 42.2 °C [8] compared with the intact virus that has a transition at 60.1 °C (see Figure 1 and Table 1). Furthermore, the enthalpy of the transition for isolated HA decreases approx. 10-fold [8], whereas that for the virus does not greatly change (Table 1). The transitions observed in the intact virus that are assigned to the unfolding of the HA protein are not detected in the DSC scans of the bromelain-treated virus (Figure 3). However, the low-temperature 'shoulder' observed at 54 °C in the DSC scan of the intact virus at pH 5 does appear as a separate peak in the bromelain-treated virus scan at this pH (see Table 2). The environment of the HA protein in the intact virus at pH 5 is thus different from that of the isolated protein. This is not surprising, since in the intact virus the HA is inserted into a membrane and may also interact with surrounding proteins.

The CD spectra for the intact virus (Figure 4) also exhibit differences in response to varying the temperature that are similar to those for the ectodomain of HA [6] and the intact HA molecule [8] at neutral pH, showing an abrupt decrease in the magnitude of the CD signal at approx. 60 °C. Although the HA in the various states (intact virus, isolated HA and ectodomain of HA) exhibit the same behaviour as monitored by both DSC and CD, these two methods are measuring different processes. Although measured independently, the abrupt change in CD at pH 7.4 occurs at a temperature approx. 8 °C lower than that of the calorimetric endotherm observed by DSC. This abrupt change in CD signal is not observed at pH 5, but occurs over a broad range of temperatures for the intact virus (see Figure 4), at a high temperature for the isolated HA [8], and very little change with temperature is observed for the ectodomain fragment of HA [6]. These results also indicate that there is a difference in behaviour at acidic pH between the intact virus and the isolated HA or its ectodomain, and that the observed endotherm in DSC is not a consequence of the conformational change leading to activation.

The enthalpy of the HA transition in the intact virus at the two pH values is similar, and the temperatures of the transitions differ

by only a few degrees. The extended coiled-coil structure is formed at pH 5 at temperatures well below that of the calorimetric transition, and is followed by denaturation [16–18]. At pH 7.4, the conformational transition observed by CD spectroscopy is also followed by inactivation of the virus [19,20]. This initial unfolding also correlates with the temperature range that leads to an increased rate of fusion [5], and is probably a consequence of the formation of an extended coiled-coil in the HA2 subunit. The calorimetric transition is observed at a higher temperature, and does not involve a further change in secondary structure. The observed DSC transition therefore must be caused by a change in tertiary and/or quaternary structure. Thus the fusogenic conformation of the protein at pH 7.4 is not a completely unfolded, thermally denatured structure. The calorimetric transition is not likely to be caused by a complete unwinding of the coiled-coil structure, since, in contrast with our results, that process in other proteins is accompanied by a larger enthalpy change and a co-operative unit of the size of a trimer [21]. At pH 5, the trimer is destabilized, as shown by the fact that the disulphide bond becomes accessible to reducing agents [22]. This agrees with our observation that most of the HA at pH 5 is in the monomeric form (Figure 10). The 'loosening' of the quaternary structure of HA at acidic pH has been proposed to be a consequence of electrostatic repulsion among the polypeptide chains [23].

The enthalpy of unfolding of the HA protein at pH 7.4 observed in both the virus (Table 1) and in HA rosettes [8] is approx. 18.8 kJ/g. This value is intermediate between that observed for membrane proteins and for soluble globular proteins. Examples of the unfolding enthalpy of membrane proteins include GLUT1, having a transition enthalpy of 11.7 kJ/g [24], unbleached rhodopsin (13.0 kJ/g; [25,26]), $Ca^{2+}$-ATPase (15.1 kJ/g; [27]) and bacteriorhodopsin (15.5 kJ/g; [28]). This is much lower than the value of 32.6 kJ/g observed for the unfolding of most soluble globular proteins at 67 °C [29]. The fact that the unfolding enthalpy of HA is intermediate between that of many integral membrane proteins and that of globular proteins can be accounted for by the structure of HA, which is largely extramembranous with only a single transmembrane segment. The lower transition enthalpy of membrane proteins is thought to be a consequence of the fact that these proteins do not unfold completely in the denatured state. This is probably the case for the transmembrane segment of HA, and possibly also for other regions of the protein. It is thus not surprising that the denaturation enthalpy is between that of a globular protein and a membrane protein that has a greater fraction of its structure embedded in the membrane.

It is clear from our DSC results that the formation of this fusogenic intermediate does not involve an exothermic process, corresponding to releasing the energy from a kinetically trapped high-energy intermediate. In addition, despite the higher temperature, the rate of fusion observed at neutral pH is much lower than that observed at acidic pH [5]. It therefore appears that, although the conformational rearrangement leading to the formation of the extended coiled-coil in HA2 is required for rapid fusion, there is a further large enhancement in the rate of fusion brought about by protonation of HA. The pH-dependence of the rate of fusion is not a consequence of changes in the affinity of HA for sialic acid [30,31], the viral receptor. The requirement for protonation for rapid fusion at lower temperatures is also observed with the extended coiled-coil structure of the ectodomain of HA2 [32,33].

At acidic pH, the increase in fusogenic potency of HA is not accompanied by a change in secondary structure, but it is correlated with a change in tertiary structure, resulting in altered spectral properties of the aromatic amino acids [34]. Acidification also results in a greater susceptibility of HA to digestion by protease K [23], as well as greater susceptibility to reducing agents [22] and a loss of stability of the trimeric form of the protein in the presence of SDS (see Figure 8). This is also consistent with our finding that the co-operativity of the calorimetric transition is lower at acidic pH than at neutral pH (Table 1). The analysis of Herrmann and colleagues shows that the trimeric form is destabilized at acidic pH because of electrostatic repulsion [23]. It is possible that, prior to inactivation, there is a release of energy as a consequence of movement of HA monomers away from each other to reduce electrostatic repulsion. This exothermic energy would not be observed in the DSC studies, since it would be released within seconds after acidification. This energy could be coupled with that required to drive the fusion reaction.

In summary, we can assign an endothermic transition in DSC scans of the intact virus to a denaturation of the HA protein. The ability of the virus to fuse at neutral pH is not a result of the release of energy as a consequence of the unfolding of a kinetically trapped high-energy intermediate. At low pH, the trimeric form of the HA protein is no longer stable in SDS, demonstrating a decrease in the stability of the trimer. However, the HA has a more folded structure in the intact virus compared with the isolated HA, indicating that this protein interacts with other components of the virus, resulting in greater structural stability.

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