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THE KINETICS OF THE ACID-INDUCED CONFORMATIONAL CHANGE OF INFLUENZA VIRUS HAEMAGGLUTININ CAN BE FOLLOWED USING 1,1'-BIS(4-ANILINO-5-NAPHTHALENESULPHONIC ACID)

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1,1'-Bis(4-anilino-5-naphthalenesulphonic acid) (bis-ANS) has been shown by fluorescence spectroscopy to bind to bromelain-cleaved influenza haemagglutinin (BHA). The fluorescence intensity of 1.2μM bis-ANS in the presence of BHA in its low-pH conformation is twenty-fold higher than in the presence of BHA in its neutral-pH conformation. The use of this probe provides a sensitive method for investigating the kinetics of the irreversible conformational change of BHA induced by low pH. At pH5.0 the reaction is described by a rapid burst followed by a double exponential increase in the fluorescence of bis-ANS, with rate constants of $5.2\pm0.9\times10^{-3}sec^{-1}$ and $6.7\pm1.9\times10^{-4}sec^{-1}$. This reaction is sensitive to the presence of *tert*-butylhydroquinone, an inhibitor of the conformational transition of BHA. The dependence of the reaction rate on pH indicates that the acid-induced conformational change is dependent upon the multiple protonation of the neutral-pH conformation of BHA.

Influenza haemagglutinin (HA) is a homotrimeric glycoprotein located on the outer surface of the virus particle (1). Each monomer is proteolytically cleaved prior to viral assembly to give two polypeptides, HA_1 and HA_2, which remain disulphide-linked. HA binds to cell surface sialic acids, which results in the adsorption of the virus onto the cell. The virus then enters the cell via clathrin-coated pits, localizing the virus within nascent endosomes. As the pH within the endosome falls below pH5.5, the HA undergoes an irreversible conformational change, which exposes the hydrophobic N-terminus of HA_2, the fusion peptide (2-4). This region of the protein inserts into the endosomal membrane, mediating the fusion of the viral membrane with the endosomal membrane and thus the release of the viral nucleic acids into the cytoplasm and the initiation of viral replication. Compounds which inhibit the conformational change of HA have the potential to exert a therapeutic effect against influenza. Although the principal structural

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Abbreviations used: BHA, bromelain-cleaved haemagglutinin; bis-ANS, 1,1'-bis(4-anilino-5-naphthalenesulphonic acid); DMSO, dimethylsulphoxide; HA, haemagglutinin; PAGE, polyacrylamide gel electrophoresis; pfu, plaque-forming unit; SDS, sodium dodecylsulphate.

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alterations associated with the change in conformation have recently been elucidated (4), the lack of a sensitive continuous assay has hindered the study of the kinetics of this reaction.

The affinity of bromelain-cleaved HA (BHA) for a number of detergents has been demonstrated to increase after the protein has undergone the conformational change at low pH (5). In the light of this observation we chose to investigate whether the fluorescent probe 1,1'-bis(4-anilino-5-naphthalenesulphonic acid) (bis-ANS) could be used to monitor this process. This compound is known to have a high affinity for non-polar cavities in proteins and a quantum yield which is very much higher in such hydrophobic environments than in aqueous solution. These properties have been used to analyse the conformational changes of a number of different proteins (6,7). The results of this study demonstrate that bis-ANS binds specifically to the low-pH conformation of BHA. Fluorescence spectroscopy can be used to monitor this binding and hence the kinetics of the change in conformation and the effects of inhibitors of this process. These data extend a recent report that the enhanced binding of bis-ANS to influenza virions at low pH reflects the conformational change of HA (8).

Materials and Methods

Materials: 1,1'-Bis(4-anilino-5-naphthalenesulphonic acid) was obtained from Molecular Probes Inc. (Eugene, Oregon, USA). Sucrose-fractionated influenza reassortant X31, with a viral titre of 4.3×10^8 pfu ml^{-1}, was obtained from Evans Medical Ltd. (Speke, UK). Antisera against influenza sialidase were the generous gift of Dr. Alan Hay (National Institute for Medical Research, Mill Hill, UK). 2-O-(4-methylumbelliferyl)-N-acetylneuraminic acid (Sigma Chemica Co., Poole, UK) was used as the substrate in sialidase assays, which were carried out according to the method of Potier *et al.* (9).

Purification of BHA: BHA was purified using a modification of the method of Brand and Skehel (10). All procedures were carried out at 4°C unless otherwise noted. Influenza reassortant X31 (3.4×10^{10} pfu) was spun for 3 hours at 130,000*g*. The resulting pellet was resuspended in 25mM Tris, 0.25mM EDTA, 12.5mM 2-mercaptoethanol, pH7.2 containing 1mg ml^{-1} bromelain (100ml) and incubated at 37°C overnight. The mixture was spun for 1 hour at 130,000*g*. The supernatant was decanted and concentrated to a volume of 50ml using a Centriprep-30 concentrator (Amicon, Beverley, Massachussetts, USA). This was applied to a Superdex 200 column (85×5cm) and eluted with 0.1M sodium phosphate pH7.4 at a flow rate of 10ml min^{-1}. Fractions (50ml) containing BHA were identified by SDS-PAGE and pooled. The yield of BHA was determined to be 104mg, based on an extinction coefficient for BHA at 280nm of 1.5$cm^{-1}(mg/ml)^{-1}$ (11). Traces of sialidase activity were removed from the preparation by applying 10mg aliquots of BHA to an anti-sialidase immunoaffinity column generated by binding antisera raised against influenza sialidase to a HiTrap-protein A column (Pharmacia, Milton Keynes, UK). Sialidase-free BHA was eluted with 0.1M sodium phosphate pH7.4, concentrated to 1mg ml^{-1} and stored at 4°C in 0.1M sodium phosphate pH7.4 containing 0.02% sodium azide.

Kinetic Studies: All kinetic studies were conducted using an LS-50B luminescence spectrometer (Perkin Elmer, Beaconsfield, UK) with the cell maintained at 25°C using a Julabo-U3 circulating water bath (Jencons, Leighton Buzzard, UK). Reaction mixtures contained 0.2M sodium acetate pH5.00, 2% (v/v) DMSO and 1.2μM bis-ANS in a final volume of 3ml unless otherwise stated. Data collection was initiated, with 1 second data points being collected every 5 seconds. The excitation wavelength was 394nm using an excitation slit width of 5nm and the emission wavelength was 500nm with an emission slit width of 10nm. After 120 seconds, sialidase-free BHA (19nM) was added and data collection continued for a further hour.

Data Analysis: Plots of fluorescence (f) against time (t) were fitted to equations describing either a single exponential fluorescence increase (equation (1)) or a double exponential increase (equation (2)).

$$f(t) = A + B.\left(1 - e^{-k_1.t}\right) \quad (1)$$

$$f(t) = A + B.\left(1 - e^{-k_1.t}\right) + C.\left(1 - e^{-k_2.t}\right) \quad (2)$$

Initial rates of fluorescence increase (d(f)/dt, v) were calculated from the derivatives of equations (1) and (2) at t=0. When analysing the inhibition of the rate of the conformational change by *tert*-butylhydroquinone, the fluorescence data were fitted to equation (1) at inhibitor concentrations above 2μM and to equation (2) when the inhibitor concentration was below 2μM. Initial rate data were fitted to equation (3)

$$v_i = \frac{v_o}{\left(1 + {}^{[I]}/_{K}\right)} \quad (3)$$

where v_i is the initial rate in the presence of inhibitor, v_o the initial rate in the absence of inhibitor and K the dissociation constant of the complex of the protein and inhibitor. All data were analysed using *Grafit* version 3.0 (Erithacus Software Ltd., Staines, UK).

Results and Discussion

The ectodomain of haemagglutinin was released from virions of influenza reassortant X31 by treatment with bromelain. The bromelain-cleaved HA (BHA) was purified by size-exclusion chromatography and the contaminating sialidase activity was removed by immunoaffinity chromatography. The use of size-exclusion chromatography instead of sucrose density gradient centrifugation (10) to separate BHA from bromelain and other protein contaminants was found to result in a five-fold increase in the yield.

Incubation of a 19nM solution of BHA (57nM in sialic acid binding sites) with 1.2μM bis-ANS at pH7.4 resulted in a two-fold increase in the fluorescence of bis-ANS. However after incubation of 19nM BHA in 0.20M sodium acetate pH5.0 for one hour in the presence of 1.2μM bis-ANS, the fluorescence intensity was twenty-fold higher than bis-ANS in the presence of BHA at pH7.4. This increase in fluorescence intensity was accompanied by a 13nm blue shift (Figure 1). The fluorescence increase was also observed when BHA was incubated at pH5.0 for one hour followed by the addition of 1.2μM bis-ANS. In the absence of protein, bis-ANS has a fluorescence intensity which is two-fold higher at pH5.0 than at pH7.4. This indicates that the majority of the fluorescence increase results from the formation of complexes between bis-ANS and the low-pH conformation of BHA.

Addition of BHA (19nM) to 200mM sodium acetate pH 5.0 containing bis-ANS resulted in a time-dependent increase in fluorescence. A representative data set is shown in figure 2A. The resulting data was multiphasic, with an initial burst phase which was complete within the first

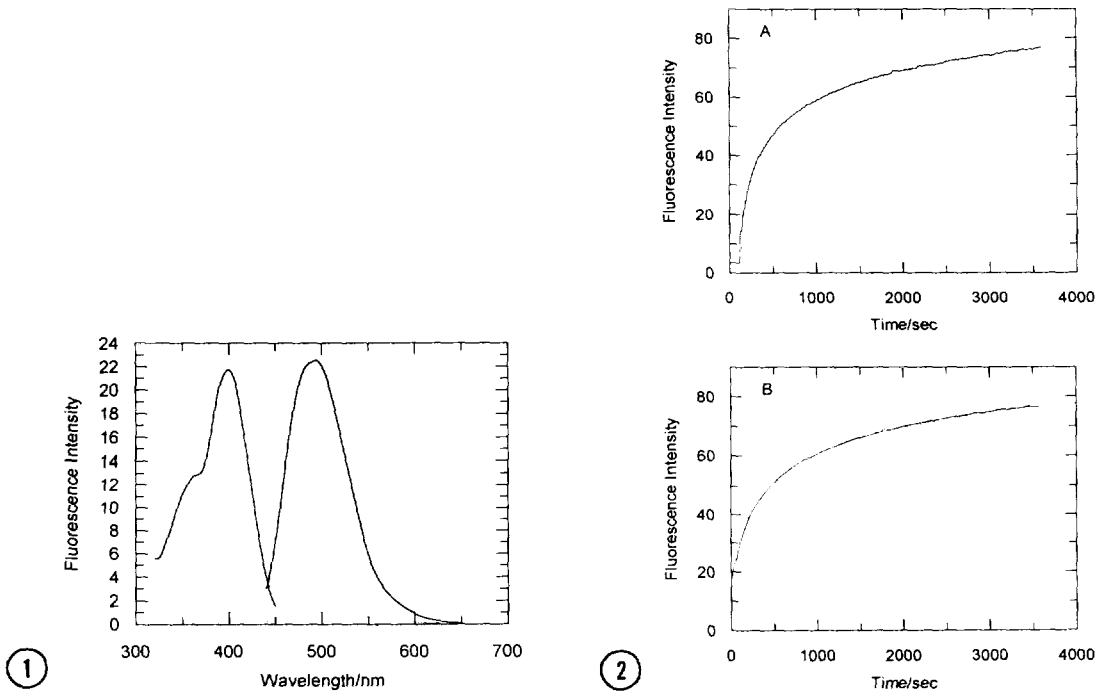


Figure 1. Bis-ANS binds selectively to the low-pH conformation of BHA.
Lower Traces: Excitation and emission spectra of bis-ANS (1.2μM) in the presence of 19nM BHA in 25mM HEPES, pH7.4, 100mM potassium chloride containing 2% v/v DMSO at 25C. Upper Traces: Excitation and emission spectra of bis-ANS (1.2μM) in the presence of 19nM BHA in 200mM sodium acetate, pH5.0, after incubation at 25C for 1 hour containing 2% v/v DMSO.

Figure 2. Time course of the conformational change of BHA.
(A) Addition of 19nM HA at t=120sec to 1.2μM bis-ANS in 200mM sodium acetate, pH5.0, containing 2% v/v DMSO at 25C. (B) Data from figure 2A were fitted to equation (2) with the time of HA addition set to t=0sec. Time points within the first minute after BHA addition were excluded from the analysis.

minute of the reaction. The remaining data could not be fitted to a single exponential, but good fits were obtained to a double exponential, equation (2), as shown in figure 2B. The mean values of the rate constants k_1 and k_2 from four independent determinations were $5.2\pm0.9\times10^{-3}sec^{-1}$ and $6.7\pm1.9\times10^{-4}sec^{-1}$ respectively. Insufficient data were collected during the burst phase to determine accurately the rate constant for this reaction, and it is likely that rapid mixing techniques will be required to separate the effects of bis-ANS binding to the low-pH form of BHA from the small increase in fluorescence which arises from the association of bis-ANS with the neutral-pH conformation of BHA. The observation of a multiphasic reaction is consistent with the differences in the rates of exposure of different antigenic regions of the low-pH conformation of BHA (12). The rate constants for the first and second observed phases (k_1 and k_2) were both found to be zero-order in bis-ANS concentration, which indicates that the measured rate constants are not due to rates of association of bis-ANS and BHA. However all three amplitudes increased with increasing bis-ANS concentration, suggesting that all the possible binding sites for this probe have not been saturated under these conditions.

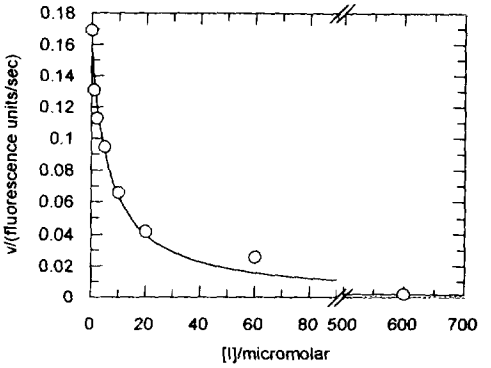
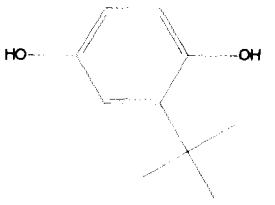


Figure 3. Inhibition of the conformational change of BHA by *tert*-butylhydroquinone. The line shows the fit of equation (3) to the data.

Structure-based inhibitor design has led to the discovery that *tert*-butylhydroquinone (I) inhibits the exposure of the fusion peptide of BHA at pH5.0 and possesses moderate antiviral activity against influenza A virus (13). The effect of this compound on the kinetics of the conformational change was investigated in order to determine the utility of this assay for the study of compounds which stabilise the neutral-pH conformation of BHA and to exclude the possibility that the observed changes in fluorescence are dependent on processes other than the exposure of the fusion peptide. As expected, the addition of (I) inhibited the rate of increase in fluorescence and the amplitude of the burst phase, but without affecting the overall amplitude of the reaction. The magnitude of k_1 at concentrations of (I) above 2μM approached the magnitude of k_2, and the data from these reactions were found to fit better to a single exponential increase (equation (1)) rather than a double exponential increase (equation (2)). This suggests that the presence of (I) inhibits the reaction described by the rate constant k_1, and that the process described by the slower rate constant k_2 follows that described by k_1. Initial rates of fluorescence increase (v) were calculated from the fitted parameters (*vide supra*). The initial rate data were fitted to equation (3), as shown in Figure 3. The dissociation constant of the complex of BHA and (I) was determined to be 6.7±1.0μM. This is in good agreement with the IC_{50} value of 5μM reported for the inhibition by (I) of fusion peptide exposure at pH5.0 (13).

The effect of pH on the conformational change was investigated in 200mM potassium phthalate buffer containing 200mM potassium chloride in the pH range 5.0 to 5.4. Control experiments indicated that the small ionic strength differences over this pH range did not result in significant changes to the rates of amplitudes of the reaction. At all pH values in this range the



Scheme I

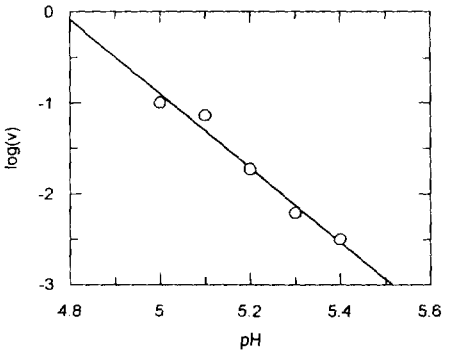


Figure 4. The pH-dependence of the initial rate of the conformational change of BHA.

data were described by a double exponential (equation (2)). A plot of the logarithms of the initial rates of fluorescence increase against pH was linear with a slope of -4.1±0.4, as shown in Figure 4. The magnitude of the slope is consistent with the proposal that multiple protonation of the neutral-pH conformation of BHA is required to trigger the conformational change (3). The observed variation of the initial rate with pH was a consequence of the dependence of the amplitudes of both phases on pH, with the rate constants k_1 and k_2 found to be independent of the proton concentration. This observation, which is consistent with previous studies on the variation of the rates of exposure of antigenic sites on the low-pH conformation of BHA with pH (14), as well as with an earlier study using bis-ANS (8), suggests that the pH environment influences a partitioning of the BHA between conformations rather than simply the proportion of BHA sufficiently protonated to trigger the conformational change.

The fluorescence intensity of bis-ANS in the presence of BHA in its low-pH conformation has been shown to be an order of magnitude higher than in the presence of BHA in its neutral-pH conformation. The use of this probe in kinetic studies of the conformational change has revealed a multiphasic reaction pathway which is highly sensitive to pH and provides a sensitive method for characterisation of inhibitors of this reaction.

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