

Anti-Peptide Antibodies Detect Steps in a Protein Conformational Change: Low-pH Activation of the Influenza Virus Hemagglutinin

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Abstract. At low pH, the hemagglutinin (HA) of influenza virus undergoes an irreversible conformational change that potentiates its essential membrane fusion function. We have probed the details of this conformational change using a panel of 14 anti-HA-peptide antibodies. Whereas some antibodies reacted equally well with both the neutral and low-pH HA conformations, others reacted to a significantly greater extent with the low-pH form. The locations of the

peptides recognized by the latter antibodies in the three-dimensional HA structure indicated regions of the protein that change in response to low pH. Moreover, kinetic experiments suggested steps in the conformational change. In addition to their relevance to membrane fusion, our results show that anti-peptide antibodies can be used to study some types of biologically important protein conformational changes.

MANY important biological processes are regulated by changes in protein conformation. Whereas some conformational changes have been analyzed in detail by electron microscopy (10, 29), x-ray crystallography (23, 24), or nuclear magnetic resonance spectroscopy (3), others have been less amenable to high-resolution structural analysis. An example of the latter is the irreversible low pH-induced conformational change that activates the membrane fusion function of the influenza virus hemagglutinin (HA,¹ for review see reference 34). Whereas the structure of the neutral pH form of the bromelain-released fragment of the HA (BHA) has been determined by x-ray diffraction analysis (35, 38), similar high-resolution information is not available for the acid conformation. Attempts to understand the low pH conformation have therefore employed biochemical (9, 26), electron microscopical (25), and immunological (5, 17, 32, 39) approaches. Circular dichroism studies indicate that the neutral and low-pH forms of the HA show no gross differences in secondary structure (26). Analyses of protease sensitivity (9, 26) and studies with two antiprotein mAbs (5, 17, 32, 39) suggest that the contacts between the HA monomers are partially dissociated upon acidification.

Here, we have confirmed and extended these earlier investigations using a panel of 14 HA-specific anti-peptide antibodies (16, 22, 37). Given their predetermined specificity for particular peptide segments, we have been able to probe

regions of the molecule not examined in the prior studies. In this manner we have gained more precise information about the structure of the HA at low pH. Moreover, we have used several of the anti-HA-peptide antibodies to monitor the kinetics and the sequence of events involved in the transition of the HA to its low-pH "fusion-active" state.

Materials and Methods

Cells, Virus, and Reagents

CV-1 cells (African green monkey kidney cells) were cultivated in DME as previously described (14). The X:31 strain of influenza virus was grown in the allantoic cavity of 11-d embryonated chicken eggs and purified as previously described (9). Iodogen was purchased from Pierce Chemical Co., Rockford, IL. Proteinase K was purchased from Boehringer Mannheim, Indianapolis, IN. [¹²⁵I]iodine and [³⁵S]methionine were obtained from Amersham Corp., Arlington Heights, IL. Zysorbin was purchased from Zymed Laboratories, South San Francisco, CA. All other biochemical reagents were obtained from the Sigma Chemical Co., St. Louis, MO.

Purification and Radiolabeling of BHA and CHA

BHA trimers were isolated from egg grown X:31 (A/Aichi/1968) influenza virus and iodinated to sp act $5-10 \times 10^5$ cpm/ μ g using the Iodogen procedure as described previously (9). The iodinated protein was then repurified on a 5-25% sucrose (wt/vol in Mes-saline buffer [MS, 20 mM 2-(N-morpholino)ethanesulfonic acid, 130 mM NaCl, pH 7]) gradient by centrifugation for 16 h at 250,000 g, 15°C in a SW41 rotor (Beckman Instruments, Inc., Fullerton, CA). The fractions from the 9S peak of this gradient (corresponding to BHA trimers) were pooled and judged to be >90% pure by SDS-PAGE. To further insure the integrity of the [¹²⁵I]BHA trimers labeled and purified in this fashion, the protein was checked for its resistance to proteinase K digestion (9). More extensive iodination resulted in the

1. Abbreviations used in this paper: BHA, bromelain released fragment of hemagglutinin; CHA, complete hemagglutinin; HA, hemagglutinin.

recovery of a significant fraction of smaller S-value BHA species that were not resistant to proteinase K (J. White, unpublished).

[³⁵S]CHA (the complete HA molecule including its transmembrane and cytoplasmic domains, nomenclature as in reference 25) trimers were prepared by infecting a monolayer of CV-1 cells with X:31 influenza virus and then labeling the cells for 16 h with [³⁵S]methionine (0.5 mCi/75 cm² tissue culture flask). The infected cells were harvested in 1 ml of lysis buffer (1% NP40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin in PBS). After removing nuclei and cell debris by centrifugation for 15 min at 12,000 g, we passed the extract over a 10-ml column of Ricin-Sepharose (9). The material that eluted with 0.2 M galactose was then concentrated, treated with 10 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone-trypsin to cleave the protein from its precursor (CHAO) to active (CHA) form, and further purified on a 5–25% sucrose gradient as just described for BHA trimers except that the gradient contained 0.1% NP40. The fractions from the 9S-CHA trimer peak were pooled.

Monomeric [¹²⁵I]BHA was prepared by acidifying a sample of [¹²⁵I]BHA trimers to pH 4.8 for 15 min at 25°C (7). The sample was then neutralized and applied to a 5–25% sucrose (wt/vol) gradient in MS buffer, pH 7, containing 0.1% NP40. After centrifugation in an SW41 rotor for 16 h at 250,000 g, 15°C, 700-μl fractions were collected from the bottom of the tube and the fractions containing the ~4S BHA monomers (about 10% of the total protein applied to the gradient) were pooled.

Acidification Reactions

Samples containing ~300 ng of [¹²⁵I]BHA per ml (~1.3 nM BHA) in MS supplemented with 0.1% NP40 (unless otherwise stated) were adjusted to the indicated pH values by the addition of predetermined amounts of acetic acid. After incubation for the indicated time and at the indicated temperature, the solutions were returned to pH 7.0 by the addition of predetermined amounts of NaOH. Solutions of [³⁵S]CHA were similarly acidified except that the concentration of [³⁵S]CHA was estimated to be ~15 nM. After incubation for the indicated time and at the indicated temperature, the samples were reneutralized with NaOH. For kinetic experiments, aliquots of acidified BHA or CHA solutions were withdrawn at specified time points and immediately dispensed into tubes containing NaOH to restore the pH to 7.0.

Immunoprecipitations

Duplicate aliquots (100 μl each) containing ~2 × 10⁴ cpm of [¹²⁵I]BHA or 4 × 10⁴ cpm of [³⁵S]CHA were incubated with the indicated antibody (50 μl diluted into MS containing 0.1% NP40) for 16 h at 4°C or, where stated, for 2 h at 25°C. The antigen-antibody complexes were then absorbed with 25 μl of Zysorbin (10% in PBS), washed two times with wash buffer (0.5 M NaCl, 0.1 M Tris, pH 8.0, 0.1% NP40), and counted. These conditions were optimized for the precipitation of [¹²⁵I]BHA with the rabbit anti-X:31 virus polyclonal antiserum. The antibodies in the anti-virus antiserum are mainly directed against antigenic sites A and B of the HA trimer (35). The anti-peptide antisera were raised against synthetic peptides from the X:47 strain of influenza virus (16, 22, 37). The X:47 and the X:31 strains are 95% homologous (2, 21, 31). In all cases, the background values (0.2–0.7% of the input counts) of HA precipitated with an antiserum against preprolactin (1:50 dilution) were subtracted. Each experiment depicted was repeated at least twice.

Results

Because the accessibility of a peptide is likely to be one of the major factors determining the reactivity between an anti-peptide antibody and an intact protein (15, 30), site-specific anti-peptide antibodies should be able to distinguish differences in the surface accessibility of peptide sequences in different protein conformations. We therefore compared the reactivities of 14 anti-peptide antibodies to the HA of influenza virus in two of its conformational states: its neutral-pH trimeric form (35, 38) and its low-pH form. Because in the low-pH conformation some of the trimeric contacts are partially disrupted (7, 9, 25, 26), one might expect certain anti-peptide antibodies to react preferentially with the molecule after acidification. Indeed, we identified several anti-HA-peptide antibodies that showed preferential binding to

the low-pH state of the molecule. We then used these conformation-sensitive probes to study the pH dependences and the apparent kinetics of several steps in the conformational change.

Anti-Peptide Antibody Binding to Neutral vs. Low pH BHA

The anti-peptide antisera used in this study were raised against synthetic peptides from almost all regions of the HA molecule (16, 22, 37). All have been shown previously to react with BHA or virus in solid-phase assays (16, 22, 37). To avoid possible conformational changes induced by adhesion of the HA to solid supports, we conducted quantitative immunoprecipitations of lightly iodinated native BHA trimers or [³⁵S]methionine labeled CHA trimers from solution. The antibody binding should then monitor the reactivity with intact protein in as close to “native” condition as possible. In all cases we have assessed the percent of the input HA precipitated. Most prior immunoprecipitation studies with anti-peptide antibodies have only presented counts of radio-labeled protein precipitated, thus making it difficult to assess the efficiency of precipitation.

Several of the antisera employed, including HA1 1-15 and HA2 15-29, are directed against peptides that are located mainly on the surface of the HA trimer (Fig. 1 *B*, bottom). Others are against peptides (see Fig. 1 *B*, top) that are relatively or completely inaccessible in the native neutral pH form of the HA either because they are buried in the trimer interface (HA1 98-106) or because they are masked in the monomer (HA1 305-328) by other peptide segments. Hence, for the latter group of peptides (Fig. 1 *B*, top), an increase in peptide exposure should lead to an increase in anti-peptide antibody binding.

The major finding presented in this paper is that the reactivities of anti-peptide antisera directed against sequences that lie partially or completely in the BHA trimer interface (Fig. 1 *B*, top) were markedly enhanced after a brief treatment of the BHA at low pH (Table I). The most striking example was the >300-fold increase observed with a mAb against a nine-amino acid determinant (HA1 98-106) that is completely buried in the trimer interface (referred to as the “interface” peptide; see Table I for peptide nomenclature [11, 16, 22, 37]). This finding agrees well with the relatively high affinity of this antibody for HA1 monomeric “tops” ($K_A = 10^6 M^{-1}$) compared with its negligible affinity for the trimer ($K_A < 10^4 M^{-1}$) (36).

Other antibodies that showed significantly enhanced reactivity after low-pH treatment of BHA were those (see Table I) against the COOH-terminus of HA1 (C-HA1), the amino terminus of HA2 (the “fusion” peptide), a “loop” peptide which is embedded in the fibrous stem of the trimer, a “hinge” peptide located in antigenic site C, a “tip” peptide located in antigenic site B near the trimer interface, a “top” peptide found in antigenic site D in the subunit interface of the globular heads, and a peptide that extends from the stem to the hinge region (HA1, residues 38–64). Calculations of the surface-accessible area (4) using a 6-Å probe showed that all of these peptides are either completely (e.g., the interface peptide) or partially (e.g., the loop peptide) buried in the native trimer interface (see Fig. 1 *B*, top; additional data not shown).

Whereas some of the antisera that bound preferentially to

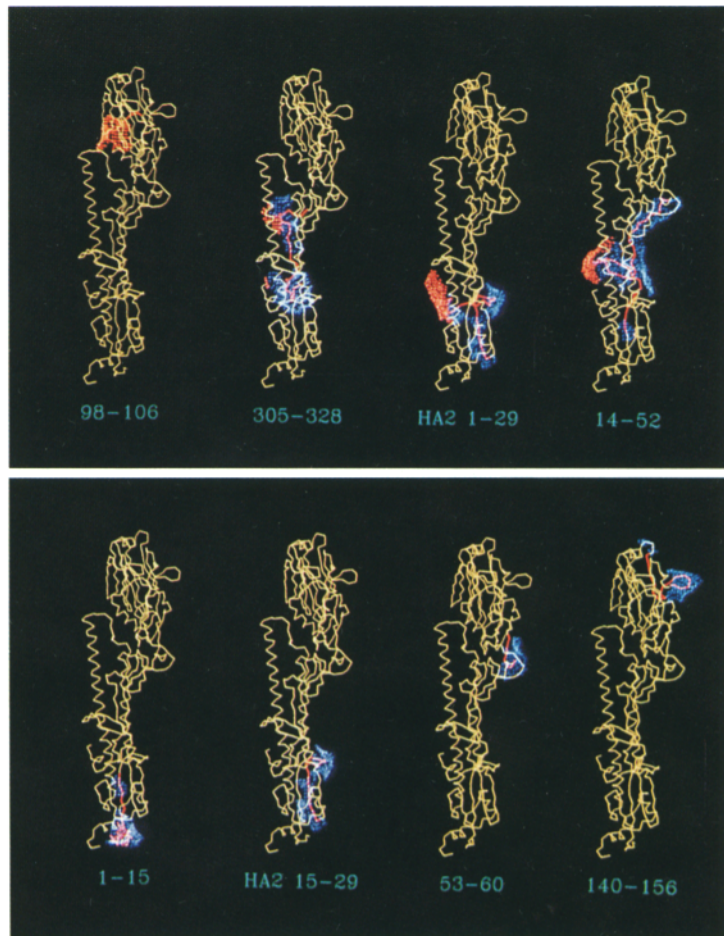
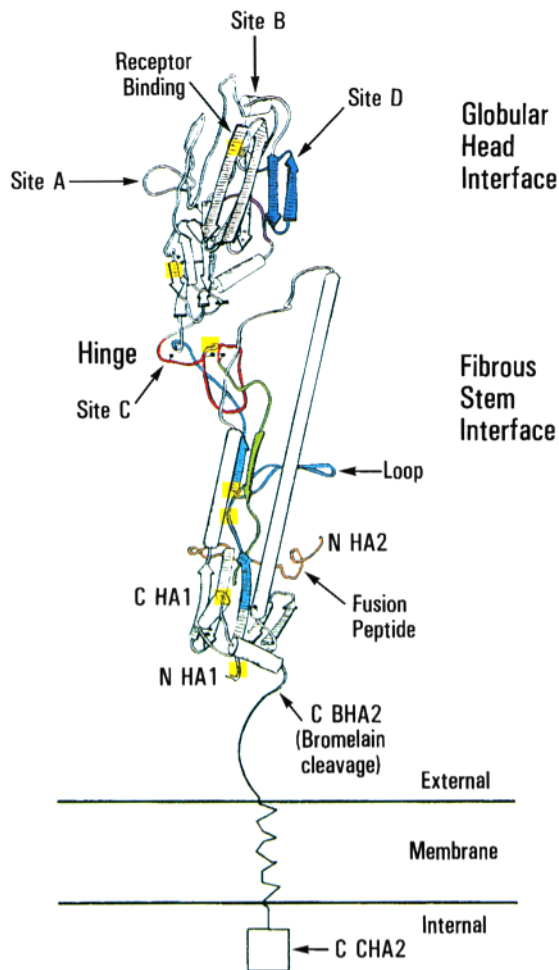


Figure 1. (Left) The location on the schematic drawing of a BHA monomer (38) of the peptide sequences that are the most reactive at low pH. The antibodies are directed against HA1 98–106 (purple), HA1 305–328 (green), HA2 1–15 (orange), HA1 14–52 (cyan), HA1 266–302 (red), and HA1 201–227 (dark blue, top). Arrows represent β -structure and rods represent α -helices. Color code is the same as in Fig. 6. (Right) Location and surface accessibility of some of the peptides used in this study. The α -carbon monomer HA coordinates of Wilson, Skehel, and Wiley (38, Brookhaven protein structure data bank) are shown in yellow; the peptide sequence used to generate the antibodies is in red. The surface-accessible area in the trimer (4) for a 6-Å probe is represented by blue dots and the area buried in the trimer is shown in red dots. Peptide sequences buried in the monomer have no dots. Conformational changes either by trimer disassembly of the globular heads (e.g., 98–106), or by peptide released from the trimer interface (e.g. HA2 1–15, HA1 14–52) would result in increased exposure of these peptides and more accessibility for antibody binding. Top panel is arranged (left to right) according to increased antibody reactivity to low pH vs. neutral pH BHA (as in Table I). Bottom panel shows those peptides with essentially no difference in antibody reactivity to neutral or low-pH BHA.

low-pH-treated BHA showed maximum binding to BHA that had been pretreated at pH 5 (e.g., the antibody against the fusion peptide), others (e.g., the mAb against the interface peptide) showed stronger reactivity with BHA that had been pretreated at pH 4.6. The former antisera are all against peptides located in the stem region of the molecule, whereas the latter are all against peptides found in the head or hinge regions of the trimer (Table I, Fig. 1 A).

The data presented in Table I were obtained using fixed dilutions of antisera (1:30 for the polyclonal antisera and 1:300 for the monoclonal ascites fluid) and under one set condition of precipitation optimized for the anti-viral antiserum. This was done for comparative purposes both among the different anti-peptide antisera and especially for comparison with the anti-virus antiserum. Several of the anti-peptide antisera could precipitate more low-pH-treated BHA by varying the

precipitation conditions. For example, for the four most extensively studied sera, the maximum amounts of low-pH-treated BHA precipitated in one round of solution immunoprecipitation were 36% with the antiserum to the interface peptide, 75% with the antiserum to the C-HA1 peptide, 39% with the antiserum to the fusion peptide, and 44% with an antiserum generated against the uncoupled loop peptide. Moreover, with the antibodies against the interface peptide and the C-HA1 peptide, higher precipitation values (89% and 92%, respectively) were obtained when precipitating low-pH-treated [35 S]methionine-labeled CHA from solution. Whether the higher degree of precipitation of CHA as compared with BHA was due to differences in the manner in which these molecules were radiolabeled, due to differences in the concentration of BHA vs. CHA in our reactions (see Methods), or whether it is a further reflection of subtle

Table I. Comparison of the Reactivity of Anti-Peptide Antibodies to Neutral and Low-pH-treated BHA

Antibody*	Residues	Location	Name	[¹²⁵ I]BHA (% precipitated)		
				pH 4.6	pH 5.0	pH 7
Virus	HA1	Site A/B	Virus	81.4	93.8	92.1
H26D08	HA1 (98-106)	Head	Interface	33.6	17.5	0.1
20	HA1 (305-328)	Stem	C-HA1	58.2	50.7	15.6
24	HA2 (1-29)	Stem	Fusion	27.2	28.1	0.5
3	HA1 (14-52)†	Stem	Loop	11.6	11.1	0.5
23	HA1 (266-302)†	Hinge (site C)	Hinge	10.6	4.8	0.5
19	HA1 (201-227)	Head (site D)	Top	8.6	3.9	1.0
17	HA1 (174-197)	Head (site B)	Tip	7.7	4.5	0.7
4	HA1 (38-64)†	Hinge	—	5.1	2.4	0.6
1	HA1 (1-15)†	Stem	N-HA1	7.5	7.6	6.4
25	HA2 (15-29)	Stem	—	6.4	6.0	6.6
2	HA1 (1-39)†	Stem	—	2.7	2.3	1.2
11	HA1 (105-139)	Head (site A)	—	1.4	1.1	0.7
15	HA1 (140-156)	Head	—	0.9	1.0	0.5
5	HA1 (53-60)	Head	—	0.9	0.4	0.3

Solutions of BHA were incubated at the indicated pH values for 15 min at 25°C, reneutralized, and then immunoprecipitated as described in the Methods section. The data shown are for a 1:300 dilution of monoclonal ascites fluid (antibody H26D08) and for 1:30 dilutions of the rabbit polyclonal antisera (all others). Values are the averages of at least four determinations. SD, 0.1-5.0%.

* Nomenclature for the anti-peptide antisera according to references 16 and 37. Site A, B, C, and D refer to the antigenic sites of the HA as defined in reference 35. † Contains Asn-X-Ser/Thr sequences which are glycosylated in A/Aichi/2/1968 (31).

differences between BHA and CHA, as suggested from other studies (7, 9, 25) remains to be determined.

Another finding depicted in Table I is that all of the antisera that reacted to the same extent with neutral and low-pH-treated BHA are directed against peptides which lie almost exclusively on the surface of the HA trimer (Table I, Fig. 1 B, bottom). These include antibodies directed against the amino terminus of HA1 (N-HA1, residues 1-15), a peptide in antigenic site A (HA1 residues 105-139), a peptide in the globular heads between sites A and B (HA1 residues 140-156), and residues 15-29 of HA2. The fact that we saw a change in reactivity of HA2 residues 1-29 whereas we saw no difference in the reactivity of HA2 residues 15-29 allowed us to localize the major change in the fusion peptide region to residues 1-15, precisely those amino acids that are buried most deeply in the trimer interface (38).

One should note that only a few of the anti-peptide antibodies were able to immunoprecipitate native pH 7 BHA trimers. These were the antibodies against the amino- and carboxy-terminal peptides of HA1 and against residues 15-29 of HA2. Even these precipitated less native pH 7 BHA trimers (6, 16, and 7%, respectively) compared with a polyclonal anti-X:31 viral antiserum (>90% pH 7 trimers precipitated). Interestingly, up to 30% of the CHA trimers have been precipitated with the C-HA1 antibody (see Fig. 5 D, legend, and Discussion).

A further demonstration of the enhanced reactivity of several antisera with peptides that lie partially or completely in the subunit interface is shown in Fig. 2. Three of the antisera depicted showed background binding to neutral-pH BHA trimers irrespective of the concentration of the antiserum used. These were the antisera against the interface (Fig. 2 a), fusion (Fig. 2 b), and loop (Fig. 2 c) peptides. All of these showed enhanced reactivity with pH 5-treated BHA. The serum dilutions for half-maximal binding (to low-pH BHA)

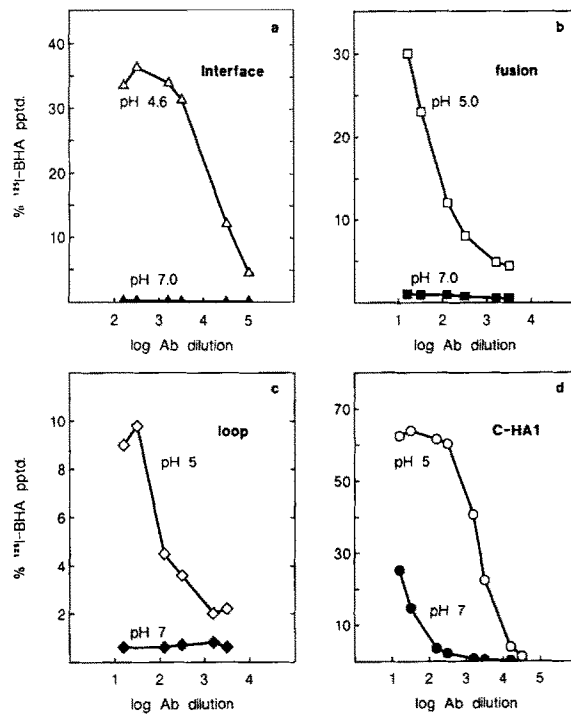


Figure 2. Titration curves for anti-peptide antisera binding to neutral and low-pH-treated BHA trimers. Incubations containing [¹²⁵I]BHA were either left at neutral pH (solid symbols) or acidified (open symbols) to either pH 4.6 (a) or pH 5.0 (b, c, and d) for 15 min at room temperature, reneutralized, incubated with the indicated dilution of the antibodies against the "interface" peptide (a; ▲, △), the "fusion" peptide (b; ■, □), the "loop" peptide (c; ◆, ◇), and the "C-HA1" peptide (d; ●, ○); nomenclature as in Table I, for 2 h at room temperature and then immunoprecipitated as described in Methods. Similar profiles (to a, b, and c) were obtained for all of the other anti-peptide antisera which showed enhanced reactivity with low-pH-treated BHA (data not shown).

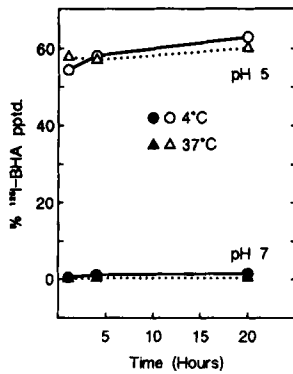


Figure 3. Time and temperature dependence of the formation of immune complexes. Samples of [125 I]BHA were either left at neutral pH (solid symbols) or treated at pH 5 (open symbols) for 15 min at room temperature, neutralized, and then immunoprecipitated with a 1:300 dilution of an antibody against the COOH-terminus of HA1. The antibody-antigen reactions were carried out at either 4°C (●, ○) or 37°C (▲, △) for the indicated times. The immune complexes were then precipitated and

washed as described in Methods. Superimposable data were obtained when the antibody-antigen reactions were conducted at 25°C (not shown).

were 1:12,000 (interface), 1:90 (fusion), and 1:150 (loop), respectively. Although the antiserum against the COOH-terminus of HA1 (at high concentration) precipitated a significant fraction of the native BHA trimers (presumably because the five most COOH-terminal residues of HA1 lie on the surface of the trimer [38]), it precipitated substantially more pH 5-treated BHA (Fig. 2 *d*). Moreover, this antiserum showed a significantly higher affinity for the low-pH form; the dilutions for half-maximal binding were 1:2,400 for pH 5-treated BHA compared with 1:36 for pH 7 BHA trimers (Fig. 2 *d*).

Experiments were carried out to show that the observed anti-peptide antibody binding was not a result of time- or temperature-dependent general denaturation of the protein and that the antibodies were not inducing the conformational change. For example, at a 1:300 dilution, the antibody against the COOH-terminus of HA1 showed no time (30 min–24 h) or temperature (4°C, 25°C, 37°C) dependence for formation of immune complexes with either neutral or pH 5-pretreated BHA (Fig. 3). Similar results were obtained with the antibody against the interface peptide (not shown). In addition, the reactivity of the anti-peptide antisera actually decreased on treatment of BHA with SDS (data not shown).

pH Dependences of the Conformational Changes

Having found that several of the anti-peptide antisera bound preferentially to the low-pH form of the HA, we next used these sera to analyze the pH dependences with which individual peptide segments became exposed in conformations suitable for antibody binding. Our results show that the optimum pH at which the BHA trimers had to be pretreated to obtain maximum reactivity was distinct for each peptide (Fig. 4 *a*). For example, half-maximal binding was obtained after preincubation of BHA trimers at pH 5.7 for the fusion peptide, pH 5.4 for the C-HA1 peptide, and pH 5.0 for the interface peptide (Fig. 4 *a*). As expected, the polyclonal anti-viral antiserum (which recognizes mainly antigenic sites A and B, Fig. 4 *a*) and a monoclonal anti-viral antiserum directed against site A (data not shown) reacted with BHA trimers in a pH-independent fashion. In all experiments shown, the samples were reneutralized before the addition of antibodies. The irreversible nature of the conforma-

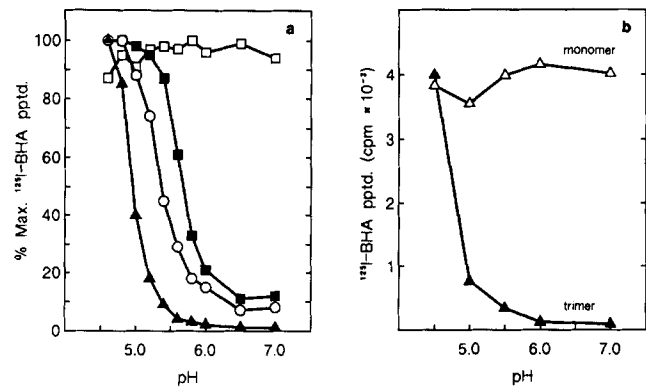


Figure 4. (a) pH dependences of the conformational changes as revealed by enhanced anti-peptide antisera reactivity. Fig. 3 *a*: Aliquots of [125 I]BHA trimers were adjusted to the indicated pH value, incubated for 15 min at room temperature, reneutralized, and immunoprecipitated with the antisera against the virus (1:75), the fusion peptide (1:30), the C-HA1 peptide (1:300), or the interface peptide (1:300) as described in Methods. The data are presented as a percentage of the maximum amount of input [125 I]BHA precipitated under these conditions with the indicated serum: antiviral, 95% (□); anti-fusion peptide, 30% (■); anti-C-HA1, 64% (○); anti-interface peptide, 33% (▲). (b) Comparison of the binding of the mAb against the interface peptide to BHA monomers and trimers. Samples of BHA monomers (△) or trimers (▲) were processed as described above. Data presented are the total counts precipitated (33% of the input for both monomeric and trimeric BHA).

tional changes, as previously suggested from biochemical studies (9, 26), was confirmed by demonstrating that the pH dependences as assessed by anti-peptide antibody binding were the same whether or not the samples were reneutralized before immunoprecipitation (not shown). In addition, identical pH profiles were obtained when the experiment was conducted in the absence of 0.1% NP40 (data not shown).

The results presented in Fig. 4 *b* illustrate that the striking increase in reactivity (300-fold) of the mAb directed against the interface peptide is the result of low-pH-induced dissociation of the three globular heads. Whereas binding of this antiserum to BHA trimers was strictly pH dependent, binding to either BHA monomers or to monomeric HA1 "tops" (26) was pH independent (Fig. 4 *b*). As noted above, these findings are in agreement with our previous study showing that, in solution, this antibody binds to monomeric HA tops but not to intact trimers (36).

For the experiments depicted in Fig. 4, samples were acidified for 15 min at 25°C. When the samples were acidified at 37°C, the pH dependence for the apparent exposure of the interface peptide shifted ~ 0.2 pH units in the more basic direction, with 50% maximal antibody binding occurring for BHA pretreated at pH 5.2. The pH dependences for the apparent exposures of the C-HA1 peptide and the fusion peptide were the same at 37°C as they were at 25°C. The pH profile for the apparent exposure of the C-HA1 peptide was identical to the pH dependence previously recorded for the proteinase K sensitivity of BHA, and for the ability of BHA to bind to preformed liposomes (9, and unpublished results). The pH dependence for enhanced reactivity of the interface peptide (at 37°C) coincides most closely with the actual pH

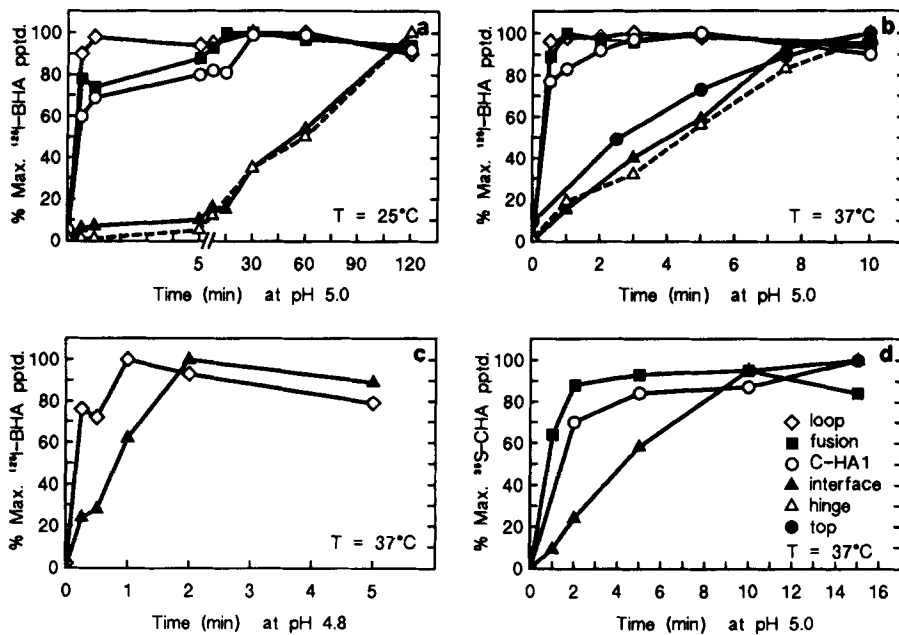


Figure 5. Kinetics of the conformational changes as revealed by enhanced anti-peptide antisera reactivity. [125 I]BHA (a-c) or [35 S]CHA (d) containing mixtures were adjusted to pH 5.0 at 25°C (a), pH 5.0 at 37°C (b and d), or pH 4.8 at 37°C (c). Samples were withdrawn at the indicated times, reneutralized, and immunoprecipitated as described in Methods. Antisera dilutions for the antibodies against the fusion peptide, the C-HA1 peptide, and the interface peptide were as indicated for the data in Fig. 4 a. For the additional antisera tested, the following dilutions were used: antiloop, 1:30; antihinge, 1:30; antitop, 1:75. As in Fig. 4 a, the data are plotted as percentage of the maximum amount of BHA or CHA precipitated by a given serum. For BHA trimers these values were: anti-fusion peptide, 30%; anti-C-HA1 peptide, 64%; anti-interface peptide, 33%; anti-loop peptide, 13%; anti-hinge peptide, 14%; anti-top peptide, 9%. For the experiments with [35 S]CHA trimers (d), in

addition to the preprolactin control values, the amount of pH 7 CHA trimers precipitated were also subtracted (anti-C-HA1, 28%; anti-fusion peptide, 1%; anti-interface peptide, 1%). The maximum amounts of [35 S]CHA precipitated were: anti-fusion peptide, 30%; anti-C-HA1, 92%; and anti-interface peptide, 89%. Symbols for the different sera are listed as an inset in d.

dependence for cell-cell fusion of BHK cells (at 37°C) with the X:31 strain of influenza virus (9).

Kinetics of the Conformational Changes

To analyze the apparent kinetics of the conformational changes, BHA (Fig. 5, a-c) or CHA (Fig. 5 d) trimers were treated at pH 5, 25°C (Fig. 5 a), pH 5, 37°C (Fig. 5, b and d), or pH 4.8, 37°C (Fig. 5 c). At the specified time intervals, aliquots were withdrawn, immediately reneutralized, and immunoprecipitated with the indicated antisera. Because the maximum amount of HA precipitable by the different sera differed greatly (see also Table I), the data have been expressed as the percentage of the maximum amount of low-pH-treated HA precipitated. Presentation of the data in this fashion was justified based on experiments that indicated that the differences in the maximum amount of HA precipitated by the different antisera likely reflect differences in the affinities of the sera for low-pH-treated HA rather than heterogeneity of the HA molecules. Firstly, sequential precipitations with the same antibody consistently precipitated additional HA. Secondly, immunoprecipitations with combinations of the different sera never precipitated a higher percentage of HA than either antiserum alone (data not shown). This latter finding suggested that the population of low-pH-treated HA molecules was not heterogeneous. Therefore, the data should accurately reflect the time required at low pH to expose the peptides in conformations suitable for antibody binding.

At 25°C, pH 5, the half times for apparent exposure of the fusion peptide and the COOH-terminus of HA1 were 15 and 30 s, respectively. The interface peptide apparently became exposed with a half-time of ~50 min after a lag of 5 min (Fig. 5 a). Whereas the half-times for exposure of the fusion peptide and the COOH-terminus of HA1 were relatively unchanged by elevating the temperature to 37°C, the half-time

for apparent exposure of the interface peptide was significantly reduced from 50 min at 25°C (Fig. 5 a) to 4 min at 37°C (Fig. 5 b). At pH 4.8, 37°C, the half-time for exposure of the interface peptide was further decreased to 45 s (Fig. 5 c). The predicted correlation between the pH dependences and the kinetics of the conformational changes as monitored by anti-peptide antibody binding was confirmed. For example, the fusion peptide became exposed for reaction with its corresponding antiserum at both a higher pH and more quickly than the interface peptide (Figs. 4 a and 5).

We also characterized the apparent kinetics for the release of three other peptides: the loop peptide (located in the stem interface), the top peptide (located in the globular head interface), and the hinge peptide (located in the hinge region). Based on the crystal structure of the BHA trimer (38) we had predicted: (a) to observe greater reactivity of the HA1 COOH-terminus, the loop peptide would have to move out from the trimer interface before and in addition to the fusion peptide; and (b) if the globular heads of the molecule open up by bending about the hinge region, then the kinetics with which the hinge peptide, the top peptide, and the interface peptide become more accessible for antibody binding should be similar. Indeed, the loop peptide became accessible measurably sooner than the COOH-terminus of HA1. At pH 5, 37°C, the half-time for the loop peptide was 7.5 s whereas the half-time for the C-HA1 peptide was 30 s (Fig. 5 b). As demonstrated at both 25°C (Fig. 5 a), and at 37°C (Fig. 5 b), the hinge, interface, and the top peptides apparently became exposed (in conformations suitable for antibody binding) in concert, suggesting that a single major low-pH-induced structural alteration in the BHA could be responsible for the increased antibody binding to discrete regions within the globular heads. Because three different probes for the globular head region required that the HA be acidified for similar lengths of time to obtain maximal antibody binding,

we believe that the kinetics mainly reflect changes in peptide accessibility rather than additional low-pH-induced changes in peptide structure required for optimal interaction with the corresponding peptide antiserum.

Because BHA appears to behave differently in some subtle, as yet unidentified ways from CHA in response to low pH (7, 9, 25), the kinetics of the apparent exposure of several peptides were compared for BHA and CHA trimers. The relative order for the exposure of these peptides was indeed found to be the same for both CHA and BHA trimers (Fig. 5 *d*). Although the half-times for apparent exposure of the fusion and C-HA1 peptides were somewhat slower for CHA than for BHA, the half-time for apparent exposure of the interface peptide at pH 5, 37°C was the same, 4 min, for CHA and BHA trimers.

Steps in the Conformational Change

These data suggest a possible unfolding pathway for the low-pH-induced conformational changes in the HA. There appear to be two major aspects of the transition in which structural alterations in the stem region are followed by changes in the globular head region of the trimer (Fig. 6). In the first step, the stem interface loop becomes more accessible, implying release from the trimer interface, quickly followed by the fusion peptide. These changes would expose more residues of the COOH-terminus of HA1 (Fig. 6 *b*). Sometime later, a second step occurs in the globular heads of the trimer such that the interface, top, and tip peptides become more accessible to their corresponding antisera; in this manner more of the hinge region would be exposed (Fig. 6 *c*). The results presented in Fig. 5 indicate that this second major step in the conformational change is highly dependent on both temperature and pH; it occurs faster at 37°C than at 25°C (Fig. 5 *b* vs. Fig. 5 *a*) and faster at pH 4.8 than at pH 5.0 (Fig. 5 *c* vs. Fig. 5 *b*). We would like to emphasize that Fig. 6, *a-c* are intended to be highly schematic and represent only a working model based on how the three-dimensional structure of the intact trimer would have to change to account for the anti-peptide antibody binding data. Nonetheless, the sequence of changes that we have proposed is sterically feasible based on examination of the three-dimensional structure of the neutral pH trimer (38). Moreover, the alterations we have monitored here are in agreement with those postulated from prior investigations (7, 17, 25). It is likely that other changes occur in the molecule that we have not detected in this study, either because certain segments of the protein might be masked by carbohydrate (27) or due to lack of anti-peptide antibody probes. For example, Helenius and co-workers favor a model where the coiled-coil alpha helices in the HA2 stem interface (for which we do not have a probe) dissociate upon acidification (7, 8).

Discussion

The low-pH-induced conformational change that activates the membrane fusion function of the influenza virus hemagglutinin (HA) has been the subject of extensive investigation (5-9, 14, 17, 25, 26, 32, 33, 39). These studies have led to a model in which the heads of the HA trimer come apart but remain globular, the stem region remains trimeric with some thinning, and no major alterations occur in overall secondary structure (7, 9, 17, 25, 26). In this study, we have used a panel of 14 anti-HA-peptide antibodies to compare the surface

structures of the native neutral-pH trimers and the low-pH conformation of the molecule. The results obtained by this analysis are fully consistent with the model arrived at by more conventional approaches. We believe that this excellent agreement validates the approach taken here and shows the utility of anti-peptide antibodies as probes for protein conformational changes.

By using anti-peptide antibodies, we have been able to extend the prior observations in two ways. Firstly, due to the defined specificities of the anti-peptide antibodies, we have been able to identify more precisely the regions of the HA which change in response to low pH. For example, we have provided the most definitive evidence that the fusion peptide comes out from the trimer interface and identified for the first time a similar conformational change for the loop peptide. Lysine 27 of this loop was previously shown to be susceptible to trypsin cleavage only after low-pH treatment (26). Secondly, by using these probes in kinetic studies we have been able to dissect the conformational change (previously envisioned as a concerted process) into at least two major transitions, with changes in the stem region (Fig. 6 *b*) preceding changes in the globular head region (Fig. 6 *c*).

The role of these conformational changes in viral fusion events is still largely unknown. Although isolated BHA and CHA respond to acidification in a manner similar to HA in the virus membrane (9, 25), the possibility still exists that the changes that we and others (5-9, 25, 26, 32, 39) have observed with isolated HA molecules in solution may be different for viral HA and/or when a virion confronts the target membrane in an *in vivo* fusion situation. To the extent that we can extrapolate from experiments in solution to the *in vivo* fusion reaction, a notable finding is that both in terms of its pH dependence and its kinetics, the fusion peptide appeared to be liberated from the stem trimer interface relatively early in the conformational change. This observation supports two previous speculations. (*a*) The fusion peptide may require additional pH-dependent modifications to initiate a fusion reaction (14, 19, 20). (*b*) Dissociation of the globular heads may be required for membrane fusion to occur (8, 26, 34).

At pH 5 and 37°C, conditions optimal for promoting extensive membrane fusion with the X:31 influenza virus (9), the globular heads appeared to separate from each other somewhat slowly (half-time, ~4 min) compared with the kinetics of fusion (half-time, ~30 s for the X:47 influenza virus at pH 5 [28]). However, by reducing the pH to 4.8 (where full fusion activity can still be obtained [9, 28]), we recorded kinetics for the dissociation of the globular heads (half-time, 45 s) in good agreement with the fusion kinetics. Perhaps this stage of the conformational change occurs more rapidly at pH 5 in virions and/or in the presence of a target membrane. Alternatively, the globular heads may have to separate further to permit antibody binding than to allow fusion. These speculations warrant future investigations.

Perhaps more important than their relevance to membrane fusion, our results have implications regarding the use of anti-peptide antibodies in general and as probes for protein conformational changes. Regarding the general usage of anti-peptide antibodies, two important findings emerged from this study. Firstly, compared with a polyclonal anti-viral antiserum, the anti-HA-peptide antibodies (even those directed against accessible sites) were not as efficient at precipitating neutral pH 7 trimers. This result was not unex-

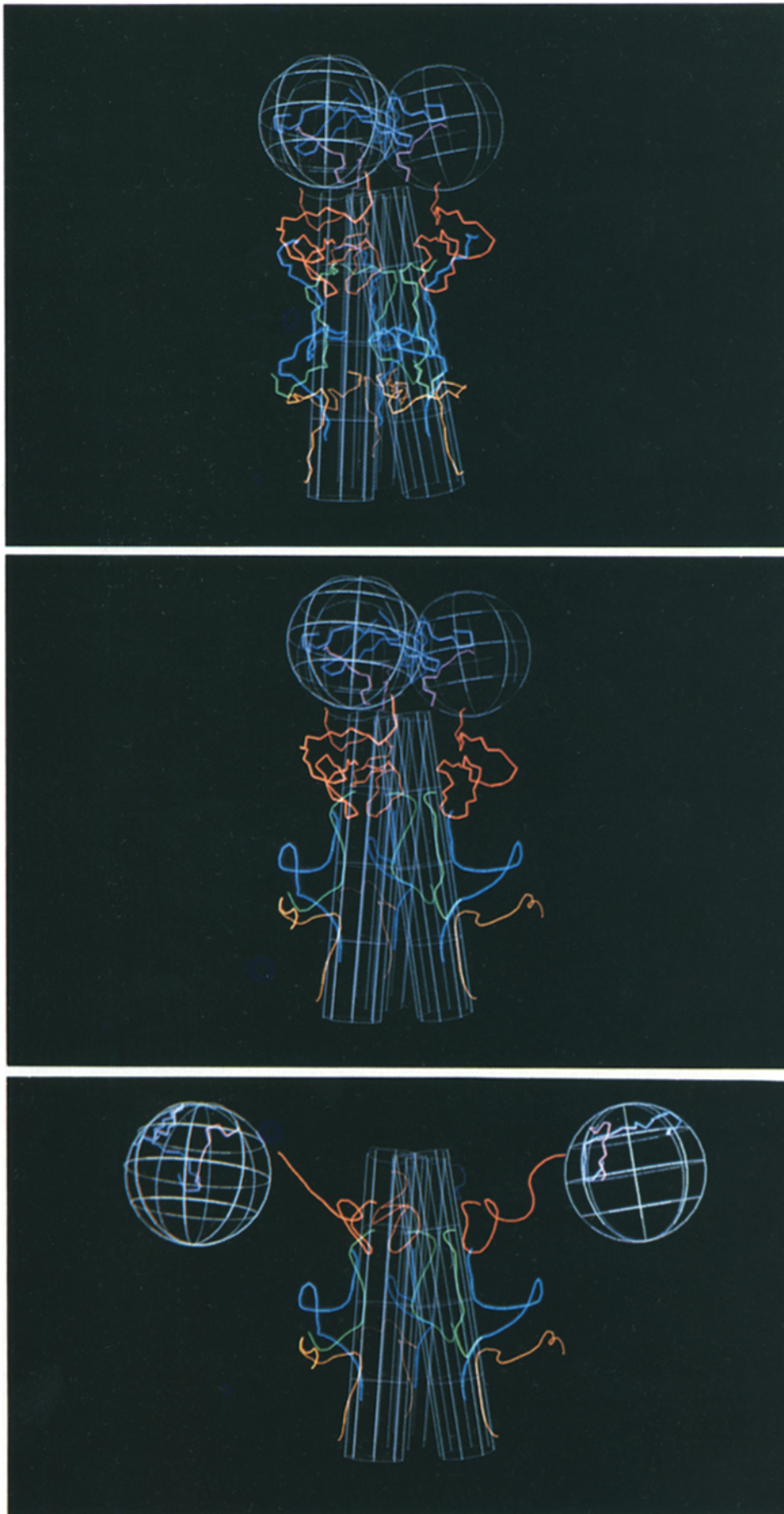


Figure 6. Schematic representation of the low-pH-induced conformational changes in the HA detected with anti-peptide antibodies. Depicted are the neutral pH form (*top*), a proposed intermediate (*middle*), and a proposed low-pH form (*bottom*). As noted in the text, additional changes most likely occur. The peptides in the stem region are proposed to be exposed first (loop peptide, cyan; fusion peptide, orange; C-HA1 peptide, light green) followed by peptides in the trimeric globular heads (top peptide, dark blue; interface peptide, purple) which separate by bending at the hinge region (hinge peptide, red). Peptide color code is similar to Fig. 1. The molecular coordinates are enclosed in spheres and cylinders to emphasize the highly schematic nature of this figure.

pected given that anti-peptide antibodies are essentially monovalent compared with the multivalency of polyclonal anti-protein antibodies. Other factors may contribute to the poorer reaction of the anti-peptide antibodies with neutral pH HA. For example, there are several amino acid changes in the major antigenic sites (A-D) between the X:47 and the X:31 viruses (2, 21, 31, 35). One should note that high-affinity anti-peptide antibodies can be generated. The mAb used in this study represents one of a panel of high-affinity anti-peptide antibodies ($K_A = 10^8 \text{ M}^{-1}$ for peptide; $K_A = 10^6 \text{ M}^{-1}$ for protein [HA "tops," 36]). Moreover, in a recent study of the antigenic sites of myohemerythrin it was found that four out of six monoclonal anti-peptide antibodies bound the intact protein with equal or higher affinity than an anti-protein antibody reactive with the same region of the molecule (12).

A second finding regarding the general use of anti-peptide antibodies is that our results on the ability of the anti-peptide antisera to immunoprecipitate pH 7 trimers appear to conflict with the strong reaction observed with the same antisera in solid phase assays (16, 22, 37). This is especially pronounced for antibodies directed against parts of the molecule that are buried in the trimer. In light of the findings presented here the behavior of the HA in solid-phase assays is more easily understood. Given that the HA is designed to partially dissociate, then it may do so (even at pH 7) when attached to a solid support. (This possibility was raised previously [37].) Although we cannot generalize our results to other proteins, our data highlight the necessity to conduct analyses in solution when examining questions of protein conformation.

Several findings regarding the use of anti-peptide antibodies as probes for protein conformational changes also emerged from this study. Most importantly, we observed that once the HA was allowed to undergo its fusion-inducing conformational change, several antisera (those directed against peptides that lie either completely or partially in the trimer interface) reacted more extensively, and in several cases quite well, with the HA. This finding highlights the obvious importance of the accessibility of any given peptide sequence in a protein for reaction with its corresponding anti-peptide antiserum. However, our data also show that among those antisera that reacted preferentially with low-pH-treated HA, both the maximum amount of low-pH HA precipitable as well as the apparent affinity of the anti-peptide antibodies for low-pH HA differed greatly for different sera. These observations strongly suggest that additional factors, such as local peptide unfolding or changes in mobility (15, 30), contribute to the overall reactivity between the anti-peptide antisera and the intact protein. In this respect it is worthy of note that at both neutral and low pH, the most reactive peptides are those which are both surface accessible and either (a) 'untethered' (e.g., terminal peptides) or (b) not highly structured (e.g., the interface peptide).

In summary, we have documented the use of anti-peptide antibodies for studying low-pH-induced alterations in the influenza virus hemagglutinin. Schoolnik and co-workers have reported a similar use of anti-peptide antisera in their studies on calmodulin. They found differential binding of four polyclonal anti-peptide antisera to calmodulin in its Ca^{2+} -free compared with its Ca^{2+} -saturated form (13). Collectively our data suggest that anti-peptide antibodies can be powerful reagents for monitoring the structural details and dynamics of certain biologically important protein confor-

mational changes (1, 18, 40). This type of analysis will be most meaningful when the three-dimensional structure of at least one conformational state of the protein of interest is known.

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References

- Balkema, G. W., and U. C. Dräger. 1985. Light-dependent antibody labeling of photoreceptors. *Nature (Lond.)*. 316:630-633.
- Both, G. W., and M. J. Sleight. 1981. Conservation and variation in the hemagglutinins of Hong Kong subtype influenza viruses during antigenic drift. *J. Virol.* 39:663-672.
- Campbell, I. D., C. M. Dobson, and R. J. P. Williams. 1985. The study of conformational states of proteins by nuclear magnetic resonance. *Biochem. J.* 231:1-10.
- Connolly, M. L. 1983. Solvent-accessible surfaces of proteins and nucleic acids. *Science (Wash. DC)*. 221:709-713.
- Daniels, R. S., A. R. Douglas, J. J. Skehel, and D. C. Wiley. 1983. Analyses of the antigenicity of influenza haemagglutinin at the pH optimum for virus-mediated membrane fusion. *J. Gen. Virol.* 64:1657-1662.
- Daniels, R. S., J. C. Downie, A. J. Hay, M. Knossow, J. J. Skehel, M. L. Wang, and D. C. Wiley. 1985. Fusion mutants of the influenza virus hemagglutinin glycoprotein. *Cell*. 40:431-439.
- Doms, R. W., and A. Helenius. 1986. Quaternary structure of influenza virus hemagglutinin after acid treatment. *J. Virol.* 60:833-839.
- Doms, R. W., M.-J. Gething, J. Henneberry, J. White, and A. Helenius. 1986. Variant influenza virus hemagglutinin that induces fusion at elevated pH. *J. Virol.* 57:603-613.
- Doms, R. W., A. Helenius, and J. White. 1985. Membrane fusion activity of the influenza virus hemagglutinin. The low pH-induced conformational change. *J. Biol. Chem.* 260:2973-2981.
- Durham, A. C. H., and J. T. Finch. 1972. Structures and roles of the polymorphic forms of tobacco mosaic virus protein. II. Electron microscope observations of the larger polymers. *J. Mol. Biol.* 67:307-314.
- Dyson, H. J., K. J. Cross, R. A. Houghton, I. A. Wilson, P. E. Wright, and R. A. Lerner. 1985. The immunodominant site of a synthetic immunogen has a conformational preference in water for a type-II reverse turn. *Nature (Lond.)*. 318:480-483.
- Fieser, T. M., J. A. Tainer, H. M. Geyson, R. A. Houghton, and R. A. Lerner. 1987. Influence of protein flexibility and peptide conformation on the reactivity of monoclonal anti-peptide antibodies with a protein alpha-helix. *Proc. Natl. Acad. Sci. USA*. In press.
- Garipey, J., T. A. Mietzner, and G. K. Schoolnik. 1986. Peptide antisera as sequence-specific probes of protein conformational transitions: calmodulin exhibits calcium-dependent changes in antigenicity. *Proc. Natl. Acad. Sci. USA*. 83:8888-8892.
- Gething, M.-J., R. W. Doms, D. York, and J. White. 1986. Studies on the mechanism of membrane fusion: site-specific mutagenesis of the hemagglutinin of influenza virus. *J. Cell Biol.* 102:11-23.
- Geysen, H. M., J. A. Tainer, S. J. Rodda, T. J. Mason, H. Alexander, E. D. Getzoff, and R. A. Lerner. 1987. Chemistry of antibody binding to a protein. *Science (Wash. DC)*. 235:1184-1190.
- Green, N., H. Alexander, A. Olson, S. Alexander, T. M. Shinnick, J. G. Sutcliffe, and R. A. Lerner. 1982. Immunogenic structure of the influenza virus hemagglutinin. *Cell*. 28:477-487.
- Jackson, D. C., and A. Nestorowicz. 1985. Antigenic determinants of influenza virus hemagglutinin. XI. Conformational changes detected by monoclonal antibodies. *Virology*. 145:72-83.
- Kielian, M., and A. Helenius. 1985. pH-induced alterations in the fusogenic spike protein of semliki forest virus. *J. Cell Biol.* 101:2284-2291.
- Lear, J. D., and W. F. DeGrado. 1987. Membrane binding and conformational properties of peptides representing the NH_2 terminus of influenza HA-2. *J. Biol. Chem.* 262:6500-6505.
- Maeda, T., and S. Ohnishi. 1980. Activation of influenza virus by acidic media causes hemolysis and fusion of erythrocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 122:283-287.
- Min-Jou, W., M. Verhoeven, R. Devos, E. Saman, R. Fang, D. Huy-lebroeck, and W. Fiers. 1980. Complete structure of the hemagglutinin gene from the human influenza A/Victoria/3/75 (H3N2) strain as determined from cloned DNA. *Cell*. 19:683-696.
- Niman, H. L., R. A. Houghton, L. E. Walker, R. A. Reisfeld, I. A. Wilson, J. M. Hogle, and R. A. Lerner. 1983. Generation of protein-reactive antibodies by short peptides is an event of high frequency: implications

- for the structural basis for immune recognition. *Proc. Natl. Acad. Sci. USA.* 80:4949-4953.
23. Phillips, D., C. C. F. Blake, and H. C. Watson. 1981. The Enzymes of Glycolysis: Structure Activity and Evolution. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 293:1-214.
 24. Robinson, I. K., and S. C. Harrison. 1982. Structure of the expanded state of tomato bushy stunt virus. *Nature (Lond.)*. 297:563-568.
 25. Ruigrok, R. W. H., N. G. Wrigley, L. J. Calder, S. Cusack, S. A. Wharton, E. B. Brown, and J. J. Skehel. 1986. Electron microscopy of the low pH structure of influenza virus haemagglutinin. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:41-49.
 26. Skehel, J. J., P. M. Bayley, E. B. Brown, S. R. Martin, M. D. Waterfield, J. M. White, I. A. Wilson, and D. C. Wiley. 1982. Changes in the conformation of influenza virus haemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc. Natl. Acad. Sci. USA.* 79:968-972.
 27. Skehel, J. J., D. J. Stevens, R. S. Daniels, A. R. Douglas, M. Knossow, I. A. Wilson, and D. C. Wiley. 1984. A carbohydrate side chain on haemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 81:1779-1783.
 28. Stegmann, T., D. Hoekstra, G. Scherphof, and J. Wilschut. 1986. Fusion activity of influenza virus. A comparison between biological and target membrane vesicles. *J. Biol. Chem.* 261:10966-10969.
 29. Unwin, P. N. T., and P. D. Ennis. 1984. Two configurations of a channel-forming membrane protein. *Nature (Lond.)*. 307:609-613.
 30. Van Regenmortel, M. H. V. 1986. Which structural features determine protein antigenicity? *Trends Biochem. Sci.* 11:36-39.
 31. Verhoeven, M., R. Fang, W. Min-Jou, R. Devos, D. Huylebroeck, E. Saman, and W. Fiers. 1980. Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75. *Nature (Lond.)*. 286:771-776.
 32. Webster, R. G., L. E. Brown, and D. C. Jackson. 1983. Changes in the antigenicity of the haemagglutinin molecule of H3 influenza virus at acidic pH. *Virology.* 126:587-599.
 33. White, J., A. Helenius, and M.-J. Gething. 1982. Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. *Nature (Lond.)*. 300:658-659.
 34. White, J., M. Kielian, and A. Helenius. 1983. Membrane fusion proteins of enveloped animal viruses. *Q. Rev. Biophys.* 16:151-195.
 35. Wiley, D. C., I. A. Wilson, and J. J. Skehel. 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature (Lond.)*. 289:373-378.
 36. Wilson, I. A., K. F. Bergmann, and E. A. Stura. 1986. Structural analysis of antipeptide antibodies against influenza virus haemagglutinin. In *Vaccines '86, New Approaches to Immunization: Developing Vaccines Against Parasitic, Bacterial and Viral Disease.* R. A. Lerner, R. Chanock, and F. Brown, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 33-37.
 37. Wilson, I. A., H. L. Niman, R. A. Houghten, A. R. Cherenon, M. L. Connolly, and R. A. Lerner. 1984. The structure of an antigenic determinant in a protein. *Cell.* 37:767-778.
 38. Wilson, I. A., J. J. Skehel, and D. C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature (Lond.)*. 289:366-373.
 39. Yewdell, J., W. Gerhard, and T. Bachi. 1983. Monoclonal anti-haemagglutinin antibodies detect irreversible antigenic alterations that coincide with the acid activation of influenza virus A/PR/834-mediated hemolysis. *J. Virol.* 48:239-248.
 40. Zhao, J.-M., and E. London. 1986. Similarity of the conformation of diphtheria toxin at high temperature to that in the membrane-penetrating low-pH state. *Proc. Natl. Acad. Sci. USA.* 83:2002-2006.