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Fusion Mutants of the Influenza Virus Hemagglutinin Glycoprotein

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Summary

The influenza virus hemagglutinin (HA) mediates viral entry into cells by a low pH induced membrane-fusion event in endosomal vesicles. Mutant viruses with altered pH dependence for both hemolysis and the HA conformational change required for fusion were selected for their ability to grow in cells treated with amantadine hydrochloride, which raises the endosomal pH. The amino acid sequence and three-dimensional location of 19 substitutions on the HA are reported. The mutations fall into two groups, one that results in the destabilization of the pH 7.0 location of the hydrophobic N-terminal HA_2 peptide, and a second that results in the alteration of intersubunit contacts, suggesting a large distortion or disruption of these contacts in the "fusion-active" conformation.

Introduction

The acidic conditions in endocytic vesicles have been found to facilitate receptor–ligand complex dissociation (e.g., Brown et al., 1983), toxin-membrane interactions, which lead to toxin subunit transmembrane movement (e.g., Donovan et al., 1981), and fusion of virus and endosomal membranes during the initiation of infection by viruses such as Semliki Forest virus and influenza (e.g., White et al., 1981). In the case of influenza viruses the pH optima of membrane fusion and hemolysis mediated by a number of antigenically distinct viruses are between pH 5.0 and 6.0 and the virus component involved in fusion and activated by acidification appears to be the hemagglutinin glycoprotein of the virus membrane (Maeda and Ohnishi, 1980; Huang et al., 1981; White et al., 1981).

It has been observed that at the pH optimum for fusion, hemagglutinin structure is modified and the molecules display properties that suggest that in the process of fusion they may interact directly with the endosomal membranes. Specifically, on incubation at about pH 5.0, soluble hemagglutinins released from virus particles by bromelain digestion (Brand and Skehel, 1972) acquire the ability to bind to lipid vesicles, to bind detergents, or to aggregate in lipid- and detergent-free solutions. The molecules also become susceptible to proteolysis, but CD analyses indicate that the structural modifications implied by these observations do not involve denaturation; they are more consistent with the relative movement of molecular domains that retain their individual secondary structure (Skehel et al., 1982).

To investigate further these changes in structure and to obtain information on the basis of their pH dependence we have made a comparative study of hemagglutinins from viruses with different pH optima for hemolysis. These viruses were specifically selected for their ability to grow in the presence of amantadine hydrochloride, which is known to increase the pH of intracellular vesicles (Gonzalez-Noriega et al., 1980) and as a consequence their hemagglutinins undergo the characteristic pH-dependent conformational change at higher pH than does the hemagglutinin of wild-type virus, and all of them lyse erythrocytes at higher pH than wild-type virus. We report here the amino acid sequences of the hemagglutinins of these amantadine-resistant mutants and an analysis of the significance of the amino acid substitutions detected in them with reference to the three-dimensional structure of the X-31 hemagglutinin (Wilson et al., 1981).

Results

The pH Dependence of Hemolysis by Amantadine-Hydrochloride-Resistant Mutants

The hemolytic activities of mutants of X-31 and Weybridge influenza viruses were determined between pH 5.0 and pH 7.0 and the pH values at which hemolysis was half-maximum are listed in Table 1. An example of the pH dependence of hemolysis by a number of mutants is given in Figure 1. Fifty percent hemolysis occurred at pH 5.75 for wild-type X-31 virus, at pH 5.7 for Weybridge, and between pH 5.8 and pH 6.4 for the amantadine-hydrochloride-resistant mutants.

Changes in the conformations of the hemagglutinins of the resistant mutants also occurred at pH values different from those at which the wild-type hemagglutinins were modified. This was assessed by determining the pH at which the hemagglutinins became susceptible to proteolysis and involved analyses by polyacrylamide gel electrophoresis of the products resulting from tryptic digestion of hemagglutinins at pH 7.0 and 20°C after incubation for 10 min at different pHs between 5.0 and 7.0 (Skehel et al., 1982). An example of the results obtained for the mutants of X-31 is shown in Figure 2. In all cases examined, which included viruses 1a, 5a, 4X, 6X, and aa2 listed in Table 1, the differences in pH at which wild-type and mutant hemagglutinins became susceptible to trypsin correlated with the differences in the pH at which the viruses lysed erythrocytes. In all cases, the actual pH at which hemolysis occurred was higher than the pH at which the HA was found to be maximally sensitive to proteolysis. This differ-

Table 1. Amino Acid Substitutions in Mutants That Lyse Erythrocytes at Increased pH

Mutants	Residue		Amino Acid Substitution	ΔpH
X-31				
1		HA_2 6	I→M	0.3
2	HA_1 207 +	HA_2 6	E→K; I→M	0.35
1a		HA_2 112	D→G	0.4
2a		HA_2 54	R→K	0.25
5a		HA_2 114	E→K	0.6
6a		HA_2 57+163	E→K; R→I	0.4
8a	HA_1 102	HA_2 6	V→M; I→M	0.5
4x		HA_2 81	E→G	0.3
6x		HA_2 9	F→L	0.6
12x		HA_2 112	D→N	0.35
aa1		HA_2 47	Q→R	0.35
aa2	HA_1 17		H→Q	0.25
aa4		HA_2 105	Q→K	0.3
aa9		HA_2 112	D→E	0.25
Weybridge				
4		HA_2 69+78	E→G; Q→D	0.15
12	HA_1 91		R→Q	0.1
18		HA_2 81	I→S	0.1
2Y	HA_1 20+31		V→A; E→V	0.2
4Y		HA_2 112	D→G	0.4
A1	HA_1 113	HA_2 82	R→I; N→D	0.3
A2	HA_1 101	HA_2 43	K→E; S→L	0.1
A4	HA_1 32		R→G	0.2
A5	HA_1 91		R→L	0.3
A6	HA_1 300		R→S	0.3
A7	HA_1 324	HA_2 139	P→S; E→D	0.25
A8		HA_2 54	R→K	0.3
A9	HA_1 32	HA_2 82	R→G; N→Y	0.25
B1		HA_2 54+160	R→K; S→N	0.2
B5		HA_2 51+91	K→R; R→Q	0.45
B7		HA_2 114	E→K	0.5
B8		HA_2 67+102	D→N; M→R	0.1
B9	HA_1 221	HA_2 114	P→S; E→K	0.45
C5		HA_2 3	F→L	0.4
C6		HA_2 54	R→G	0.1
C7		HA_2 47	Q→L	0.45
C10		HA_2 54	R→S	0.1
C13		HA_2 51	K→N	0.2
C15	HA_1 205	HA_2 47	G→E; Q→K	0.3

The residue numbers for the Weybridge mutants are related to the X-31 hemagglutinin sequence.
ΔpH values are simple differences between the pHs at which hemolysis by the mutants and wild-type virus are 50% of maximum.

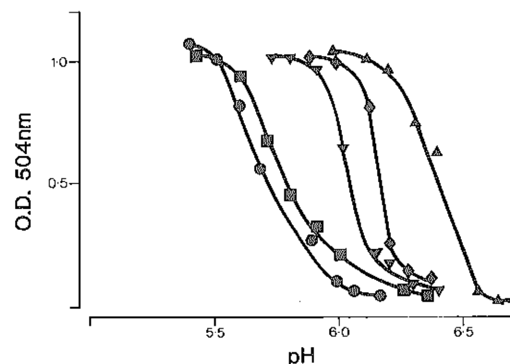


Figure 1. The pH Dependence of Hemolysis by the Wild-Type Viruses and Representative Amantadine-Hydrochloride-Resistant Mutants

Hemolysis was assayed as described in Experimental Procedures. From these data the pH of 50% hemolysis by X-31 ■ was pH 5.75; of Weybridge virus ● was pH 5.7; of the X-31 mutants 4X ▼ and 6X ▲ was pH 6.05 and pH 6.35, and of the Weybridge mutant C7 ◆ was pH 6.15. Similar experiments provided the ΔpH data shown in Table 1 for the other mutants.

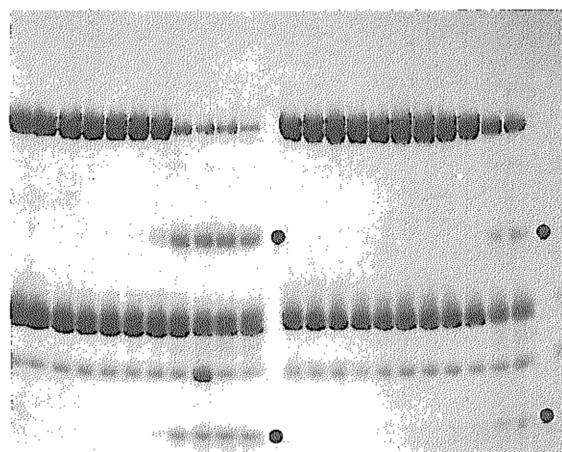


Figure 2. pH-Dependent Changes in the Conformation of X-31 and X-31 Mutant 1a Hemagglutinins

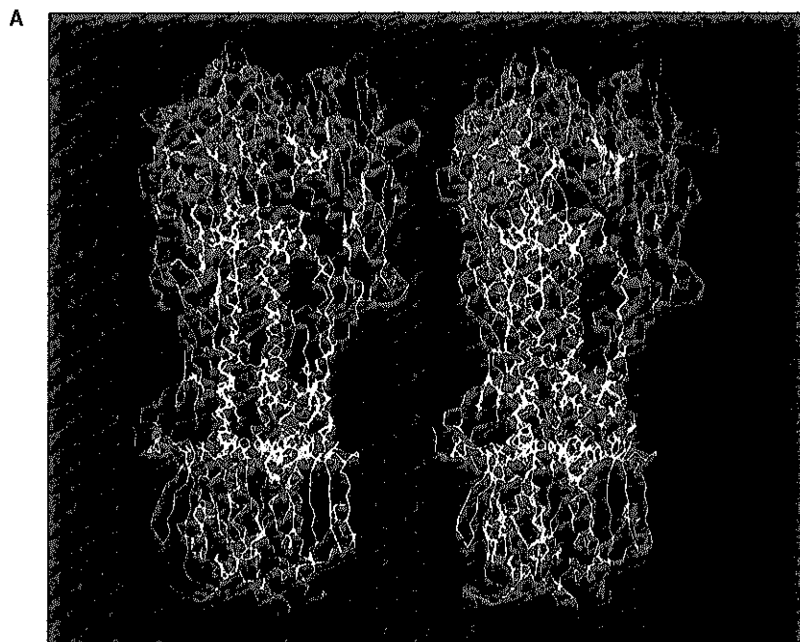
The sensitivity of bromelain-released hemagglutinins to digestion with trypsin was used as an assay of conformation changes in the molecules. After 10 min incubation at the appropriate pH hemagglutinin solutions were adjusted to pH 7 and made 1% w/w with respect to trypsin. After 10 min at 20°C an equal amount of soybean trypsin inhibitor was added to stop digestion, and products were boiled in 1% SDS, .05 M β-mercaptoethanol, 8M urea and analyzed by polyacrylamide gel electrophoresis in SDS-containing buffers (Daniels et al., 1983).
The characteristic products of HA_1 digestion of approximate molecular weights 25,000 and 15,000 are indicated by ●. The pH of incubation was decreased from pH 7.0 to pH 5.0 in 0.2 pH unit steps shown from left to right. The 11 lanes on the left contained the products of digestion of X-31 mutant 1a BHA and the 11 on the right, the products of digestion of X-31 BHA. The lane on the right end of the gel contained trypsin inhibitor only.

ence is assumed to be a reflection of the relative sensitivities of the assay procedures.

The Amino Acid Sequences of the Mutant Hemagglutinins

Amino acid sequences were deduced from the nucleotide sequences of the virus RNA genes for hemagglutinin, which were determined by the dideoxynucleotide chain-terminating procedure of Sanger et al. (1977) using 5′ ^{32}P-labeled synthetic oligodeoxynucleotides to prime reverse transcription of virus RNA. In all cases the complete sequences of the coding regions for the amino-terminal signal peptide and the HA_1 and the HA_2 polypeptides were determined and the amino acid substitutions detected are given in Table 1. For the X-31 mutants the majority of amino acid changes (15 out of 17) were in the HA_2 polypeptide and involved charged residues in 11 out of the 14 hemagglutinins examined. The molecular locations of the changes and their possible structural consequences are considered below. Of the Weybridge mutants, a greater proportion contained amino acid substitutions in the HA_1 polypeptide (11 out of 24) and again the majority of changes (28 out of 35) involved charged amino acids. More of these mutants also contained two amino acid sub-

A



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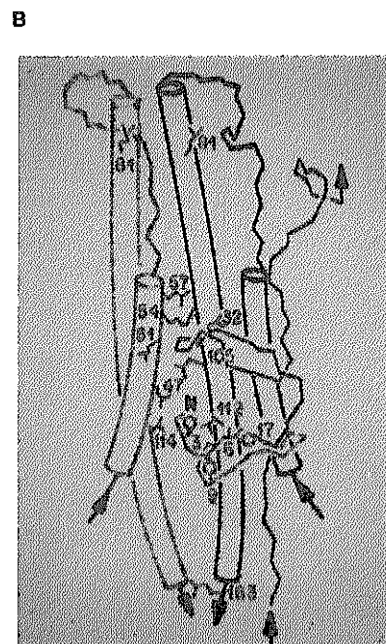

Figure 3. The Locations in the Hemagglutinin of Amino Acid Substitutions Detected in the Mutant Viruses

(a) A stereo α-carbon trace of the HA trimer (HA_1, blue; HA_2, red). The locations of single-substitution mutations from X-31 and Weybridge mutants are shown in yellow. The double-substitution mutants are purple (see text for details). Figures 3a, 4, and 5 were produced with the molecular graphics program, HYDRA, written by Rod Hubbard.

(b) An α-carbon drawing of a detail from (a), showing the helical section of two HA_2 subunits (green and black) and a segment of HA_1 (blue). The N-terminal peptide of the black HA_2 subunit is shown in red, tucked into the interface between the two HA_2 subunits. The amino acids at the position of single-substitution mutants are shown (red).

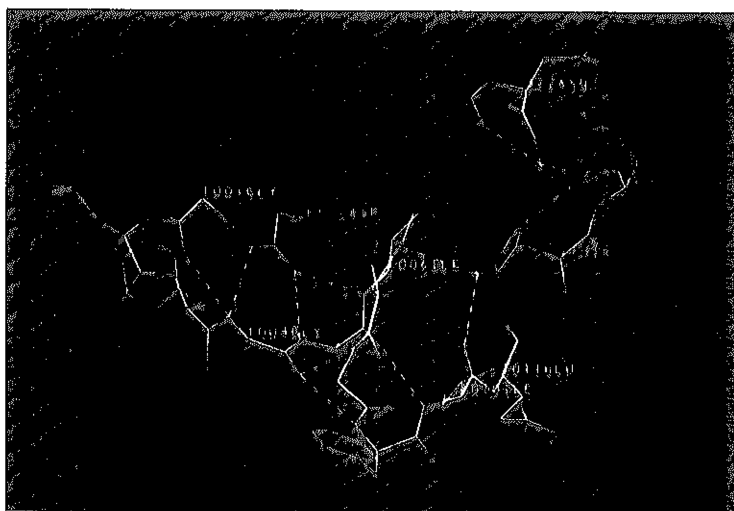

Figure 4. The Structure of the N-Terminal Peptide of HA_2 Showing Some of the Interactions That Are Altered in the Mutants

The N-terminal peptide of HA_2 (white) is shown wrapped across Asp 112-HA_2 and His 17 of HA_1. Hydrogen bonds are green; oxygen, red; nitrogen, blue; carbon, white or yellow; x = water molecule. Amino acid numbers in HA_2 subunits begin at 1001 for the N-terminal glycine. Mutations in 1112, 17, 1003, 1006, 1009 raise the pH at which hemolysis and the HA conformational change occur (see Table 2 and text for details).

stitutions (11 out of 24, compared with 3 out of 14 X-31 mutants).

The Molecular Locations of Amino Acid Substitutions in the Mutant Viruses

The 11 amino acid substitutions found in mutants of X-31 virus, listed in Table 1, are shown in yellow on the three-dimensional structure of the X-31 HA trimer in Figure 3a. Also shown in yellow are substitutions in the Weybridge strain mutants with single amino acid changes and, in purple, Weybridge strain mutants with double amino acid substitutions. Some of the substitutions (3, 51, 69, 112) in the Weybridge mutants involve amino acids conserved in all hemagglutinin sequences reported, while others (47, 54, 112, and 114) are in the same location as substitutions observed in X-31 mutants, and both are therefore readily placed on the X-31 structure. The molecular locations of the remainder (Figure 3a) (91, 300, 32, as well as a number of double substitutions) are based on previously reported considerations that the three-dimensional structures of all influenza hemagglutinins will share certain features (Wilson et al., 1981; Wiley et al., 1981). The positions of single

Table 2. The Location and Probable Structural Consequences of the Amino Acid Substitutions Found in the Mutant Viruses

Position	Substitution From	to	+ΔpH	Location and Probable Effect of Mutation
I.				
112-HA_2	Asp	Gly Asn Glu Glu	.41 .35 .25 .25	Asp 112, on the long helix of HA_2, is completely buried behind residues 1–6 of the N-terminal peptide of HA_2. The two Δ oxygens form four hydrogen bonds to the Amide nitrogens of residues 4, 5, and 6, and the amino nitrogen of residue 1, presumably neutralizing the terminal amino group (it is also possible that the hydrogen bonds are due to the amide nitrogens of residues 3, 4, 5, and 6, indistinguishable at the current level of refinement) (Fig. 4). The observed substitutions would distort or destroy these interactions, destabilizing the position of the amino-terminal peptide of HA_2 in the pH 7.0 conformation.
114-HA_2	Glu	Lys	.6	Glu 114, on the long helix of HA_2, forms a hydrogen-bonded salt link to Lys 117 of the same chain, which is in van-der-Waal contact with the terminal amino group from a second subunit of HA_2. The substitution of Lys for Glu (114) would replace a neutralized salt bridge GLU-114:LYS-117, with two positively charged Lysines (114, 117), one of which (117) is in contact with the amino terminal of HA_2 of another subunit. This would electrostatically destabilize the position of the amino terminus in the pH 7.0 conformation.
17-HA_1	His	Gln Arg	.25 .7	His 17 of HA_1 is almost completely buried by amino acids 6–14 from the amino-terminal peptide of HA_2. NE2 of His 17 forms hydrogen bonds via a water molecule to the carbonyl oxygens of both residues 6 and 10 of HA_2 (NE1 forms a hydrogen bond to the carbonyl of His 18 (HA_1), which is linked to the C-terminal region of HA_1 through residues 321 and 323) (Fig. 4). The substitutions of both Gln and Arg for His would disrupt some of these polar contacts and because His 17 is nearly buried, distort this region due to the large size (and/or charge) of the substituted residue.
6-HA_2	Ile	Met	.3	Ile 6 of the N-terminal peptide of HA_2 is buried in contact with His 17 (HA_1, see above) and residues 22, 112, and 115 of HA_2. The relatively conservative substitution of Met for Ile, which increases the side chain length by one atom, would disturb the tight packing with His 17-HA_2 and may distort the position of Tyr 22-HA_2. Thus the substitution would destabilize the current location of the amino-terminal peptide both directly and, possibly, indirectly by effects on the positions of His 17 HA_1 and Asp 112 HA_2.
9	Phe	Leu	.6	Phe 9 of the amino-terminal peptide is packed near Ile 10 and Ala 5 of the N-terminal peptide of HA_2, Phe 119 of the HA_2 long helix, and with another HA_2 subunit's Arg 124. The substitution to Leucine, which introduces a tetrahedral carbon in place of a planar carbon at the γ position has the potential to destabilize the position of the amino-terminal peptide directly, via interactions with residues 5 and 10, and may also distort the salt link of Glu 132 (of one subunit of HA_2), and Arg 124 (of another subunit of HA_2).
II.				
81	Glu	Gly	.3	Glu 81 and Glu 74 at the top of the long helix of HA_2 (45 Å from the N terminal of HA_2) form a hydrogen-bonded salt link with Arg 76 HA_2 at the top of the long helix of a second subunit of the trimer (Fig. 5b). This pattern is repeated 3-fold by the symmetry of the trimer. The substitution of Gly for Glu would destabilize this interaction, which appears to be one of the important salt links stabilizing the trimeric arrangement of α-helices.
54	Arg	Lys	.25	Arg 54 of HA_2 is in the interface between one HA_2 subunit and the HA_1 and HA_2 chains of another subunit of the trimer. It forms a hydrogen-bonded salt link to the totally conserved Glu 97 of HA_2 of another subunit and a hydrogen bond to the carbonyl oxygen of Thr 28 of HA_1 of another subunit in the trimer. It also forms a hydrogen bond to Glu 57 of its own HA_2 chain (Fig. 5a). Because Lys has only one side-chain nitrogen, compared to three in Arg, the substitution by Lys would necessarily result in the disruption of either the salt link to HA_2 or the hydrogen bond to HA_1 on the second subunit, destabilizing the subunit contact.
163	Arg	Ile	.4	Arg 163 is near the bottom (viral membrane end) of HA_2, 35 Å along the trimer axis from the HA_2 N terminus. It forms a hydrogen-bonded salt link to Glu 131 of a second HA_2 subunit, a residue that is completely buried in the HA_2–HA_2 interface. The observed substitution would destroy this salt link between HA_2 subunits.
57	Glu	Lys	.4	Glu 57 of HA_2 is salt-linked with Arg 54 (see above), on the same subunit. The substitution of Lys for Glu, a net change in electrostatic charge of +2, would destroy this contact and may destabilize the intersubunit salt link made by Arg 54 (see above) (Fig. 5a). (Note: both substitutions, Glu 57 and Arg 163, (above entry) are found in the same mutant, so that one may be neutral.)
47	Gln	Arg	.35	Gln 47 is 3.5 Å from Glu 114 (see above and 6 Å from the terminal amino group of a second subunit of HA_2. The substitution of Arg for Gln would electrostatically destabilize both the location of the amino terminus of HA_2 and the subunit interface in the pH 7.0 conformation by one or two mechanisms: (1) direct repulsive charge interaction between Arg 47 and amino terminus of a second HA_2 subunit; (2) by neutralizing Glu 114, which would leave Lys 117 unneutralized and in van-der-Waal contact with the amino terminus of HA_2 (see 114 table entry).
105	Gln	Arg	.3	Gln 105 of HA_2 forms a hydrogen bond with the amide nitrogen of residue 29 in HA_1 and it is in van-der-Waal contact with His 106 of a second HA_2 subunit. It is also hydrogen-bonded via a water molecule to Asp 109, which in turn hydrogen bonds to residue 2 of the N-terminal peptide of HA_2. The substitution of Arg for Glu will place a positive charge in contact with His 106, destabilizing the HA_2-HA_2 subunit interface and may form a hydrogen-bonded salt link to Asp 109 of HA_2, distorting its interaction with residue 2 of the N-terminal peptide of HA_2.

amino acid substitutions (yellow in Figure 3a) are also shown in a schematic diagram depicting the interface region between two HA_2 chains (green and black) and a segment of one HA_1 chain (blue) (Figure 3b). For emphasis, the N-terminal region of the black HA_2 subunit is shown in red. What is striking in both of these figures is that mutations affecting fusion occur not only in the vicinity of the amino terminal of HA_2 (3, 6, 9, 47, 112, 114, 17-HA_1), but also in an interface above the fusion peptide, where a peptide from HA_1 fits between two subunits of HA_2 (51, 54, 57, 105,

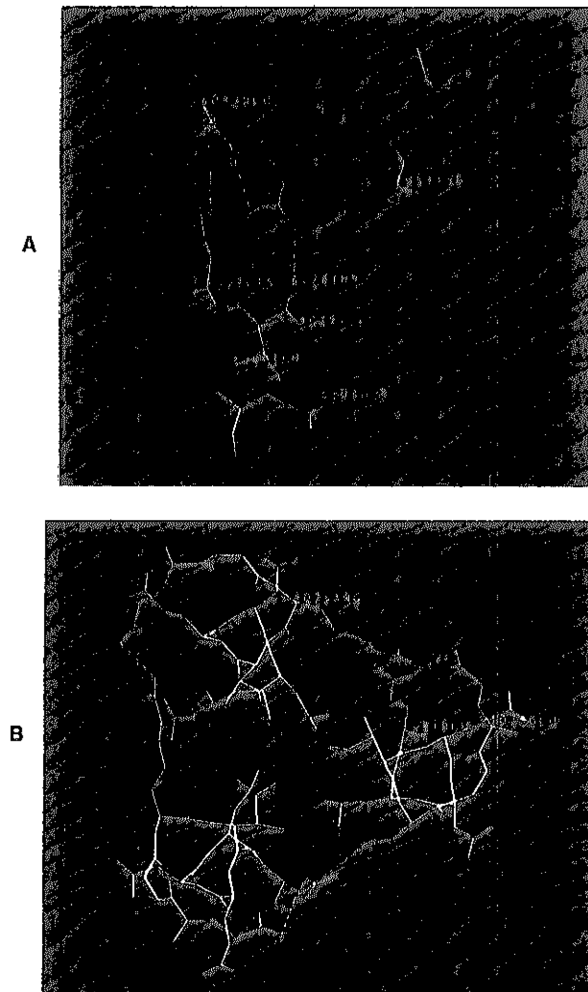

Figure 5. Portions of the Interface between Hemagglutinin Subunits That Are Altered in the Mutants

(a) A section of the interface between two HA_2 subunits (numbered from 1001 and 5001) and on HA_1 (4000's). Atom colors are as in Figure 4. Substitutions at HA_2 residues 1054, 1057, and 5105 affect the inter-subunit salt link Arg 1054–Glu 5097 and hydrogen bond 4028-HA_1:1054-HA_2. (See Table 2 and text for details).

(b) Three α helices (yellow) from 3 HA_2 subunits are linked by hydrogen-bonded salt bridges involving Glu-81, Glu-74, and Arg-76. This arrangement is shown repeated 3-fold by the trimeric symmetry of the protein. A substitution at 81 would destabilize these links, which hold the helices together 45 Å away from the N-terminal peptide of HA_2 (see Figure 3b).

32-HA_1), and at remote regions, primarily in the HA_2-HA_2 subunit interface (81, 163, and a number of double substitutions).

The noncovalent interactions made by the amino acids substituted in the mutants and the probable structural consequences of the observed substitutions are tabulated in Table 2. These structural details suggest two categories of effects.

First, substitutions in residues in the amino-terminal peptide of HA_2 (3, 6, 9) or in other residues that interact to stabilize the pH 7.0 location of the amino-terminal peptide (112, 17-HA_1) all appear to destabilize the pH 7.0 conformation that holds the N-terminal peptide in a hydrophobic crevice. For example, Figure 4 shows how the HA_2 amino terminal (1–11) wraps across Asp 112-HA_2 and His 17-HA_1, forming a network of hydrogen bonds to those residues. The observed substitutions (see Table 2 for details) would distort or destroy these interactions, destabilizing this arrangement of the N-terminal peptide and, therefore, presumably reducing the energy barrier to a conformational change, which results in the expulsion of the peptide. Furthermore, (Figure 3b), substitutions at 47 and 114 would also appear to destabilize the location of the amino terminal; in this case, by juxtaposing unneutralized positive charges next to the amino terminus (see Table 2).

Second, substitutions at the interface between HA_1 and HA_2 from one subunit with HA_2 from a second subunit (51, 54, 57, 105, 32-HA_1), and at other regions of the HA_2-HA_2 interface (81, 163), would all appear to reduce the stability of the trimeric interactions that stabilize the pH 7.0 conformation of the trimer interfaces. For example, Figure 5a shows the salt link and hydrogen bonds that Arg 54-HA_2 makes with both Glu 97-HA_2 and Thr 28-HA_1, on both of the chains of a second subunit in the trimer. Substitutions at 54, 57, and 105 (Figure 5a) would destabilize this and other (see Table 2) interactions that determine the conformation of this interface at neutrality.

Figure 5b shows the details of a hydrogen-bonded salt link involving Glu-81 and Arg-76, between the three long α-helices (yellow) of the three HA_2 chains. This interaction is located 45 Å up the trimer axis away from the N terminal of HA_2 (see Figure 3b). The observed substitution at 81-HA_2 would destabilize this salt link, which appears to be important in holding the HA_2 subunits together in the pH 7.0 conformation of the trimer. The observed substitution at Arg 163, 77 Å away from Glu-81 and near the virus membrane end (bottom) of the trimer (Figure 3b), would also destroy an HA_2-HA_2 inter-subunit salt link.

Most of the Weybridge mutants (for which no X-ray structure of the parent HA exists) fit into category 1 (3, 112, 114) or category 2 (47, 54, 81, 32-HA_1). Residues 91-HA_1 and 300-HA_1 appear to be in the HA_1-HA_2 interface between the globular portion of HA_1 and the top of the HA_2 stem, implicating that interface in the structural transition as well.

A number of double substitutions in mutants (purple in Figure 3a) cannot be interpreted with confidence at this stage because one of the substitutions may be neutral. It is interesting to note that while most of these cluster in regions defined in the categories above, a few (101, 102, 205, 221) fall in the interfaces between globular HA_1 domains at the top of the molecule.

Discussion

Evidence that the hemagglutinins of influenza viruses are involved in membrane fusion comes from a number of observations. First, viruses that contain hemagglutinins made up of unprocessed biosynthetic precursors of the HA_1 and HA_2 polypeptides are not infectious (Klenk et al., 1975; Lazarowitz and Choppin, 1975) and are unable to mediate fusion (Huang et al., 1981; White et al., 1981).

They are rendered potentially fusogenic by proteolysis in vitro, which results in the production of the HA_1 and HA_2 polypeptide components, a process reminiscent of the generation of an active fusion glycoprotein in Sendai virus (Scheid and Choppin, 1974; Hsu et al., 1981, 1982). Second, at the pH optimum for influenza virus-mediated fusion, hemagglutinins undergo an irreversible conformational change, as a result of which the molecule acquires the capacity to bind to lipid membranes. The hydrophobic region exposed at low pH is the amino-terminal portion of HA_2, the site of precursor processing mentioned above (Daniels et al., 1983), which is analogous in amino acid sequence to the amino-terminal region of the Sendai virus F glycoprotein (Gething et al., 1978; Scheid et al., 1978) proposed to interact with cell membranes during fusion (Richardson and Choppin, 1983). Third, hemagglutinins expressed on the surface of cells infected with vectors containing DNA copies of the RNA gene for hemagglutinin are able to promote cell fusion providing that they are processed proteolytically into HA_1 and HA_2 polypeptides and that the assay for fusion is conducted at approximately pH 5 (White et al., 1982).

The results presented here, indicating that the differences in pH at which the hemagglutinins of the amantadine-hydrochloride-resistant viruses undergo a conformational change, correlate with differences in the pH at which the mutants lyse erythrocytes and provide further support for the proposed role of hemagglutinin as a fusion protein. They also suggest that resistance of influenza viruses to amantadine hydrochloride at the high concentration used is a property of their hemagglutinins, and, for certain viruses, this has been observed before (Scholtissek and Faulkner, 1979; Hay and Zambon, 1983). However, it is clear that this is not the only molecular marker of resistance to this drug (Hay et al., 1979; Lubeck et al., 1978) and studies of the mechanisms of its inhibition of different influenza viruses are in progress and will be presented elsewhere.

The Conformational Change

We have previously reported that changes in the conformation of the X-31 hemagglutinin at the pH optimum for membrane fusion render the molecule susceptible to trypsin digestion at HA_1 lys-27 and that, following digestion, the distal globular domain of the HA_1 polypeptides was released as monomers. (Skehel et al., 1982; Daniels et al., 1983). Similar results using a reducing agent rather than trypsin have been reported by Graves et al. (1983). Our conclusion from these results was that the low pH conformational change, resulting in the exposure of the hydrophobic HA_2 N terminal, was accompanied by extensive breakage of intra- and inter-subunit contacts.

The location and nature of the substitutions reported here extend this picture of the low pH, fusion-active conformation of the HA. All of the mutations appear to lower the energy barrier necessary for the conformational transition to a fusion-active state. One category of substitutions destabilizes the unexposed location of the hydrophobic N-terminal peptide of HA_2, either by placing positive charges near the amino terminus of HA_2 or by altering the network of hydrogen bonds and nonpolar contacts that stabilize that part of the structure. The second category of substitutions distorts or destroys salt bridges and other contacts in the interfaces between the chains and subunits of the HA trimer. In a number of cases, the latter substitutions result in the loss per monomer of one of the salt bridges that appear to stabilize the initial pH 7.0 quarternary structure. These altered contacts are found both in a region where HA_1 and HA_2 of one subunit interact with HA_2 of a second subunit, and in regions remote from the N terminal, extending 77 Å along the length of the HA_2-HA_2 subunit interface.

Thus it appears that altering interactions between subunits of the trimer can raise the pH optimum for fusion, which indicates either that: changes in the protein conformation that uncover the hydrophobic N-terminal peptide require changes in the subunit interactions throughout the HA_2-HA_2 and part of the HA_2-HA_1 interfaces, or that partial or total disruption of the trimeric quartenary structure accompanies the low pH conformational change.

Consequences for Membrane Fusion

Examination of the pH 7.0 trimeric structure indicates that it would be possible to extract the hydrophobic N-terminal peptide of HA_2 without complete disruption of the HA_2-HA_2 subunit interfaces and that may be what occurs. However, the mutants observed here are also consistent with a disruption of the trimeric quartenary structure that may be required either to allow involvement of other regions of the molecule in membrane fusion or to bring about a juxtaposition of the two membranes involved in the fusion reaction by rearrangement of the HA, which is presumed linked by hydrophobic peptides to both membranes (Skehel et al., 1982).

The low pH conformational change of the HA appears to be another example of a proton-sensitive conformational switch like that involving the Bohr protons in the cooperative binding of oxygen by hemoglobin and the Caspar carboxyls in the assembly of Tobacco Mosaic virus (Perutz et al., 1984; Butler et al., 1972). In the case of the amantadine-hydrochloride-resistant mutants of the HA, our interpretation of the structural consequences of the observed amino acid substitutions is that alterations that lower the energy required to uncover the N-terminal peptide of HA_2 or to rearrange the subunit interfaces lower the concentration of protons required to trigger the conformational change. Which amino acids or salt bridges must bind protons to cause the conformational transition is unknown, although the current results suggest that a number of them may be involved.

In the absence of direct structural information on the low pH induced conformation, it is difficult to deduce how the structural changes discussed would facilitate fusion. Does rearrangement of the oligomeric structure of the HA molecule provide a means for bringing together two lipid bilayers and overcoming the forces between them? Does it modify the stability or local composition of the bilayers to promote fusion?

Biological Consequences of pH Mutations

The pH at which the hemagglutinins of different viruses such as the mutants described here participate in membrane fusion may have a number of biological consequences. For example, since in vitro incubation of viruses at the pH at which the conformational change in hemagglutinin occurs leads to inactivation of infectivity, it is possible that viruses with hemagglutinins that undergo the change at higher pH will be prone to inactivation under certain extracellular physiological conditions that occur during infection. It is also possible that hemagglutinin stability may be influenced by factors other than pH, such as temperature, and that changes in primary structure similar to those reported here may influence the temperature sensitivity of viruses. Preliminary results supporting this suggestion have been obtained for temperature-sensitive mutants of fowl plague virus (H. D. Klenk, personal communication).

Nevertheless, observations that different influenza viruses hemolyze at different pH have been reported (e.g., Maeda and Ohnishi, 1980; Huang et al., 1981; White et al., 1981) and we have observed that antigenically closely related isolates of human or avian origin differ in the pH at which they hemolyze by up to 0.7 pH units. We have also determined (data not shown) that the amino acid sequences of such viruses contain only small numbers of amino acid sequence differences in positions similar to those reported here for the amantadine-hydrochloride-resistant mutants. We have three examples of viruses of the H_3 subtype: two viruses recovered from the original isolate of A/duck/Ukraine/63 lyse erythrocytes at pH 5.5 or pH 5.9 and differ in their HA_2 components at three positions, residues 2 (ileu for leu), 50 (arg for gly), and 154 (asn for lys). Because of their molecular locations, the changes at 2 and 50 may be responsible for the changed pH of hemolysis. Second, a cold adapted variant of A/Alaska/1/77, CR29 (Maassab et al., 1982) was found to differ in pH of hemolysis from wild-type virus by 0.4 pH units and also to contain a single amino acid substitution in HA_2 at position 79 (asp to asn). Third, we have also determined the amino acid sequences of variants of X-31 virus adapted to growth in MDCK cells, which demonstrate differences in pH of hemolysis of 0.7 pH units (Rott et al., 1984). One such mutant contained a single amino acid substitution of His to Arg at position 17 of HA_1 (see Table 2). The complete amino acid sequences of the hemagglutinins of these viruses will be presented elsewhere; they are recorded here to show that small numbers of amino acid substitutions in hemagglutinins other than those of amantadine-hydrochloride-resistant mutants can result in changes in pH of hemolysis. The relative stability of these viruses in normal circumstances of infection is not known.

In this connection it is reasonable to note that formation of complex functional molecules such as the HA trimer is subject to a number of constraints to accommodate not only trimer stability but also processes involved in its assembly, in correct precursor cleavage, and in the structural transition during membrane fusion. With regard to this last selection pressure, a number of substitutions in the hemagglutinins of the mutants reported here occur in the hydrophobic amino-terminal decapeptide of HA_2, a region of the molecules previously found to be conserved other than at position 2, in all influenza A viruses (Skehel and Waterfield, 1975), which may be directly involved in fusion. In addition to their location, they are notable since, in contrast to amino acid substitutions, which affect the pH of hemolysis by other mutants, they do not involve charged residues (leu for phe at position 3, met for ileu at 6, and leu for phe at 9). The existence of such hemagglutinins in fusion-active virus suggests that absolute conservation of the sequence of this region is not required for the functions of the hemagglutinin and makes it unlikely that hemagglutinin–cell interactions mediated at low pH involve sequence-specific recognition of this conserved structure.

Experimental Procedures

Cells and Viruses

Primary chick embryo fibroblasts were prepared as described by Porterfield (1960). The influenza viruses X-31 (H_3N_2) (Kilbourne, 1969) and A/chicken/Germany/34 (H7N1) "Weybridge" strain, were from the stocks of the World Influenza Centre at Mill Hill. They were grown in the allantoic cavities of 10 day old embryonated hens' eggs and purified as described by Hay (1974). Mutants resistant to amantadine hydrochloride were isolated as follows. For X-31 virus, allantoic fluid from eggs infected with plaque-purified viruses was used to infect the chorioallantoic membranes of de-embryonated eggs. Two hours after infection amantadine hydrochloride was added to a concentration of 100 μg/ml and after 2 to 3 days the medium was diluted to about 1 HAU/ml and used to infect the chorioallantoic membranes of fresh de-embryonated eggs incubated in medium containing amantadine hydrochloride (100 μg/ml). Mutants harvested after 2 to 3 days were plaque-purified on chick embryo fibroblasts in the presence of amantadine hydrochloride (100 μg/ml) and trypsin (4 μg/ml) and were used to produce viruses for the studies described. For the Weybridge virus, chick embryo fibroblasts were used instead of the chorioallantoic membranes and trypsin was not required for plaquing these viruses. All of the viruses isolated by this procedure lysed erythrocytes at higher pH than the wild-type viruses and contained amino acid substitutions in their HA.

Nucleotide Sequence Analyses

Sequences were determined using the dideoxynucleotide chain-terminating procedure of Sanger et al. (1977) as described previously (Daniels et al., 1983). The 5' ^{32}P-labeled primers of reverse transcription were synthesized by the procedure of Patel et al. (1982). The primers used for the X-31 mutants were complementary to nucleotides 5–15, 191–202, 465–474, 623–632, 777–786, 971–981, 1134–1144, 1330–1340, 1431–1441, and 1614–1628 numbered according to the cDNA sequence of Verhoeyen et al. 1980. For the Weybridge mutants primers complementary to nucleotides 5–15, 229–239, 427–437, 573–583, 832–843, 1113–1124, 1315–1324, and 1526–1537 numbered according to the sequence reported by Porter et al. (1979) were used.

Hemolysis

The pH at which the different viruses lyzed human type 0 erythrocytes was determined by incubating erythrocytes to which virus was adsorbed, at appropriate pH and at 37°C for 30 min. The amounts of hemoglobin released into the cell supernatant fluid were estimated spectrophotometrically at 504 nm.

All erythrocytes were obtained from a single individual and were used within 2 days of being drawn. Wild-type virus controls were included in each series of hemolysis tests. When up to five different mutant viruses, containing identical nucleotide and amino acid substitutions, were analyzed simultaneously, maximum differences of .05 pH units in the pH at which hemolysis occurred were recorded. The range of pH at which hemolysis by wild-type control virus occurred in different tests, however, was 0.2.

Estimates of Hemagglutinin Conformational Change

These were made by determining its suceptibility to tryptic digestion as described before (Skehel et al., 1982). Hemagglutinins released from viruses by bromelain treatment were incubated at the appropriate pH for 10 min, neutralized by adding Tris, and then digested with trypsin (1% w/w) for 10 min at 20°C. The digests were analyzed by polyacrylamide gel electrophoresis as described before (Skehel et al., 1982).

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