Labeling of the Cytoplasmic Domain of the Influenza Virus Hemagglutinin with Fluorescein Reveals Sites of Interaction with Membrane Lipid Bilayers†

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ABSTRACT: The hemagglutinin (HA) glycoprotein of influenza virus was labeled in its cytoplasmic domain with fluorescein. Reactive amino groups in the external domain were blocked by modification of the intact virus with the membrane-impermeable reagent isethionyl acetimidate. The HA was then solubilized with the detergent octyl glucoside, and the single lysine in the cytoplasmic domain was reacted with fluorescein isothiocyanate. This protocol resulted in the incorporation of 1.3 mol of fluorescein/mol of HA. Using a virus strain lacking lysine in the cytoplasmic domain of HA, it was determined that 0.47 mol of fluorescein/mol of HA was located at an additional site(s). The fluorescein groups at both sites exist in an environment of reduced polarity as shown by a shift in excitation and emission maxima and a shift in the pK_a of the fluorescein groups. The fluorescence polarization and the pK_a of the fluorescein groups were greater when the HA was incorporated into liposomes than when in detergent solution. These data indicate that the fluorescein groups interact directly with the lipid bilayer, probably in the phospholipid head-group region. The fluorescence properties of the labeled HA were not responsive to the gel to liquid-crystal phase transition in the lipid bilayer. These results indicate that the boundary between the cytoplasmic domain and the hydrophobic sequence that anchors the protein to the lipid bilayer is located in the head-group region of the bilayer.

The membranelike envelope of influenza viruses contains two surface glycoproteins [reviewed extensively, e.g., see Lamb (1983)]. The hemagglutinin (HA)[1] is the major virion surface protein. It is responsible for virus attachment to sialic acid containing receptors on host cells and for fusion of the virus envelope with the membrane of endocytic vesicles during virus penetration. The neuraminidase (NA) glycoprotein is present in lesser amounts in the virus envelope and is responsible for release of the virus from receptors. Both glycoproteins are synthesized in the endoplasmic reticulum and transported to the host plasma membrane by the same pathway followed by host plasma membrane glycoproteins. The virus envelope is acquired by budding from the host membrane. During the budding process, the internal viral components associate with the cytoplasmic surface of the membrane and interact with the viral glycoproteins to form a specialized region of the membrane from which host proteins are largely excluded. The surface glycoproteins are the major antigenic species on the virus envelope and host plasma membrane to which antiviral antibody is directed. Periodic genetic variation in these glycoproteins gives rise to new virus strains that are antigenically distinct and are responsible for recurring epidemics of influenza.

The HA glycoprotein is a trimer of identical subunits. Each subunit consists of two disulfide-linked peptide chains designated HA_1 (M_r approximately 50 000) and HA_2 (M_r approximately 25 000) derived by proteolysis of a common precursor, HA_0. The HA is anchored to the envelope phospholipid bilayer by a hydrophobic sequence near the carboxy terminus of HA_2 (or HA_0). The carboxy-terminal 10–12 amino acids form a moderately hydrophilic sequence that is presumed to reside on the internal surface of the virus envelope and the cytoplasmic surface of the host plasma membrane. This sequence will be referred to as the cytoplasmic domain. Even though direct evidence for the internal disposition of this sequence has not been presented, analogy with other viral glycoproteins with a similar structure and function makes such a topography very likely. A large fragment of the HA released from the virus envelope by the proteolytic enzyme bromelain has been crystallized and its structure determined by X-ray diffraction (Wilson et al., 1981). The bromelain cleavage site is nine amino acids from the membrane-anchoring sequence. Thus, relatively little structural information beyond a knowledge of the amino acid sequence is available for the membrane anchor sequence or the cytoplasmic domain. Structural information about the interactions of this particular region of the protein with membranes is of interest as a general model for membrane protein–lipid interactions since a large number of viral and cellular membrane glycoproteins share this same basic organization. In addition, this region of the protein appears to be involved in transport of the protein to the cell surface (Sveda et al., 1982; Doyle et al., 1985), and it is probably involved in interaction with internal viral components in the budding process.

We have developed a strategy to specifically label lysine-212 of the HA_2 of the X47 strain of influenza virus with fluorescent probes in order to obtain structural information about the

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[1] Abbreviations: DEAE, diethylaminoethyl; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; HA, hemagglutinin; IAI, isethionyl acetimidate; NA, neuraminidase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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cytoplasmic domain. Lysine-212 is the first charged amino acid following the hydrophobic membrane anchor sequence (Min Jou et al., 1980). It is preceded by two polar, though uncharged, amino acids, which could conceivably extend across the head-group region of the lipid bilayer with lysine-212 exposed to the aqueous phase. Alternatively, a more condensed structure could result in lysine-212 residing within the bilayer structure. Our results favor the latter structure. The approach we used to label HA was based on that of Whiteley & Berg (1974) in their study of the topography of erythrocyte membrane proteins. Reactive lysine residues in the external domain of the HA in intact virus were modified with the membrane-impermeable reagent isethionyl acetimidate (IAI). The HA was then solubilized with detergents, and the remaining unblocked lysine(s) was (were) reacted with fluorescein isothiocyanate. As a control for labeling at other sites besides lysine-212, we used the HA of the X31 strain of influenza virus in which residue 212 is arginine instead of lysine (Verhoeyen et al., 1980). Modification with IAI does not introduce major perturbations in the structure of most proteins, since the positive charge on the modified lysines is preserved with only a small increase in ionic radius. In any event, the activity of the HA is known to be retained even after more drastic modification of its lysine amino groups (Laver et al., 1981). The results presented here indicate that lysine-212 of the X47 HA_2 exists in an environment of reduced polarity compared to the aqueous phase that probably involves direct interaction with the head-group region of the phospholipid bilayer. In addition, a site (or sites) on the HA_2 of both the X31 and X47 viruses is (are) also labeled by this procedure and also appear(s) to interact directly with the lipid bilayer.

Experimental Procedures

Modification of Influenza Virus with IAI. The X47 and X31 strains of influenza virus (originally obtained from Dr. J. Schulman, Mt. Sinai School of Medicine) were grown in embryonated eggs and purified by sucrose gradient centrifugation as described previously for Sendai virus (Lyles, 1979). Purified virus was pelletted by centrifugation at 40 000 rpm for 30 min in a Beckman 50Ti rotor and resuspended in 50 mM sodium borate and 0.9% NaCl, pH 9.5, at 1 mg of protein/mL. IAI (20 mM, Pierce Chemical Co. or Sigma Chemical Co.) was dissolved in the same buffer to which 50 mM NaOH had been added to neutralize the IAI, and an equal volume was added immediately to the virus suspension and incubated 30 min at 37 °C. The same amount of freshly dissolved IAI was added twice more followed by incubations at 37 °C for 30 min. The virus was then pelleted through a 15% sucrose cushion and resuspended in 50 mM sodium phosphate–borate and 0.9% NaCl, pH 8.0, containing 10 μg/mL aprotinin, at a concentration of 1.5 mg of protein/mL. The virus either was used immediately for preparation of labeled HA or else was stored at −70 °C. Assays for protein (Lowry et al., 1951) and viral hemagglutinin and neuraminidase activities (Scheid & Choppin, 1974) were performed as described.

Fluorescein Labeling of HA. Influenza virus modified with IAI was mixed with half its volume of 20% octyl glucoside (Calbiochem) in the same buffer and incubated 30 min at room temperature. The nucleocapsids were pelletted at 40 000 rpm for 60 min. The supernatant was made 5×10^{-4} M in fluorescein isothiocyanate (Eastman Chemical Co.) and was incubated overnight at 30 °C. Tris (0.1 M final concentration, pH 8.0) was added, and the glycoproteins were separated from unreacted fluorescein by centrifugation on a 5–20% (w/w) sucrose gradient containing 50 mM octyl glucoside at 40 000 rpm for 16 h at 5 °C in an SW41 rotor. The gradient was buffered either with 10 mM sodium phosphate and 0.9% NaCl, pH 7.4 (PBS), or with 0.1 M Tris, pH 8.8, in later experiments. The fluorescent glycoprotein band near the middle of the gradient was visualized with an ultraviolet light and collected from the bottom of the tube. The resultant glycoprotein fraction in PBS was chromatographed on Sephadex G-25 in 0.1 M Tris and 50 mM octyl glucoside, pH 8.8, prior to chromatography on DEAE-cellulose (Whatman DE52). A 2-mL DE52 column equilibrated in the same buffer was loaded with 2–4 mL of glycoprotein solution containing 0.5–1.0 mg of protein and was washed with 2 mL of buffer. The fluorescein-labeled hemagglutinin was eluted from the column with 0.1 M Tris, 0.1 M NaCl, and 50 mM octyl glucoside, pH 8.8. SDS gel electrophoresis of proteins was performed as described (Laemmli, 1970). Gels were analyzed by a dual light source (soft laser and ultraviolet) densitometer equipped for either absorbance or fluorescence detection with digital integration (Biomed Instruments, Fullerton, CA).

The fluorescein-labeled HA was tested for the presence of noncovalently bound fluorescein by denaturation in SDS and chromatography on Sephadex G-25. Following DEAE-cellulose chromatography, the HA was made 0.2% in SDS and 50 mM in dithiothreitol, boiled 5 min, and incubated 1 h at room temperature. Iodoacetamide was added to 0.1 M and incubated 30 min at room temperature. A 1-mL sample was chromatographed on a 16-mL G-25 column in 10 mM sodium carbonate and 0.1% SDS, pH 9.5. Approximately 1-mL fractions were collected and were assayed for fluorescein fluorescence and the absorbance at 280 nm. The fluorescence in the void volume peak was 400-fold above background, and the fluorescence in the inclusion volume was not detected. Therefore, contamination by noncovalently bound fluorescein was considered to be <1% of the total fluorescence.

Incorporation of HA into Liposomes. A solution of dimyristoylphosphatidylcholine (DMPC) in chloroform was evaporated to form a thin film. The lipid was dissolved at 7.5 mg/mL in 600 mM octyl glucoside in PBS and was added to a solution of HA from the DEAE-cellulose column. The final lipid concentration was 0.75 mg/mL, and the protein concentration was 100–500 μg/mL. Alternatively, the HA was centrifuged on a 5–20% sucrose gradient in PBS with 2% sodium cholate at 40 000 rpm for 16 h in an SW41 rotor. The fluorescent HA fraction from the gradient was then used to dissolve the lipid film directly to 0.75 mg/mL. The centrifugation step usually resulted in a 3-fold dilution of the protein concentration. The detergent solutions of HA and lipid were dialyzed 3 days against three changes of PBS. In early experiments, the liposomes were separated from unincorporated HA by flotation in sucrose gradients. The liposome suspension was mixed with an equal volume of 67% sucrose and was overlayed with 15% sucrose in PBS and then with PBS alone. The gradient was spun at 35 000 rpm for 16 h in an SW41 rotor, and 0.75-mL fractions were collected. The liposomes were at the interface of the 15% sucrose and PBS. Since >90% of the fluorescence was associated with the liposomes, the centrifugation step was omitted in later experiments.

Absorbance Measurements. Absorption spectra were taken with a Bausch and Lomb Spectronic 2000 spectrometer. The extinction coefficient for HA was calculated by using protein concentrations determined by the Lowry method and corrected for the difference in tyrosine content between HA and the standard (cytochrome *c*). The extinction coefficient determined, 8.77×10^4 M^{-1} cm^{-1} at 280 nm, was similar to that calculated from the tryptophan and tyrosine content of HA

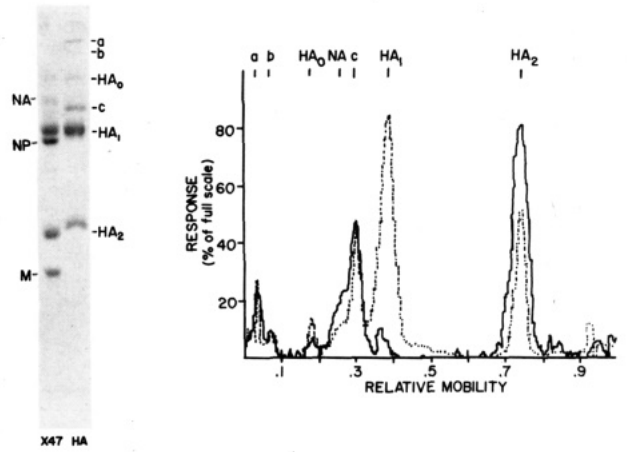

FIGURE 1: SDS gel electrophoresis of fluorescein-labeled glycoproteins of influenza virus. The X47 strain of influenza virus was reacted with IAI, and the glycoproteins were solubilized with octyl glucoside. The glycoproteins were reacted with fluorescein isothiocyanate and then separated from unreacted fluorescein by rate zonal centrifugation in a sucrose gradient containing octyl glucoside. The glycoprotein fraction of the gradient and purified X47 virus were analyzed by SDS gel electrophoresis. (Left panel) Gel stained with Coomassie blue. (Right panel) Densitometer scan of the HA lane: (—) fluorescence before staining; (---) absorbance after staining.

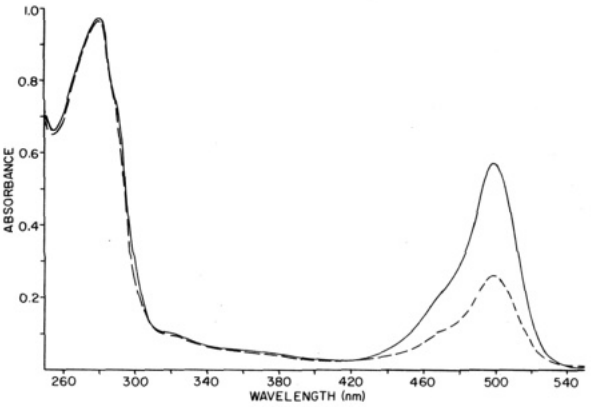

FIGURE 2: Absorption spectra of fluorescein-labeled HA of influenza viruses. Fluorescein-labeled HAs of the X47 (—) and X31 (---) viruses were prepared as described under Experimental Procedures. Spectra were obtained for HA in 0.1 M Tris, 0.1 M NaCl, and 50 mM octyl glucoside, pH 8.8. The protein concentrations were adjusted so that the 280-nm peaks were superimposable to facilitate comparison of the two samples.

(9.18×10^4 M^{-1} cm^{-1}). The extinction coefficients determined for fluorescein, 5.56×10^4 M^{-1} cm^{-1} at 495 nm and 1.59×10^4 M^{-1} cm^{-1} at 280 nm, were similar to published values (De Petris, 1978).

Fluorescence Measurements. A Spex Fluorolog fluorometer was used equipped with a 450-W xenon lamp, dual monochromators, a water-jacketed sample holder, and a single photon counting emission photomultiplier tube. Fluorescein fluorescence was routinely determined with 495-nm excitation and 530-nm emission wavelengths and 10-nm band-pass slits. Corrected excitation spectra were taken in the ratio mode using a reference photomultiplier with a rhodamine B detector. When emission was monitored at wavelengths less than 530 nm, then 5-nm slits were used. Fluorescence polarization was measured by using polarization filters supplied with the instrument and was calculated according to the equation:

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

corrected for emission monochromator anisotropy (Azzi, 1974). Polarization values of turbid samples were corrected for light-scattering effects as described (Lentz et al., 1979). Temperature was monitored in the sample by a copper–constantan thermocouple.

RESULTS

Preparation of Fluorescein-Labeled HAs. Purified virions of the X47 and X31 strains of influenza virus were reacted with the membrane-impermeable reagent IAI to block reactive amino groups on the external surface of the virus envelope. The viral glycoproteins were solubilized with the detergent octyl glucoside, and the remaining unreacted lysine residues were modified with fluorescein isothiocyanate. Modification of either virus with IAI did not result in a loss of hemagglutinating activity. In addition, the HA has been shown to retain full hemagglutinating activity and reactivity with both antisera and a panel of monoclonal antibodies after modification of the reactive lysine residues in the external domain with fluorodinitrobenzene (Laver et al., 1981). In contrast, the neuraminidase activity was completely inactivated (>98%) by modification with IAI. After reaction with fluorescein isothiocyanate, the HA was separated from unreacted fluorescein by rate zonal centrifugation in sucrose gradients containing octyl glucoside. Figure 1 shows the analysis by gel electrophoresis of the labeled glycoprotein from the X47 virus at this stage of purification. By comparison with intact virus, it can be seen that the glycoprotein fraction was free of the major internal viral proteins, NP and M. A small amount of the neuraminidase glycoprotein was still present at this stage, and a variable but small amount of HA_0, the uncleaved precursor glycoprotein, was present in all preparations. In addition, several additional bands (a–c) appeared after purification of the HA that were absent or present in small amounts when intact virus was analyzed. These represent aggregates of the HA_2 subunit that form in SDS. When the samples for electrophoresis were analyzed without boiling, the aggregates were present in much larger amounts at the expense of the amount of protein migrating with monomeric HA_2, whereas the amount of HA_1 was the same whether the samples were boiled or not (data not shown). Boiling for longer times did not reduce the amount of aggregates beyond the level seen in Figure 1. We know that higher order aggregates beyond the native trimer are not present in the glycoprotein fraction before SDS treatment, since the HA was isolated by its sedimentation velocity (approximately 10–12 S), thus removing any higher order aggregates. Figure 1 also shows results of densitometry of the gel in Figure 1 to quantitate the fluorescence of each protein species (before staining) and the absorbance after staining. As expected, most of the fluorescence is associated with HA_2 and species containing HA_2 (HA_0 and the aggregates). Only 2–5% of the fluorescence is associated with HA_1. The NA glycoprotein is also labeled and is visible as a shoulder on peak c. There is a lysine residue near the amino terminus of the NA glycoprotein that may reside inside the virus envelope (Blok et al., 1982) and be labeled by this procedure. After chromatography on DEAE-cellulose, the remaining NA was undetectable even on gels overloaded beyond that in Figure 1. The purified HA was also free of noncovalently bound fluorescein (<1% of protein-bound label) as determined by gel filtration after denaturation with SDS (data not shown). When the HA of the X31 virus was subjected to the same labeling protocol, the HA_2 subunit (and not HA_1) was also labeled with fluorescein, although to a lesser extent than that of the X47 strain. Figure 2 shows absorbance spectra of the labeled HAs of these two virus strains after the DEAE-cel-

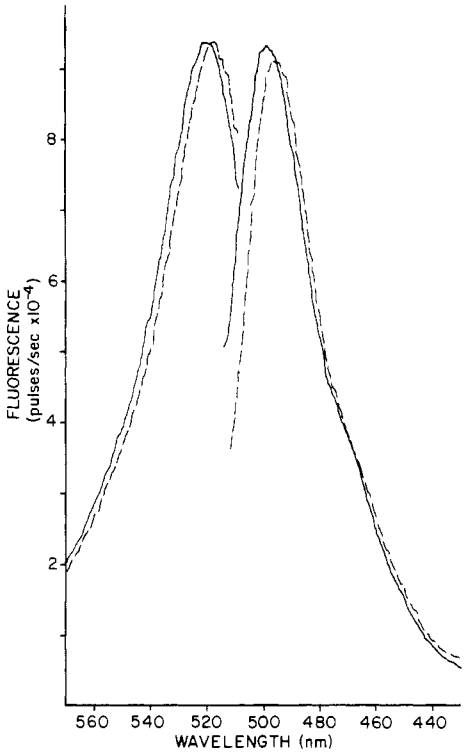

FIGURE 3: Fluorescence excitation and emission spectra of fluorescein-labeled HA. Fluorescein-labeled HA of the X47 virus incorporated into liposomes of dimyristoylphosphatidylcholine (—) was prepared as described under Experimental Procedures. Bromelain-digested HA (---) was prepared by dialysis of labeled HA (in the absence of lipids) to remove octyl glucoside followed by incubation with bromelain (1 mg/mL) for 2 h at room temperature. Samples were diluted in 0.1 M Tris, pH 8.8, to give equivalent fluorescence intensities. Corrected excitation spectra (right side of figure) were determined at 530-nm emission wavelength. Emission spectra (left side of figure) were determined at 495-nm excitation wavelength; 10-nm slits were used.

lulose chromatography step. The difference in the fluorescein absorbance peaks centered around 500 nm is due to the specific labeling of lysine-212 in the cytoplasmic domain of the X47 HA, since of the three amino acid differences in HA_2 between these two strains it is the only one capable of being modified by fluorescein isothiocyanate. From spectra similar to those in Figure 2, the HA of the X47 virus was calculated to contain 1.3 ± 0.2 mol of fluorescein/mol of HA monomer (mean ± standard deviation, $N = 5$), while that of the X31 virus had 0.47 ± 0.08 ($N = 9$) mol of fluorescein/mol of HA. Thus, 0.8 of the HA molecules of the X47 virus was modified at lysine-212, and an additional site (or sites) was (were) modified in the HAs of both strains to a lesser extent. Since the native glycoprotein is a trimer of identical subunits (Wilson et al., 1981), there are 2.4 fluoresceins at lysine-212 per trimer. The relative quantum yields of fluorescence of the labeled glycoproteins from the two viruses were the same (ϕ_F approximately 0.3 by comparison with 6-carboxyfluorescein). In the spectroscopic studies that follow, it would be possible to determine the spectral properties of the fluorescein located at lysine-212 of the X47 HA by subtracting the spectral contribution of the labeled X31 HA. In practice, the labels on the two proteins have very similar properties, indicating that they reside in similar environments, thus making such a correction a moot point.

Fluorescence Properties of Fluorescein-Labeled HAs. The fluorescence properties of the labeled HAs in three different physical states were examined: detergent micelles, liposomes, or detergent-free rosettes. As purified by DEAE-cellulose chromatography, the HA is soluble in the presence of the detergent octyl glucoside. In some experiments, sodium cholate was substituted as the detergent by rate zonal centrifugation on sucrose gradients containing cholate. Liposomes were formed from a mixture of HA and DMPC by dialysis of either detergent. Removal of the detergent in the absence of lipids results in aggregation of the glycoproteins by their hydrophobic membrane anchor sequences to form glycoprotein micelles referred to as rosettes. As noted in the data below, no differences in the spectral properties of the labeled HAs were noted between octyl glucoside and cholate. Likewise, HA in liposomes formed from either detergent had equivalent spectral properties.

Table I: Fluorescence Properties of Fluorescein-Labeled HA[a]

physical state	virus strain	excitation max (nm)	emission max (nm)	polarization
DMPC	X47	500	520	0.232 ± 0.014 (5)
	X31	501	520	0.238
octyl glucoside micelle	X47	502	522	0.158 ± 0.032 (3)
	X31	500		0.160
rosette	X47	504	523	0.208
	X31	502	520	0.189

[a] Fluorescein-labeled HA was prepared and incorporated into liposomes or rosettes as described under Experimental Procedures. Fluorescence measurements were made on samples in 0.1 M Tris, pH 8.8. Data shown are the mean of two experiments except where indicated [±SD (N)].

Figure 3 shows the excitation and emission spectra of the fluorescein-labeled HA of the X47 virus incorporated into DMPC liposomes by dialysis from octyl glucoside (solid lines) compared with those of labeled HA that has been digested with the proteolytic enzyme bromelain in the absence of lipids or detergents (dashed lines). As expected, the spectra of the proteolytically digested HA were very similar to those of fluorescein isothiocyanate (excitation and emission maxima at 495 and 515 nm, respectively). However, both the excitation and emission maxima of the intact HA incorporated into liposomes display a red shift of about 5 nm, indicating that the native protein structure maintains the fluorescein in an altered environment. This red shift was also observed for the labeled HA of the X31 virus incorporated into liposomes and also for both proteins in detergent or in the form of detergent-free protein micelles or rosettes (Table I). The emission maximum was independent of the wavelength used for excitation from 490 to 505 nm, thus providing no evidence for environmental heterogeneity. A probable explanation for the red shift in the spectra is that the fluorescence groups on these proteins reside in an environment of lower polarity than the bulk aqueous phase (Hartig et al., 1977; Stanton et al., 1984). The location of lysine-212 in the HA of the X47 virus near the hydrophobic membrane-anchoring sequence of HA_2 makes it particularly likely that the fluorescein at this position interacts with the membrane lipid bilayer when the protein is incorporated into liposomes. A similar environment would be provided by detergent when the protein is in a micelle or by the membrane-binding regions of other HA molecules in a rosette. Liposomes containing labeled HA of either the X47 or X31 virus were digested with bromelain and then subjected to flotation by centrifugation in sucrose gradients. Approximately half of the original fluorescence intensity of both HAs was removed from the liposomes by proteolysis. This indicates that some of the labels are located on the external surface of the liposomes. Thus, the HAs are probably randomly oriented in these liposomes. The fluorescent label that remained associated with the liposomes (presumably residing on the in-

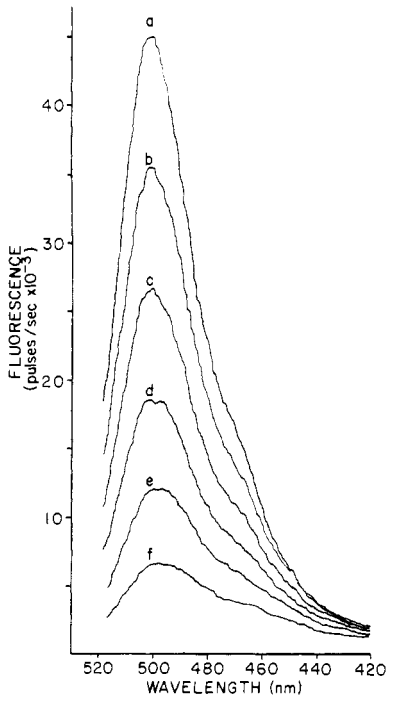

FIGURE 4: pH dependence of fluorescein-labeled HA excitation spectrum. Fluorescein-labeled HA of the X47 virus was incorporated into dimyristoylphosphatidylcholine liposomes. Excitation spectra were obtained as in Figure 3 except that the buffer was 10 mM sodium phosphate and 0.15 M NaCl titrated to the following pH with either 1 N NaOH or 1 N HCl: (a) 9.50; (b) 8.28; (c) 7.79; (d) 7.32; (e) 6.86; (f) 6.32.

ternal surface) displayed the red spectral shift, while the label released from the liposomes by proteolysis did not (data not shown).

Steady-state fluorescence depolarization data (Table I) also lend support to the hypothesis that the fluorescein interacts with the membrane lipid bilayer by indicating that the mobility of the fluorescein groups is restricted by their environment. The polarization values observed (0.15–0.25) are intermediate between the relatively unrestricted rotation of fluorescein bound to lysine residues that are exposed to an aqueous environment ($p < 0.05$) and the limiting polarization of fluorescein in the absence of rotation, $p_0 = 0.49$ (Chen & Bowman, 1965). Furthermore, the polarization is greater for labeled HA in a lipid bilayer than for HA in a detergent micelle or rosette. This result was obtained with the HAs from both the X47 and X31 viruses. These data suggest that the fluorescein groups interact directly with the lipid bilayer, rather than residing in a low polarity environment formed only by the folding of the peptide chain itself.

Protonation Reactions of Fluorescein Bound to HA. A useful property of fluorescein probes is that they undergo protonation reactions in the physiological pH range. Such reactions are influenced, of course, by the environment of the titratable groups. Thus, further information about the location of the fluorescein groups on the labeled HAs could be obtained from a determination of their apparent pK_a. Figure 4 shows the pH dependence of the excitation spectrum of the labeled HA from the X47 virus incorporated into liposomes. Two spectral features that are particularly sensitive to pH are (1) the intensity of the main transition at 495–500 nm and (2) the relative intensity of the shoulder at 460 nm compared to the intensity of the main peak. Spectra for labeled HA from both X47 and X31 viruses as a function of pH do not have an isosbestic point. However, as shown in Figure 5, the data can be fit reasonably well to a single pK_a. The lack of isosbestic

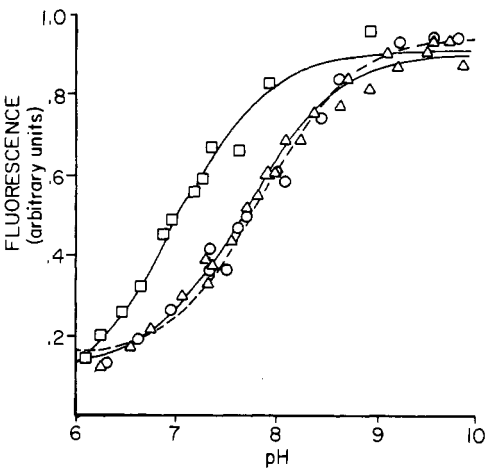

FIGURE 5: pH titration of fluorescein-labeled HAs. Fluorescein-labeled HAs were incorporated into dimyristoylphosphatidylcholine liposomes. Titrations were performed as in Figure 4. Fluorescence was monitored at 495-nm excitation and 530-nm emission wavelengths. (△) HA from the X47 virus; (○) HA from the X31 virus; (□) HA from the X47 virus to which Triton X-100 was added to a final concentration of 1%. Connecting lines are the least-squares fit to a single pK_a.

behavior in similar systems has been attributed to the proximity of the pK_a for the fluorescein dianion–monoanion reaction to that of the monoanion–neutral fluorescein reaction in environments of reduced polarity (Stanton et al., 1984).

The data shown in Figure 5 are the intensity from excitation at 495 nm as a function of pH, which undergoes a 10-fold increase over the range of the titration. The apparent pK_a determined for the fluorescein on the HA of the X31 virus, 7.8, is practically identical with that of the X47 HA, 7.7. Both pK_a's are sensitive to the physical state of the protein. The apparent pK_a determined for the HA in liposomes is 0.7–0.8 unit higher than that determined after solubilization of the liposomes with the detergent Triton X-100 (pK_a = 7.0). Furthermore, the apparent pK_a in detergents is 0.3–0.7 unit higher than published values for the fluorescein dianion–monoanion transition (Leonhardt et al., 1971; Martin & Lindquist, 1975; Stanton et al., 1984). The increase in the apparent pK_a in detergent and in liposomes is probably due largely to the lower polarity of the environment, which favors the less charged acidic forms of the fluorescein groups. The greater shift in pK_a observed in liposomes compared to detergent is consistent with the depolarization data, showing that the lipid bilayer has a larger effect on the properties of the labels than does the detergent micelle.

Temperature Dependence of the Fluorescence of Fluorescein-Labeled HA. The fluorescence properties of the labeled HA of the X47 virus incorporated into DMPC liposomes were examined to determine whether the fluorescein groups were affected by the gel to liquid-crystal phase transition of the lipid bilayer, which occurs around 23 °C. As shown in Figure 6, the fluorescence polarization values are essentially independent of temperature between 10 and 37 °C, indicating that the mobility of the labels is not greatly affected by the phase transition in the lipid bilayer. The fluorescence intensity of the labeled HA in liposomes increased with increasing temperature (Figure 6, curve a). This is due largely to a decrease in pK_a with increasing temperature (about 0.15–0.2 pH unit between 5 and 35 °C). In contrast, labeled HA in detergent solutions such as sodium cholate (Figure 6, curve b) showed a slight decrease in intensity with increasing temperature. Since the pK_a in detergent solution is relatively far removed from the pH in the experiment (7.5), the intensity is not very sensitive to changes in pK_a. The intensity decrease observed

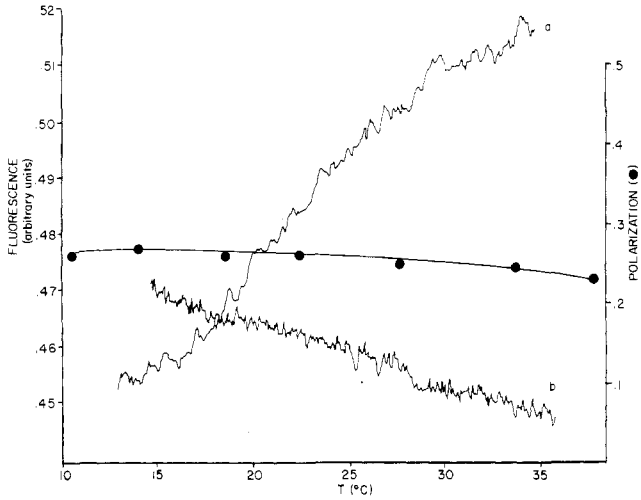

FIGURE 6: Temperature dependence of fluorescence of fluorescein-labeled HA. Fluorescein-labeled HA of the X47 virus was incorporated into dimyristoylphosphatidylcholine liposomes (a) or into 2% sodium cholate (b). Fluorescence was monitored as a function of temperature as in Figure 5. The buffer was 10 mM sodium phosphate and 0.15 M NaCl, pH 7.4 (at 25 °C). Note the expanded scale for fluorescence intensity. Polarization measurements were made on fluorescein-labeled HA in liposomes.

Table II: Protonation State of Fluorescein-Labeled HA in Liposomes[a]

	virus strain			
	X47 at		X31 at	
liposomes	35 °C	5 °C	35 °C	5 °C
DMPC	0.398	0.429	0.352	0.392
DPPC	0.382	0.424	0.365	

[a] Data shown are ratios of fluorescence intensity from 460-nm excitation to intensity from 495-nm excitation. Samples were in 10 mM sodium phosphate and 0.15 M NaCl, pH 7.4. Data are averages of two experiments.

is probably due to an increase in nonfluorescent mechanisms of relaxation from the excited state (Peske et al., 1971).

The increase in intensity (decrease in pK_a) with increasing temperature observed when labeled HA is incorporated into liposomes occurred without any notable discontinuity in the region of the phase transition. This suggests that the temperature dependence of pK_a is not due to the change in physical state of the lipid bilayer. Further support for this conclusion is provided by the data in Table II. The data shown are the ratios of fluorescence intensities obtained from excitation at 460 vs. 495 nm. This ratio is indicative of the protonation state of the fluorescein, as can be seen in the spectra in Figure 4. Changes in the extent of protonation at a given pH (7.4 in this experiment) will reflect changes in the pK_a of the fluorescein groups. The temperature dependence of the fluorescein pK_a of the labeled HA in DMPC liposomes is shown by the higher intensity ratio at 5 °C compared to 35 °C (Table II). Labeled HA incorporated into DPPC liposomes also had a higher ratio at 5 °C compared to 35 °C, similar to HA in DMPC liposomes, even though the DPPC liposomes are in the gel state in both temperatures. These data show that the temperature dependence of the fluorescein pK_a is not due to changes in the physical state of the lipid bilayer.

Discussion

The data presented here provide evidence for two sites on the HA of influenza virus that appear to interact with phospholipid bilayers. One is lysine-212 of the X47 HA_2. The other is the site or sites also labeled on the X31 HA. Lysine-212 is usually considered to be the beginning of the cytoplasmic domain of HA, since it is the first charged amino acid following the membrane anchor sequence. Experiments to test the function of the cytoplasmic domain have been designed on the basis of this premise [e.g., see Doyle et al. (1985)]. Thus, our results suggest that the cytoplasmic domain actually begins in the head-group region of the lipid bilayer. The data that suggest an interaction with lipid bilayers are the red shift of the excitation and emission maxima (Figure 3) and the shift in the pK_a of the fluorescein labels (Figure 5), which indicate that the labels reside in environments of lower polarity than the aqueous phase, and the fluorescence polarization data, which indicate that the mobility of the probes is restricted by their environment (Table I). Many fluorescent molecules display a blue shift in their fluorescence spectra upon incorporation into a nonpolar environment, due to the greater polarity of the excited state compared to the ground state (Pesce et al., 1971). However, fluorescein probes have been observed to show the opposite effect, i.e., a red shift (Hartig et al., 1977; Stanton et al., 1984). The magnitude of the red shift we observe is similar to that observed for fluorescein dissolved in methanol vs. water. An environment with such an intermediate polarity would be expected to be found in the head-group region of a lipid bilayer or detergent micelle. The shift in pK_a is also due primarily to the effect of a lower polarity medium, which favors the less charged protonated species (Fernandez & Fromherz, 1977). Under our conditions of relatively high ionic strength (0.15 M), local effects of other charged groups on the pK_a of the fluorescein would be minimized (McLaughlin, 1977). The effect of a lower polarity environment on the protonation of a neutral base, such as the original lysine amino group, would be the opposite of that for the negatively charged fluorescein; that is, the pK_a would be lowered due to the fact that the uncharged base would be favored in a less polar environment.

The altered environment and mobility of the fluorescein groups on HA could also be explained by the labels residing in a low polarity region of the protein, separate from the lipid bilayer. However, the magnitude of the pK_a shift and the degree of immobilization are greater when the protein is in a lipid bilayer than when it is in a detergent micelle. This could conceivably result from a difference in the conformation of the protein when it is in a bilayer vs. a micelle. The more likely explanation is that the labels interact directly with the lipid bilayer, since the cytoplasmic domain of HA is comprised of only nine amino acids beyond lysine-212, and this sequence is not markedly hydrophobic. We also considered that the incorporation of labeled HA into liposomes could alter the apparent pK_a by introducing a permeability barrier to protons for fluorescein groups that reside inside the liposomes. The proton permeability of liposomes containing HA must be high enough that all of the fluorescein groups are able to be titrated (Figure 5). If this were not the case, the fluorescence intensity increase over the pH range of the titration would be much less than that observed in the presence of detergent, when in fact they are the same.

While the fluorescein groups on the HA appear to interact directly with the lipid bilayer, it is unlikely that they extend into the hydrocarbon-like region of the lipid fatty acyl chains. The insertion of fluorescein or the original lysine residues into a hydrocarbon-like environment is highly unfavorable, since the partition coefficient of fluorescein from water into hexane is immeasurably low ($<10^{-4}$) (Stanton et al., 1984). Thus, unless constrained by the protein structure, a location of the

labels on the interior of the bilayer would not expected. As pointed out above, the extent of the red shift is consistent with an environment of intermediate polarity between that of water and that of hydrocarbons. Further support for the hypothesis that the fluorescein groups on HA interact with the phospholipid head groups is provided by comparing our results with those of Stanton et al. (1984), who described the properties of fluorescein conjugated directly to the head group of phosphatidylethanolamine through a short linker group. They showed that the conformation of the fluorescein head group was in a reversible equilibrium between at least two states. The extremes of the conformational difference were represented by the fluorescein being fully extended away from the lipid bilayer and by the fluorescein being sequestered in the head-group region of the bilayer. In fact, the properties of our fluorescein-labeled HA are remarkably similar to those of the sequestered head-group label with respect to emission and excitation maxima, fluorescence depolarization, and pK_a. A location in the head-group region of the bilayer would make sense for lysine-212 of the X47 HA_2, in which the hydrophobic membrane anchor sequence occupies a position in the bilayer next to the fatty acyl chains, so that the first few polar amino acids may be held in the polar head-group region of the bilayer. However, such a position for lysine-212 was not a forgone conclusion, since it is preceded by two polar, though uncharged, amino acids (Min Jou et al., 1980), and thus could conceivably have extended into the aqueous phase. The reason for the interaction of the other fluorescence group also found on the X31 HA with the lipid bilayer will become clearer when the site or sites of labeling are determined. We are currently preparing cleavage fragments of HA_2 suitable for Edman degradation to find the labeled position in the amino acid sequence. The possibilities for the site of labeling of the X31 HA include a residue other than lysine in the cytoplasmic domain or else a lysine in the external domain. If the latter is true, then the lysine amino group must be protected from reaction with IAI in the virus envelope but accessible to reaction with fluorescein after detergent solubilization. There are several lysine residues in the external domain near the hydrophobic membrane anchor sequence, which may be protected from reacting with IAI by interacting with the envelope lipid bilayer or by the protein structure and thus may occupy a position analogous to lysine-212 in the cytoplasmic domain. Label in these positions would be expected to have properties similar to those at lysine-212.

The mobility and pK_a of the fluorescein groups on HA appear to be relatively insensitive to the phase transition of the phospholipid bilayer (Figure 6, Table II). It is possible that the lipids in contact with the protein do not participate in the phase transition, since it is well documented that integral membrane proteins perturb the thermotropic behavior of the bilayers in which they reside [discussed by Owicki & McConnell (1979)]. The vesicular stomatitis virus G protein, which is structurally similar to HA, has been estimated from differential scanning calorimetry studies to remove 270 molecules of lipid per molecule of protein from participation in the main phase transition when incorporated into DPPC bilayers (Petri et al., 1980). This lipid perturbation is further supported by a series of fluorescence studies on the same system (Petri et al., 1981).

The influenza virus HA glycoprotein is typical of a large class of membrane glycoproteins that have a large external domain, a single membrane-spanning sequence, and a small cytoplasmic domain. The observation that the boundary between the membrane anchor sequence and the cytoplasmic domain is actually located in the head-group region of the lipid bilayer may be a general feature of this class of membrane proteins. The head-group region of a phosphatidylcholine bilayer from the fatty acyl carboxyl group to the choline methyl groups may only be about 5 Å wide (Büldt et al., 1979), since the choline base probably lies parallel to the plane of the bilayer. Thus, the observation that the fluorescein groups on the labeled HA reside in the head-group region of the bilayer provides relatively precise information about the location of the labeled sites relative to the membrane. In future studies, these labels may be used to determine the effect of bilayer width on the membrane-spanning region of the protein. In addition, they may be able to detect interactions between HA and other virion proteins by techniques such as resonance energy transfer.

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Physicochemical Characterization of the α-Peptide of the Sodium Channel from Rat Brain†

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ABSTRACT: The α-peptide of the rat brain sodium channel of apparent molecular weight 260K has been purified to homogeneity in order to determine its structural and chemical properties. By negative-stain electron microscopy, the molecule morphology of the solubilized channel protein appears as a stack of disks or rouleaux whose dimensions are 40 Å × 200 Å. Measurement of the secondary structure by circular dichroism shows that the α-peptide is a conformationally flexible polypeptide that contains mostly β-sheet and random-coil in mixed detergent–phospholipid micelles and folds into a conformation that has approximately 65% α-helix after reconstitution into phosphatidylcholine vesicles. Preparative polyacrylamide gel electrophoresis was used to obtain a chemically homogeneous peptide to analyze the amino acid and carbohydrate composition. The amino acid composition shows a reasonably high content of acidic amino acids with no striking excess of hydrophobic amino acids, while carbohydrate analyses show that carbohydrate is 31% by weight of the protein with sialic acid representing over 50% of the total carbohydrates. The high α-helical content, the amino acid composition, and the large carbohydrate mass are similar to those of the eel electroplax sodium channel and appear to be general features of the sodium channels which have been analyzed structurally and chemically to date.

Propagation of the action potential in nerve and muscle cells is the result of a transient increase in the membrane permeability to sodium ions. These permeability changes are controlled by a transmembrane protein channel, the sodium channel, which is gated to a sequence of resting, opened, and closed states during excitation. Although the electrical properties of this membrane channel have been extensively investigated, only until relatively recently has the molecular composition of this system been described. Current evidence from photoaffinity labeling, radiation inactivation, and purification and reconstitution suggests that sodium channels from eel electroplax (Agnew et al., 1978, 1983), rat bran synaptosomes (Beneski & Catterall, 1980; Hartshorne & Catterall, 1981; Barhanin et al., 1983a,b), and rat sarcolemma (Barchi et al., 1984) are composed of a glycoprotein of M_r ~260K (α). The sodium channel isolated from mammalian brain includes additional subunits of M_r 39K (β1) and 37K (β2) in which β2 is apparently linked to α by disulfide bonds (Hartshorne et al., 1982). However, the functional substructure of the rat brain sodium channel complex, as revealed by radiation inactivation techniques, is an assembly composed of only the 260-kilodalton (kDa)[1] (α) and 39-kDa (β1) polypeptide components (Barhanin et al., 1983b; Angelides et al., 1985). These proteins appear to be sufficient in mediating transmembrane ion flux after reconstitution into lipid bilayers and respond to pharmacological agents in a fashion similar to sodium channels in situ (Tamkun & Catterall, 1981; Weigele & Barchi, 1982; Rosenberg et al., 1983; Hartshorne et al., 1985). Recently, highly purified sodium channels isolated from rat brain and eel electroplax consisting of only the α-peptide have been shown by single-channel techniques to be pharmacologically- and voltage-regulated after reconstitution into defined lipid membranes (Hanke et al., 1984; Rosenberg et al., 1984). This indicates that the 260-kDa peptide is unique in that many of the conducting and regulatory functions of the channel are associated with a single polypeptide chain.

Through the application of fluorescent techniques, some structural information has appeared describing the arrangement of the neurotoxin receptor sites of the in situ channel (Angelides & Nutter, 1983a,b). These measurements have shown that many of the functional sites on the α component are topologically distant and therefore must communicate through alterations in the polypeptide matrix of a conformationally flexible α-chain (Darbon & Angelides, 1984; Angelides & Brown, 1984).

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; STX, saxitoxin; TTX, tetrodotoxin; SDS, sodium dodecyl sulfate; MAP-2, microtubule-associated protein 2; NF1, -2, and -3, neurofilament triplet proteins 1, 2, and 3, respectively; Css II, toxin II from *Centruroides suffusus suffusus*; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; WGA, wheat germ agglutinin.

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