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ASSOCIATION OF THE INTERNAL MEMBRANE PROTEIN WITH THE LIPID BILAYER IN INFLUENZA VIRUS

A STUDY WITH THE FLUORESCENT PROBE 12-(9-ANTHROYL)-STEARIC ACID

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SUMMARY

The lipid fluorescent probe 12-(9-anthroyl)-stearic acid was introduced into the lipid bilayer of influenza virus particles. Fluorescent energy transfer was observed from the viral protein to the probe. This transfer persisted after removal of the glycoprotein spikes which cover the outside of the viral particle, demonstrating that the energy donor was an internal protein. It was concluded that the energy donor was the non-glycosylated membrane protein (M protein), the major protein component of the spikeless particle. Analysis of the emission spectrum of the spikeless particle excited at 275 nm shows that a substantial portion of the fluorescence arises from tyrosine residues, in contrast to most other proteins which contain both tryptophan and tyrosine.

It is suggested that the donor residue(s) are located no more than 11 Å exterior to the bilayer surface, and that a portion of the M protein may penetrate into the bilayer.

INTRODUCTION

Although all biological membranes are known to consist chiefly of protein and lipid, the very large number of different proteins which are present in most membranes has until recently frustrated attempts to study the nature of protein–lipid interactions in these structures.

The enveloped viruses offer several interesting experimental systems for the study of naturally occurring protein–lipid interactions. Influenza virus is typical of these viruses in many important respects. Specifically, the proteins of influenza virus particles are all coded by the viral genome, i.e. no host cell proteins are present in the completed virion [1,2]. The viral lipids, on the other hand, are incorporated from preexisting host cell lipids [3,4] and are organized into a bilayer [5]. Each

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virus particle contains a very limited number of polypeptide chains [1,6–8] and evidence has been obtained for a defined location in the completed virion for each polypeptide species [9–11]. The non-glycosylated internal membrane protein (the M protein) and the glycoprotein spikes are the only proteins which appear to be in close association with the lipid bilayer. While there is very strong evidence regarding the location of the glycoprotein spikes on the external surface [1,6,12] the evidence for locating the M protein on the inner surface of the lipid bilayer is more indirect. It is based mainly on the following findings: (1) An electron-dense layer immediately underneath the unit membrane structure in the influenza viral envelope [13,14]; (2) A major protein of about 26000 molecular weight which is not associated with the nucleocapsid and is completely resistant to proteolytic digestion [1,6]; (3) The demonstration that electron micrographs of glutaraldehyde-fixed particles from which lipids have been extracted have a membrane-like layer surrounding the nucleocapsid [10]. The location of the M protein in the virion is only inferred in a very approximate way by these findings. As one alternative to the association of the M protein with the lipid bilayer, Nermut [15] has observed an electroluminescent space between the internal "core" structure and the unit membrane in electron micrographs, and has suggested that this corresponds to a space between the bilayer and the internal structure of the intact virion.

In this paper we present spectrofluorometric observations showing that the M protein is in very close proximity to the lipid bilayer, and may penetrate into the bilayer.

MATERIALS AND METHODS

Virus. The WSN strain of influenza A_0 virus was grown in Maden–Darby bovine kidney cells [16] and purified as previously described [5].

Spikeless virus particles. The glycoproteins which constitute the spikes seen on the outer surface of the viral particles were completely removed by a modification of the treatment with bromelain previously described [1]. This was achieved by incubating the particles with bromelain for 4 h as described previously, and then adding a second, equal aliquot of bromelain solution and continuing the incubation overnight. No glycoproteins remained with the particles after purification on a 5–40% potassium tartrate gradient [1].

Red cell membranes were prepared from fresh heparinized human blood by the procedure of Dodge et al. [17].

Bovine serum albumin Type F, essentially fatty acid free, was obtained from Sigma Chemical Co.

12-(9-Anthroyl)-stearic acid originally described by Waggoner and Stryer [18], was conveniently prepared by the method of Parish and Stock [19]. To 444 mg (2 mmoles) of anthracene-9-carboxylic acid (Aldrich Chemical Co., Milwaukee, Wisc.) in 10 ml of benzene was added 1.2 ml of trifluoroacetic acid anhydride. To the resulting clear solution was added 630 mg (2.1 mmole) of 12-hydroxystearic acid. After allowing the reaction mixture to stand for about 30 min at room temperature, a portion was applied to a Schleicher and Schuell 1500 silica gel thin-layer plate (activated for at least 1 h at 110°C). The mixture was separated in chloroform–acetonitrile (5:1, by vol.). 12-(9-Anthroyl)-stearic acid was the slowest moving

blue fluorescent band (R_F approx. 0.5), which was scraped from the plate, eluted and dried. It was identified by cochromatography with authentic 12-(9-anthroyl)-stearic acid (courtesy of Dr A. Waggoner) and by the identity of its absorption spectrum with that previously reported [18].

Incorporation of 12-(9-anthroyl)-stearic acid into viral particles. A 25-ml Erlenmeyer flask was wrapped in carbon paper to decrease light. About 300 μg of 12-(9-anthroyl)-stearic acid dissolved in ethanol were evaporated to a thin film at the bottom of the flask. About 1 ml of purified virus dialyzed into phosphate buffered saline (approx. 200 μg protein/ml) and 1 ml of phosphate-buffered saline were added, and the suspension was shaken gently for 3–4 h at room temperature. The particles were then either purified on a potassium tartrate gradient or treated with bromelain to remove the spikes and then repurified. The integrity of intact and spikeless labeled particles was confirmed by electron microscopy.

Comparable observations to those reported in this paper were obtained if the particles were labeled after spike removal and repurification. However, spikeless particles required much less 12-(9-anthroyl)-stearic to achieve comparable incorporation. Thus, 1 ml (approx. 100 μg protein/ml) of purified spikeless particles were shaken with 5–60 μg of 12-(9-anthroyl)-stearic acid for 3–4 h, and then repurified on a potassium tartrate gradient.

Fluorescence measurements were made on a Cary differential spectrofluorometer equipped with a Rhodamine B quantum detector located within the sample compartment. Corrected spectra are obtained with this instrument. Concentrations of virus were kept within the linear response region of emission intensities from the viral protein excited at 275–295 nm, and from 12-(9-anthroyl)-stearic acid excited at 361 nm.

12-(9-Anthroyl)-stearic acid concentration in a sample of virus particles was measured from the fluorescence of a mixture of 0.5 ml of sample, 0.5 ml of 10% sodium dodecylsulfate and 0.5 ml of ethanol. The emission was measured at 470 nm (excited at 361 nm) and compared to a standard under the same conditions. The addition of large amounts of virus to the standard had no effect on the 12-(9-anthroyl)-stearic acid fluorescence, showing that this measurement is independent of virus concentration. 12-(9-Anthroyl)-stearic acd in the viral particles accounted for 3 mole per cent or less of the viral lipid in the experiments reported in this paper. For comparative purposes within a single virus preparation, the amount of 12-(9-anthroyl)-stearic acid was determined directly from its emission intensity at 440 nm upon excitation at 361 nm.

RESULTS

A. Energy transfer from internal viral protein to 12-(9-anthroyl)-stearic acid

The results presented in this paper arose from the initial observation that, when 12-(9-anthroyl)-stearic acid is introduced into influenza virus a marked transfer of fluorescence is observed from viral protein to the probe. This is seen by comparing the emission spectra, excited at 275 nm, of labeled and unlabeled viral particles (Fig. 1). A typical protein spectrum is seen from the unlabeled viral particles (maximum at approx. 328 nm, Fig. 1a), while the labeled particles give a spectrum in which a second, smaller peak with a maximum at approx. 430 nm is observed

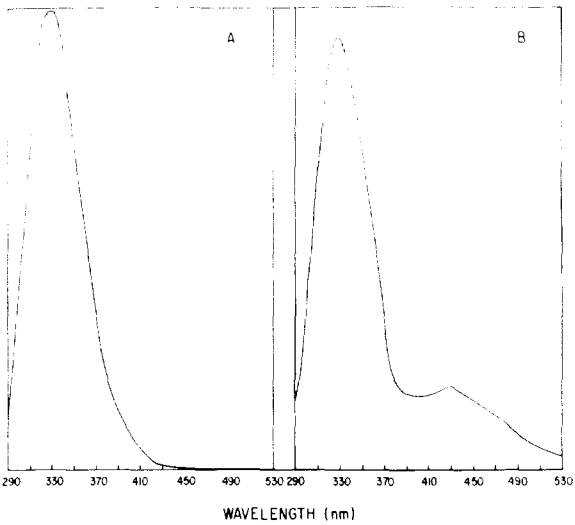

Fig. 1. Emission spectra of influenza virus particles in phosphate-buffered saline, excited at 275 nm. (*A*) Unlabeled. (*B*) Labeled with 12-(9-anthroyl)-stearic acid. Emission is in arbitrary units, and differs for A and B.

(Fig. 1b). The absorption (i.e. excitation) spectrum of 12-(9-anthroyl)-stearic acid is not measurably different in benzene and ethanol. It was therefore assumed that the excitation spectrum was independent of the dielectric constant of the environment over this range, and that this spectrum was an accurate reflection of the excitation spectrum in the viral particle. This excitation spectrum shows a marked minimum at 275–305 nm. Thus, the emission peak centered around 430 nm is not due to direct excitation of 12-(9-anthroyl)-stearic acid by the light source; correction for direct excitation of 12-(9-anthroyl)-stearic acid was generally less than 10% of the total height of the peak at 430 nm. In confirmation of this, no energy transfer was found under the same excitation conditions if 12-(9-anthroyl)-stearic acid was incorporated into human red cell membranes. Quite surprisingly, the binding of 12-(9-anthroyl)-stearic acid to fatty acid-free bovine serum albumin, while causing substantial quenching of protein fluorescence (as has previously been reported to result from fatty acid binding [20]) did not give rise to measurable energy transfer. The observation was thus specific for influenza virus, and could not be made in two other systems in which lipid–protein interactions occur.

That energy transfer was in fact the basis of the observation in influenza virus was further confirmed by disrupting the virus with sodium dodecylsulfate. In the disrupted virus the emission at 430 nm upon excitation at 275 nm fell below the detectable level, i.e. energy transfer disappeared. Thus, the transfer arose from a non-covalent relationship between the donor and the acceptor.

The identity of both the donor and acceptor species was established by fractionation of the virus particles. The characteristic absorption spectrum of 12-(9-anthroyl)-stearic acid was used to follow its distribution. The labeled virus preparation was dissolved in 1% sodium dodecylsulfate and extracted with *n*-butanol. Under these conditions the lipids are extracted into the butanol phase [21]. All the 12-(9-anthroyl)-

stearic acid was found in this phase, with none detectable in the aqueous phase or in the insoluble interphase which formed on extraction. After concentrating the butanol phase to dryness, it was applied to a thin-layer chromatogram. A single fluorescent spot was found, which migrated identically with authentic 12-(9-anthroyl)-stearic acid. Thus, no covalent modification of the probe occurs when it is introduced into the virus.

Experiments were carried out to exclude the possibility that any of the fluorescence arose from viral lipid or RNA. A butanol extract, similar to the one described above did not show a significant amount of the fluorescence characteristic of the unlabeled virus. Neither did the RNA, extracted by a phenol method [22]. Thus, the fluorescence of the viral particles arises from its protein rather than its lipid or RNA components.

We next tried to identify which of the viral proteins contained the donor fluorophore(s). Accordingly, the glycoprotein spikes which are attached to the outer surface of the viral bilayer were removed by proteolytic digestion. This procedure does not cause loss of any viral lipid [9], or any modification of the internal viral proteins [1].

The fluorescence in different samples was compared by normalization to counts of [^{3}H]uridine incorporated into RNA. While about half of the intrinsic viral fluorescence was lost upon spike removal, the amount of transfer lost was much less (about 30%). Thus, most of the energy transfer occurred between an internal viral protein and 12-(9-anthroyl)-stearic acid (Fig. 2).

There is persuasive inferential evidence that 12-(9-anthroyl)-stearic acid incorporated into influenza virus particles is localized in the viral bilayer. It has been demonstrated previously that spin-labeled stearic acid derivatives analogous to 12-(9-anthroyl)-stearic acid are incorporated into the viral bilayer [5,23], and are not measurably bound to viral proteins. 12-(-9-Anthroyl)-stearic acid is incorporated to about the same extent under the same condition. No 12-(9-anthroyl)-stearic acid

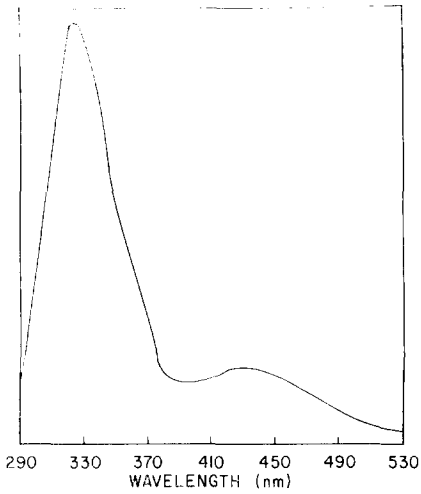

Fig. 2. Emission spectrum of 12-(9-anthroyl)-stearic acid-labeled influenza virus after spike removal, excited at 275 nm. Emission is in arbitrary units.

is lost from the particle upon spike removal, which would be expected since no lipid is lost during this process [9]. Nor can 12-(9-anthroyl)-stearic acid be bound to isolated viral nucleocapsid, even after prolonged incubation. On the other hand, 12-(9-anthroyl)-stearic acid is much more easily introduced into spikeless than intact particles (see Materials and Methods), an observation which is readily explained by the greater accessibility of the viral bilayer surface after spike removal. Finally, previous studies of 12-(9-anthroyl)-stearic acid in lipid bilayers using both fluorescence [18,24] and X-ray diffraction [25] have shown that 12-(9-anthroyl)-stearic acid is incorporated into bilayers, with the anthroyl fluorophore buried, as expected, in the hydrophobic interior of the structure. The emission maximum of 12-(9-anthroyl)-stearic acid incorporated into intact or spikeless particles is consistent with its localization in a highly non-polar environment. We thus conclude that 12-(9-anthroyl)-stearic acid is localized in the viral bilayer.

B. Intrinsic fluorescence of viral structures

In order to characterize further the fluorescent energy donor, the intrinsic fluorescence properties of several viral preparations were compared. The intact virus, excited at 275 nm showed an emission maximum at 328 nm. The spikeless particle showed a pronounced blue shift, with an emission maximum at 322 nm. The isolated nucleocapsid resembled the intact particle in having an emission maximum at 328 nm. These results suggested that tyrosine residues, which emit light at shorter wavelengths than tryptophan, may be responsible for a substantially greater part of the fluorescence of the M protein than of the spikes or the NP protein.

Observations which support this contention were made by separating the contributions of tyrosine and tryptophan fluorescence in the spikeless particle. This was done by measuring emission spectra when the particles were excited at 278 and 295 nm. The 295-nm spectrum arises essentially completely from tryptophan residues, while the 278-nm spectrum arises from both tyrosine and tryptophan

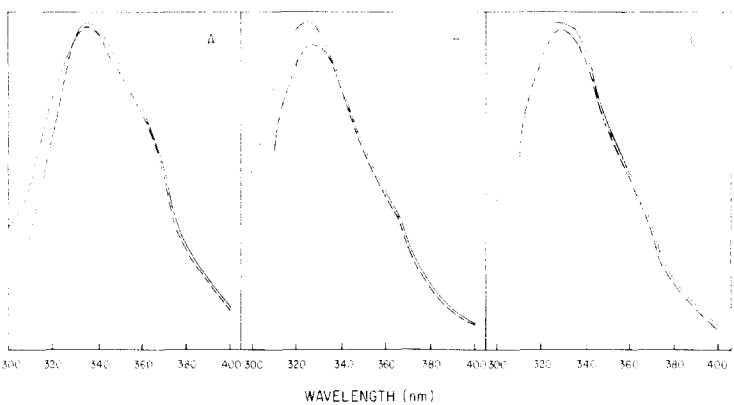

Fig. 3. Emission spectra, excited at 278 nm (——), 295 nm (– – – –), normalized to emission at 370 nm. (A) Bovine serum albumin, essentially fatty acid-free, in 0.008 M, phosphate, pH 7.4. (B) Spikeless influenza virus particles in phosphate-buffered saline. (C) Human red blood cell membranes in 0.008 M phosphate, pH 7.4. Emission is in arbitrary units and differs for A, B and C.

residues. Thus, if the two spectra are normalized at 370 nm (where all the emission arises from tryptophan at either exciting wavelength) the difference between the two represents emission from tyrosine residues [26]. The sensitivity of this technique was demonstrated using bovine serum albumin. This protein is typical of most proteins containing both tyrosine and tryptophan residues in that the overwhelming majority of its fluorescence emanates from the tryptophan residues, despite the fact that there are 10 times as many tyrosines in the molecule. A very small contribution from tyrosine fluorescence has been identified in the spectrum of this protein, however, [26,27] and is clearly seen by this technique (Fig. 3a).

In contrast, the spikeless virus particles showed a much higher proportion of their total fluorescence arising from tyrosine residues (Fig. 3b). That this was not a general property of membrane proteins was demonstrated by comparing the results with human red blood cell membranes (Fig. 3c). Again, a much smaller contribution from tyrosine residues was observed despite the fact that tyrosine residues are present in substantial amounts in red cell membrane proteins [28].

DISCUSSION

The results reported here demonstrate energy transfer between an internal viral protein and a fluorescent probe localized in the viral bilayer. There are two major internal proteins in the viral particle, the NP protein and the M protein [1,6,8]. The NP protein is part of a helical ribonucleoprotein structure. Such structures are readily isolated from disrupted virus particles and contain the NP protein and RNA [29,30]. Electron microscopy has demonstrated their helical structure, and they are believed to exist in this form in intact virus particles [31]. Even if such helical structures were in close apposition to the bilayer, only a very small number of the NP protein subunits could be sufficiently close to the bilayer to permit energy transfer. Thus, the donor in the energy transfer system we have described appears to be predominantly or exclusively located within the M protein.

It has been shown that the efficiency of energy transfer of this type depends upon the inverse sixth power of the distance between donor and acceptor [32–34]. This relationship has provided the basis for measurement of the distance between two fluorophores in several different proteins [35–37]. Such a distance might be measurable in the present case only if a number of conditions could be fulfilled: (1) a single donor species could be unambiguously identified; (2) introduction of 12-(9-anthroyl)-stearic acid did not alter the spatial relationship of the donor with the bilayer. Our evidence suggests that neither of these conditions are fulfilled. However, a maximum value for the donor–acceptor distance can be estimated.

Preliminary amino acid analysis of purified viral proteins, and tryptophan estimations based on acrylamide gels of virions doubly labeled with [^{14}C]leucine and [^{3}H]tryptophan showed that the M protein is quite poor in tryptophan, containing a single tryptophan residue per 26000 molecular weight (unpublished observation). The M protein thus provides only about one-third of the tryptophan residues in the spikeless particle while accounting for two-thirds of the total protein [1]. If, as our evidence suggests, most of the tyrosine fluorescence but only a proportional amount of the tryptophan fluorescence of the spikeless particle arises from the M protein, then the tyrosine fluorescence from the M protein is very large, and

may even predominate in the emission spectrum of this protein excited at 275 nm. Substantial energy transfer is still seen when the labeled spikeless particle is excited at 295 nm, suggesting that tryptophan is an energy donor in this system, but the possibility that one or more of the seven or eight tyrosine residues of the M protein may also serve as energy donors cannot be excluded.

It also appears that introducing 12-(9-anthroyl)-stearic acid into the bilayer affects the conformation of the M protein. We consistently observed that, despite the clear demonstration of energy transfer by the criterion of sensitized emission centered around 430 nm, a corresponding quenching of intrinsic fluorescence from intact or spikeless particles was not observed. On the contrary, an increase of fluorescence of about 10% was more commonly seen after introducing 12-(9-anthroyl)-stearic acid into the viral bilayer. This increase in quantum efficiency upon labeling the spikeless particle suggests that a conformational change is occurring in the donor protein.

The R_0 value (i.e. the distance between donor and acceptor at which transfer efficiency is 50%) was calculated [32] for this system using the following assumptions: (1) The donor emission spectrum is the same as the spectrum of the unlabeled spikeless particle; (2) The acceptor excitation spectrum is the same in the spikeless particle as in ethanol or benzene; (3) The donor quantum yield is 0.2; (4) The refractive index of the medium between the donor and acceptor is 1.40; (5) The orientation factor is 2/3, i.e. both donor and acceptor groups undergo rapid free rotation relative to one another. A value of 22.6 Å was obtained for R_0 under these assumptions. It should be noted that this value would be only about 10% smaller if a quantum yield of 0.1 were assumed. Since energy transfer cannot generally be demonstrated at a greater donor–acceptor distance than 1.7 R_0 [35], the distance between the donor moiety and the 12-(9-anthroyl)-stearic acid in the bilayer does not exceed 38.5 Å.

The anthroyl group is sufficiently large that a precise localization within the hydrophobic region of the bilayer is not possible. To a first approximation, therefore, the anthroyl group can be considered to occupy an average position at the center of the bilayer, or 28 Å from the surface if the bilayer is 56 Å thick [38]. This means that the portion of the protein containing the donor residue(s) cannot be more than approx. 11 Å external to the bilayer surface. However, the finding that introducing 12-(9-anthroyl)-stearic acid into the spikeless particle increases the quantum yield of an internal protein suggests that the M protein is closer to the bilayer than this maximum value, and may actually penetrate the bilayer. The introduction of 12-(9-anthroyl)-stearic acid in high concentrations into a synthetic bilayer has been shown to alter its electron density profile quite considerably [25].

The reason for the apparently high quantum efficiency of the M protein tyrosine residues is not known, but one interesting possibility is that the tyrosine residues are in a helical segment of the molecule interacting with lipid. High quantum efficiencies for tyrosine have been observed from proteins dissolved in a helicogenic solvent [26] or detergent [39]. Further studies are in progress to test this hypothesis and to elucidate more precisely the nature of the bilayer–protein interaction.

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