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Photolabile and Paramagnetic Reagents for the Investigation of Transmembrane Signaling Events

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To investigate the dynamics of membrane processes that may be integral components of specific transmembrane signaling events we have synthesized several novel paramagnetic probes and their photoreactive counterparts. The structure of these probes was designed to (1) restrict "flipping" across the membrane bilayer; (2) contain paramagnetic or photoreactive moieties that could be placed at specific depths within the bilayer; (3) provide information about membrane structure as well as dynamics of protein movement; and (4) in the case of the photoreactive probes, be of high specific radioactivity.

The molecules described in this paper consist of amino acid, dipeptide, or carbohydrate groups attached to arylazide- or nitroxide-bearing fatty acids. The synthesis and initial characterization of these membrane probes is described.

Key words: photoreactive probes, ESR spin labels, membranes

Mitogens, toxins, antigens, and ionophores are all groups of cell effectors whose common mode of action is thought to involve transmembrane signaling events. Work currently in progress in this laboratory is directed at the elucidation of the temporal sequences of some of these signaling events. Several investigators [1–4] have begun to explore the possibility of using photoreactive probes to monitor the structure and dynamics of membrane components. Our specific goal has been to prepare photoreactive derivatives of fatty acids that will insert spontaneously into the surface monolayer of sealed membrane systems. By substituting the fatty acid chain at various positions with a photoreactive azide or arylazide moiety and coupling this substituted chain to a polar restraining group we are able to detect and localize proteins that reside within or penetrate a specific zone

ABBREVIATIONS: NDV – Newcastle disease virus; TLC – thin-layer chromatography; ESR – electron spin resonance; 12NS – 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy; 12NS-GA, 12NS-GG, 12NS-T, 12NS-GT – glucosamine, glycylglycine, tyrosine & glycyltyrosine derivatives of 12NS respectively; 12-APS – 12-(4-azido-2-nitrophenoxy)-stearic acid; 12-APS-GA, 12-APS-GG, 12-APS-GT – glucosamine, glycylglycine and glycyltyrosine derivatives of 12-APS.

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of the lipid bilayer. One such study in which 12-APS-GA was used to label cholera toxin proteins as they penetrated a membrane bilayer will be described in a forthcoming paper. In this report we describe several photoreactive probes, together with the synthesis of their structural counterparts containing paramagnetic centers. Use of these latter molecules has enabled us to make valid predictions regarding the localization of the photoreactive probes within the plane of the membrane bilayer. Since the sensitivity of detection of proteins labeled after activation of the probes is directly proportional to the specific radioactivity of the probe used, we describe a number of derivatives of photoreactive and paramagnetic probes that are capable of being derivatized with radioactive iodine. Investigations to date suggest that a dipeptide linkage is sufficiently polar to restrict a fatty acid bearing this group from "flipping" between the inner and outer monolayers of intact membrane systems. Evidence to support this observation is described.

MATERIALS AND METHODS

Synthetic Procedures

The hydrocarbon spin label 12NS was purchased from Syva, Palo Alto, California, and was purified by TLC before use. Solvents were redistilled as required. Carrier-free $Na^{125}I$ was purchased from Amersham-Searle. 12NS-GA was synthesized as previously described [5]. 12NS-GG was synthesized using the succinimide activated ester procedure of Lapidot et al [6]. 12NS (10 mg) was dissolved in dry ethyl acetate (40 μl) and added to a solution of N-hydroxysuccinimide (3 mg) in dry ethyl acetate (100 μl). To this mixture was added a solution of dicyclohexylcarbodiimide (5.4 mg) in dry ethyl acetate (10 μl), and the reaction mixture was stirred at 15°C for 10 h. Dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness under a stream of nitrogen. The dried residue was redissolved in a minimal volume of chloroform/methanol (2:1) and purified by TLC on silica gel G plates using a solvent system of benzene/ethyl acetate/petroleum ether/acetic acid (100:14:10:1). The product moved as a discrete band (Rf: 0.44) and was identified by paramagnetic absorption and by reaction to hydroxylamine and ferric chloride [7]. This product was dissolved in tetrahydrofuran (2 ml) and added to a solution of glycylglycine (10 mg) and sodium bicarbonate (2 mg) in water (1 ml). The mixture was stirred overnight at 15°C, acidified with 1 N HCl, and diluted with cold water (10 ml). Organic solvent was removed by distillation under diminished pressure, the product being retrieved from this aqueous suspension by filtration through Whatman No. 2 paper and purified by TLC on silica gel G plates using a developing solvent system consisting of chloroform/methanol/acetic acid (85:5:2). The product moved as a diffuse band (Rf: 0.13), and was identified by its response to o-tolidine [8]. The yield was 67% (determined by ESR absorption).

12NS-T and 12NS-GT were synthesized by a procedure similar to the one described for 12NS-GG (above) with either tyrosine methyl ester or glycyltyrosine replacing glycylglycine, and with a reaction solvent of dry dimethylformamide. The reaction products were purified by TLC (as above) and identified by their responses to o-tolidine [8], by paramagnetic spectroscopy, and by the presence of a phenolic absorption band in the ultraviolet spectrum.

12-APS was prepared according to the method of Chakrabarti and Khorana [1] with a modified reaction solvent consisting of diethyl ether which had been redistilled from sodium benzophenone.

12-APS-GA was prepared as previously described [4]. 12-APS-GT was synthesized essentially as described for 12NS-GT, using the succinimide ester of 12-APS. The product was purified by TLC with a solvent system of benzene/ethyl acetate/petroleum ether/ acetic acid (100:14:10:1). The product moved as a sharp band (Rf: 0.21) and was identified by its response to o-tolidine and by the presence of an absorption peak at 360 nm in the ultraviolet spectrum (nitrophenylazide).

Iodinated probes based on the phenolic derivatives of 12NS and 12-APS were synthesized using Chloramine-T to liberate ICl from carrier-free and carrier diluted $Na^{125}I$, respectively, as described by Brown and Reith [9]. This method permits iodination to be effected on a very small scale and with only brief exposure of the nitroxide moiety to oxidizing conditions. It was observed that even under these mild conditions there was a 40% decrease in spin intensity following iodination. The products of iodination were purified by paper chromatography and identified by autoradiography. Using a developing system consisting of n-butanol/2 M acetic acid (1:1 v/v), di-iodo-12NS-T-Me moved as a sharp band (Rf: 0.97). Under the conditions described above, 12-APS-GT could be iodinated to high specific radioactivity with no concomitant loss of active azide (as determined by infrared spectroscopy).

Electron Spin Resonance Spectroscopy

ESR spectra were obtained on a Varian E104 spectrometer operating at X band and equipped with a Varian variable-temperature accessory. Temperature was monitored ($\pm$ 0.1°C) with a copper-constantan thermocouple attached to a Doric digital thermocouple indicator. Each spectrum represents a 4-min scan over a 100 g range (power = 20 mW). Multiple scans were taken at each temperature from approximately 4° to 45°C. Deviations from the mean were within the circumference of the data point. ESR running time was about 4 h/sample.

A 12 μl aliquot of a 12NS-GG stock solution (10^{-3}M) was placed in a glass sample tube and dried under nitrogen. To this tube was added 60 μl of a suspension of NDV (132 μg as protein) in Tris-saline buffer (pH 7.4) containing 1 mM ethylenediamine tetra-acetic acid. The strain of virus used was HP16 derived from embryonated chicken eggs. The tube was rotated at room temperature for 15 min and then the contents were transferred to a capillary tube and spun at 6,000 X g for 8 h at 5°C. ESR spectra were obtained at temperature intervals of 1°C, and the parameter h_H/h_P (shown in Fig 2) plotted against 1/°K. The discontinuities in the Arrhenius plots were located by computer analysis [5].

RESULTS AND DISCUSSION

Numerous investigators have begun to explore the possibility of using photoreactive molecules to monitor the structure and dynamics of membrane components [1–4]. Typically, these probes consist of a stable, relatively inert molecule which may be inserted

into the membrane, and which can be converted subsequently into a highly chemically reactive species (eg, nitrene or carbene) upon irradiation with light of an appropriate wavelength. After the generation of this active species, the probe becomes covalently attached to membrane components with which it is in close contact. In the case of the nitrene-containing probes, the specificity of the linking reaction appears to be very loose, and it is unlikely that the probe discriminates between the potential targets on the basis of any chemical characteristics.

Our approach to the study of transmembrane signaling has been to develop paramagnetic and photoreactive probes for use in analyzing the disposition, dynamics, and organization of membrane structure at specific depths within the membrane bilayer. We have begun to synthesize a series of complementary probes bearing either a nitroxide (paramagnetic) or arylazido (photoreactive) group at a defined position along a fatty acid chain. Since there is a spin-label counterpart for each photoreactive probe (Fig 1), we have been able to collect data that enable us to make valid predictions of the location, mobility, and behavior of both classes of probe. For example, we have synthesized 12NS-GG and 12-APS-GG. Evaluation of the behavior of the spin-label 12NS-GG in the membrane bilayer of NDV particles demonstrates that its thermotropic behavior is capable of revealing two characteristic discontinuities in the Arrhenius plots (Fig 2) of spectral data based on the proportion and mobility of the probe in membrane hydrocarbon versus aqueous milieux. We have previously shown that the discontinuities apparent at approximately 14° and 30° reflect characteristic features of the outer monolayer of sealed right-side-out membrane systems [5].

Our spin-label studies with 12NS-GA in right-side-out (RSO) and inside-out (ISO) sealed vesicles prepared from human erythrocyte ghosts show that if a preparation of ISO vesicles is contaminated to the extent of 15% with RSO vesicles, then four discontinuities are detected rather than just the two discontinuities characteristic of ISO vesicles. We therefore predict that if the 12NS-GG described here is flipping across the bilayer of the viral envelope, then the amount that flips is less than 15% during the 4–6 h needed to scan the 5°–45°C temperature range depicted in Figure 2.

In our present work, we use photoreactive fatty acid probes coupled to ^{14}C-glucosamine (eg, 12APS-GA) since our experimental approach requires that we be able to identify proteins that become covalently attached to the probe during photoactivation. Since labeling of membrane proteins after irradiation of samples containing these probes has shown between 0.1% and 10% efficiency, we have had to resort to oxidative combus-

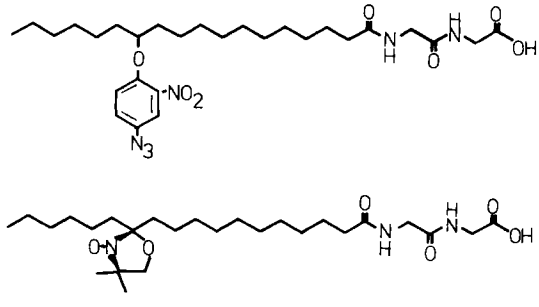


Fig 1. Typical photoreactive probe, 12-APS-GG (top) and paramagnetic probe, 12NS-GG (bottom) described in this paper. Chain substitutions may be made at positions other than position 12.

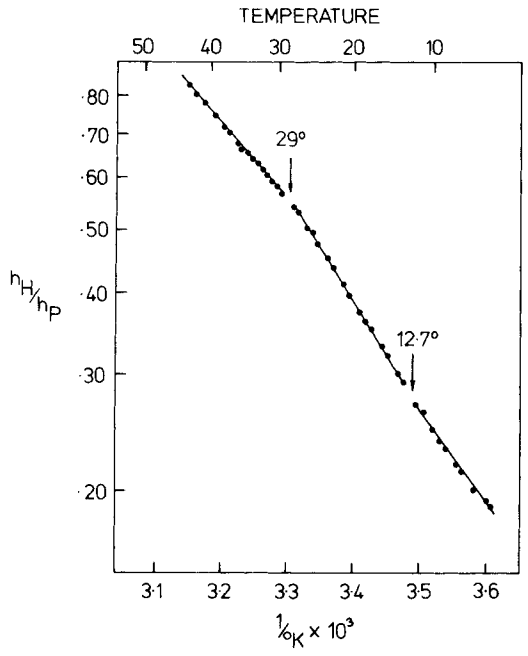
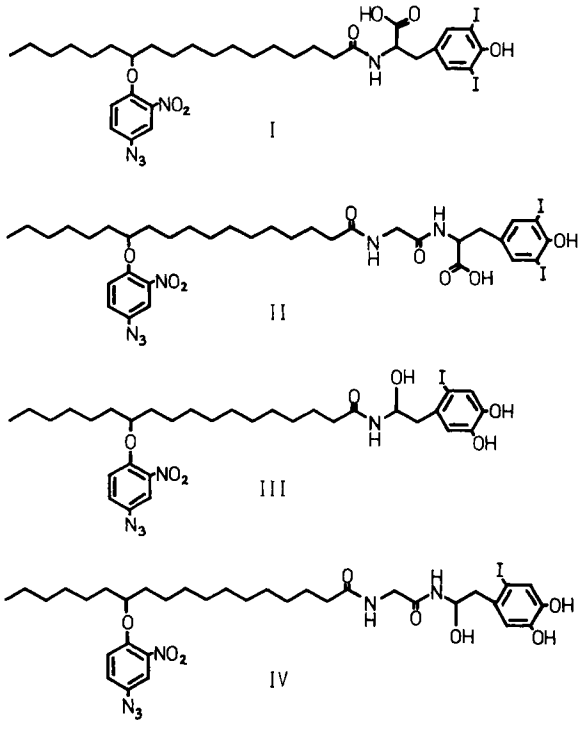


Fig 2. Arrhenius plot derived from ESR spectra of 12NS-GG in an intact membrane-enveloped viral preparation. The two discontinuities evident in the plot result from changes occurring with temperature in the outer monolayer of the lipid bilayer enveloping the viral particles; disrupted preparations show four discontinuities in similar plots (data not shown for freeze-thawed NDV suspensions). The results indicate that the paramagnetic probe is restricted in its distribution to a single membrane monolayer of sealed membrane systems.

tion of gel slices, followed by scintillation counting of the resultant $^{14}CQ_2$ to detect the low levels of radioactivity associated with each protein band within a reasonably short time. Since incorporation of the radioisotope ^{125}I into the structure of the probes would facilitate the rapid exposure of autoradiographs made from these gels, we have developed methods for the preparation of a number of derivatives of the membrane probes which lend themselves to iodination with $Na^{125}I$ under simple experimental conditions (Fig 3). For example, we have synthesized a methyl ester derivative of 12NS-T that contains ^{125}I as a meta ring substituent. This molecule was shown by ESR spectroscopy to be much more hydrophobic than 12NS-GG or 12NS-GA, since there was no indication of a hydrophilic component in the ESR spectrum of spin-labeled NDV even after extensive dilution (Fig 4). In this respect, 12NS-$T^{125}I$ may be more similar to the naphthalene-based probes described by Gitler and Klip [3]. Since it was not possible to obtain the ESR partitioning parameter h_H/h_P used in graphing the Arrhenius plots which enable us to locate the characteristic temperatures of a given membrane sample, we plotted the low-field hydrocarbon peak height (h_{+1}) versus 1/°K obtained from NDV spin labeled with 12NS-$T^{125}I$ and observed two clear breaks at 14°C and 20°C, together with two less obvious, but significant, breaks at 30°C and at 38°C. On this basis we postulate that the probe shows no discriminating specificity between the inner and outer monolayer components of the membrane bilayer of NDV. It is still possible that the photoreactive counterpoint of the probe will remain restricted to a single monolayer for the 1-min period employed for photoactivation. Since the 12NS-



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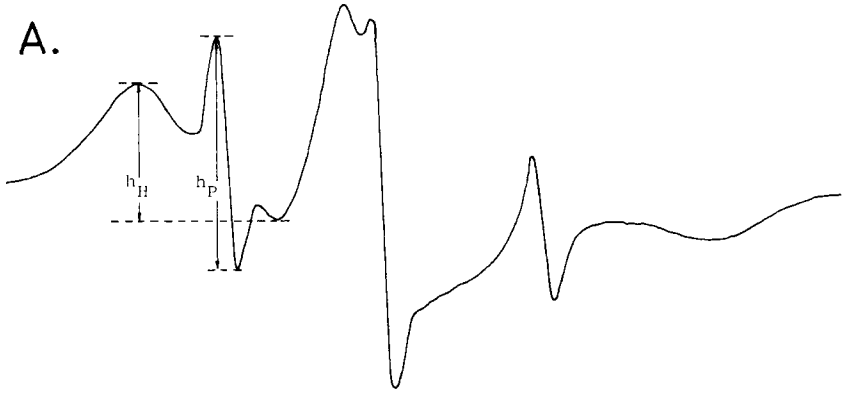

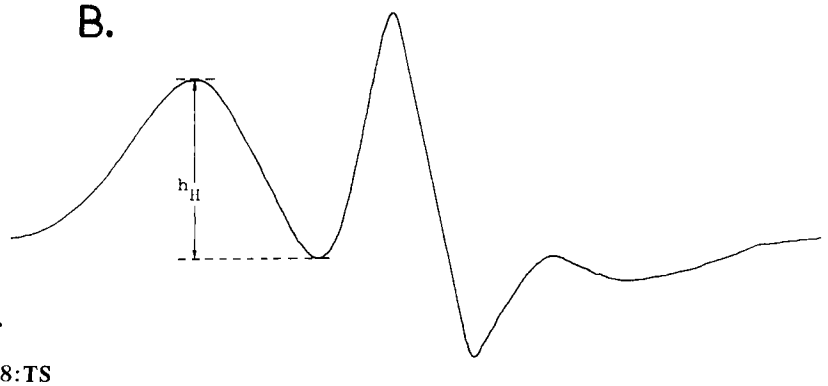

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GG showed appreciable discrimination between the two monolayers of the viral membrane, being restricted to the outer compartment, it was clear that other dipeptides such as 12NS-GT and 12-APS-GT would offer the property of being surface-restricted, together with the potential for being iodinated to a high specific radioactivity. Infrared spectral analysis of 4-fluoro-3-nitrophenyl azide showed that under the experimental conditions used to catalyze iodination of 12NS-T with Chloramine-T, no decrease in absorbance at 2100 cm^{-1} (N_3 asymmetric stretch) was observed, indicating that the iodination procedure has no effect on the integrity of the arylazide substituent of the photoreactive probes 12-APS-T and 12-APS-GT.

A direct assessment of the spatial positioning of 12-APS-GT, 12-APS-GA, and 12-APS-T within the membrane is being conducted using sealed lipid vesicles incorporating the coat protein of M13 coliphage. In this experimental system the precise symmetric distribution of the 5,000 dalton protein across the lipid bilayer is known [10], and activation of the photoreactive probes inserted into these vesicles, followed by selective cleavage of protein and identification of the fragments showing radioactivity, permits precise identification of the location of the probe within the membrane. This work will be described in a separate communication.

More polar characteristics have been incorporated into some fatty acid probes with the replacement of tyrosine with norepinephrine (see Fig 3) or dopamine (not shown). These probes can also be iodinated [11], although in this case the approach is more complicated than the simple methods utilizing oxidizing agents such as Chloramine-T. Probes based on the structures described have been used in our laboratory to identify the location of a variety of integral membrane proteins [4] and also to illustrate the penetration of proteins (eg, cholera toxin) into the lipid matrix of a viral membrane envelope.

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Fig 3. Structures of photoreactive probes discussed in this paper. 12-APS-T (I), 12-APS-GT, (II), 12-APS-norepinephrine (III), and 12-APS-glycylnorepinephrine (III), and 12-APS-glycylnorepinephrine (IV). The varying amphipathic properties of these probes are a function of the hydrophobic hydrocarbon chain, which becomes inserted into the lipid matrix of a membrane, and the polar peptide linkages and ring substituents that remain exposed to the aqueous environment of the supporting medium.

Fig 4. A: ESR spectrum of 12NS-GA spin label (10^{-4}M) in a suspension of NDV particles (150 μg as protein in 10 μl PBS) at 22°C. B: ESR spectrum of 12NS-T^{125}I (10^{-4}M) in a suspension of NDV particles (150 μg as protein in 10 μl PBS) at 22°C. The lower spectrum lacks three of the absorption peaks present in the upper spectrum. The three missing peaks are those attributable to absorptions from spin label in an aqueous environment. The broad absorption peaks present in both spectra result from spin label resident in a hydrophobic (membranous) domain. PBS: 5 mM phosphate-buffered saline.

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