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Photoreactive labeling of M13 coat protein in model membranes by use of a glycolipid probe

(photogenerated nitrene/membrane mapping/membrane restriction)

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ABSTRACT Coliphage M13 coat protein in synthetic bilayer membranes was labeled by use of 12-(4-azido-2-nitrophenoxy)stearoyl[1-^{14}C]glucosamine, a photoreactive glycolipid probe that spontaneously inserts into membranes. In this model system, the probe preferentially labeled the proteins over the lipids. Experiments designed to test the probe's restriction to integral membrane proteins revealed that extrinsic proteins as well as external peptide fragments of integral membrane proteins were not accessible to the photogenerated nitrene on the fatty acid chain. Only integral membrane peptides were labeled by the membrane-bound probe. These results indicate that protein labeling can be effected specifically from within the hydrocarbon milieu of a model membrane system.

Recently, we described the synthesis of several photoreactive probes designed for the high-resolution mapping of membrane proteins (1, 2). In addition, we demonstrated the ability of one of these probes, 12-(4-azido-2-nitrophenoxy)stearoyl[1-^{14}C]-glucosamine, designated 12-APS-GlcN, to specifically label the envelope proteins of Newcastle disease virus (3) and to follow the transmembrane dynamics involved in cholera toxin activity (4). These studies prompted further investigation into the nature of the interaction of the probe with membrane proteins in an effort to define the region of the probe's reactivity and to verify the probe's restriction to integral membrane proteins. Toward this goal, we have constructed phospholipid vesicles containing the 5000-dalton coat protein of the coliphage M13, which orients itself unidirectionally across the membrane bilayer under appropriate conditions (5). Because the amino acid sequence as well as the hydrophobic region of this protein are known, it should be possible to determine the position of the probe in the membrane by using the hydrophobic amino acids of the protein as a gauge of membrane depth.

In this report, we show that M13 coat protein incorporated into dimyristoyllecithin (DML) vesicles is indeed labeled by the photoreactive probe (12-APS-GlcN) upon irradiation. Furthermore, evidence is presented that indicates that the probe does not label externally added coat protein, nor does it label amino acid residues of the protein that are shown to be outside of the phospholipid bilayer by trypsin cleavage experiments.

MATERIALS AND METHODS

DML was obtained from Sigma or Calbiochem; trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) was purchased from Worthington. Both were used without further purification.

Preparation of M13 Coat Protein. M13 virus was grown and isolated as described by Wickner (6) with slight modifications. *Escherichia coli* strains K-37 and LA-9 were used for growing unlabeled and [^{3}H]proline-labeled virus, respectively. The bacteria were cultured either in YT medium or in M63 minimal medium (7) to optimize the uptake of label. The M13 coat protein was extracted from whole virus by established procedures (8) and stored at either +4°C or −20°C in 50 mM Tris buffer (pH 7.5).

Preparation of Coat Protein Vesicles. The M13 coat protein vesicles were prepared by using DML according to a cholate dilution protocol established by Wickner (5). Briefly, 0.5 mg of coat protein in 1% sodium cholate was added to 0.2 ml of a 2% sonicated DML solution. The resulting solution was incubated for 0.5 hr at 20°C followed by the addition of 15 ml of 0.1 M potassium phosphate buffer (pH 7.4) at 20°C. Large vesicles (≈1 μm in diameter) were formed during overnight incubation at 20°C; 0.02% sodium azide was present to prevent bacterial growth. The vesicles were collected by centrifugation at 44,000 × *g* for 0.5 hr at 4°C. Any free, unincorporated protein was removed from the vesicle sample by pelleting the resuspended vesicles onto a 25% sucrose pad. The free protein penetrated the sucrose layer; the vesicles were pelleted on top and could be easily removed with a pasteur pipette. Sucrose was removed by diluting the vesicle band in 15 ml of buffer and repelleting the vesicles at 44,000 × *g* for 0.5 hr. The vesicle pellet was resuspended in buffer (5 μg of protein and 95–200 μg of DML in 0.6 ml) and used directly for photoreactive labeling experiments described below.

For the trypsin cleavage experiments, the specific activity of [^{3}H]proline-labeled coat protein in vesicles was 14 Ci/mol (1 Ci = 3.7×10^{10} becquerels).

Preparation of DML Vesicles. DML vesicles were prepared by cholate dilution as above, omitting protein. These vesicles were collected by centrifugation at 44,000 × *g* for 0.5 hr at 4°C and were approximately 3000 Å in diameter.

Photoactivation Protocol. The synthesis of 12-APS-GlcN has been described (1, 2); it has a specific activity of 50 Ci/mol. Usually $0.4\text{–}1.1 \times 10^{-2}$ μCi of probe was used per μg of coat protein. In general, probe in ethanol was dried and resuspended in a few microliters of ethanol. The ethanol never exceeded 1% of the volume of the final vesicle suspension. Appropriately diluted vesicle suspensions were then added to the probe and the samples were incubated for 15 min at 37°C. The samples were divided into 0.6-ml portions (typically containing 5–10 μg of protein and 95–200 μg of DML) and irradiated for 2–5 min at 360 nm with a mineral lamp (UV Products, San Gabriel, CA). Dark controls consisted of unirradiated samples. The vesicles were pelleted at 44,000 × *g* in a Beckman SW50.1 rotor equipped with 0.7-ml adaptors for 0.5 hr at 4°C and resuspended in a small amount of sodium dodecyl sulfate (NaDodSO$_4$) sample buffer for analysis by NaDodSO$_4$/polyacrylamide gel electrophoresis or thin-layer chromatography.

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Abbreviations: 12-APS-GlcN, 12-(4-azido-2-nitrophenoxy)-stearoyl[1-^{14}C]glucosamine; DML, dimyristoyllecithin; NaDodSO$_4$, sodium dodecyl sulfate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

In experiments involving trypsin treatment of the vesicles, unbound probe was removed by washing the vesicles several times. All of these procedures were carried out under red safety lights.

Trypsin Digestion of Vesicle-Bound M13 Coat Protein. Twenty-five microliters of 0.5% TPCK-treated trypsin was added to each 0.6-ml aliquot of irradiated or unirradiated vesicles containing approximately 38 μg of [^{3}H]proline-labeled coat protein. The samples were incubated for 1 hr at 37°C, then centrifuged at 115,000 × *g* for 0.5 hr at 4°C. The pellets were resuspended in 25 μl of 0.1 M phosphate buffer (pH 7.4) and spotted on 500-μm silica gel-G thin-layer chromatography plates (20 × 20 cm) for separation of the labeled components. The supernatants were removed. Radioactivity was either measured directly or the supernatants were dried, resuspended in 25–50 μl of buffer, and applied to thin-layer plates in order to separate free probe from any ^{14}C-labeled peptide fragments. Controls for these experiments were (*i*) unirradiated and trypsinized, (*ii*) irradiated and untrypsinized, and (*iii*) unirradiated and untrypsinized samples.

Thin-Layer Chromatography. The plates containing pellet fractions were developed in $CHCl_3$/MeOH/HOAc/H_2O, 50:25:8:4 (vol/vol), whereas those containing supernatants were run in $CHCl_3$/MeOH/NH_4OH, 65:35:5 (vol/vol). In both cases, protein and peptide fragments remained at the origin and were well separated from lipids and free probe. The thin-layer plates were exposed to no-screen x-ray film or scraped into Triton/toluene scintillation fluid for measurement of radioactivity on a Beckman model LS-100c liquid scintillation counter.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Slab-gel electrophoresis was carried out in the dark on 10/15% polyacrylamide discontinuous (10 ml of each) or gradient gels with a 2.5% acrylamide stacking gel and a 1% $NaDodSO_4$/Tris/glycine running buffer. The samples were boiled for 2 min in reducing sample buffer before application. After electrophoresis, gels were stained with Coomassie blue and prepared for fluorography by established procedures (9, 10).

RESULTS AND DISCUSSION

Fig. 1 shows the results of an experiment in which a suspension of M13 vesicles containing the photoreactive probe, 12-APS-GlcN, was irradiated for 5 min. The band corresponding to the M13 coat protein on an autoradiogram of a slab gel after electrophoresis is radioactively labeled in the irradiated sample (lane B), whereas the unirradiated control shows no corresponding band (lane A). The large radioactive spot just below the protein band represents radiolabeled lipid and free probe in the irradiated sample and simply free probe in the control. The radioactive material at the top of the gel in the irradiated sample (lane B) is probably aggregated M13 coat protein and not material (protein) that has been crosslinked by irradiation; none of this radioactive material is seen at the top of Fig. 3, in which lanes B and C both contain irradiated protein samples. M13 coat protein is notorious for remaining at the top of $NaDodSO_4$/polyacrylamide gels, especially when more than 3–4 μg of protein is applied. We therefore attribute the material at the top of the gel in Fig. 1 to aggregated protein. We have also noticed that there is usually less of this aggregation when the M13 coat protein is added from the outside of the vesicles, perhaps because there is less competition between $NaDodSO_4$ and fatty acid chains for coat protein in this situation. We have no cause to believe that protein–protein crosslinking is effected by irradiation at 366 nm. A more effective separation of the protein, lipid, and probe is accomplished by thin-layer chromatography. In the autoradiogram shown in Fig. 2, the labeled protein remains at the origin in the same pattern as applied and is well separated from the labeled lipid and free probe. As seen in Table 1, there is more than a 10-fold increase in the [^{14}C]-probe-associated counts in the protein spot of the irradiated sample over those in the control. This demonstrates that the photoreactive probe is indeed capable of labeling proteins in model membrane systems at least as efficiently as those in natural membranes (1).

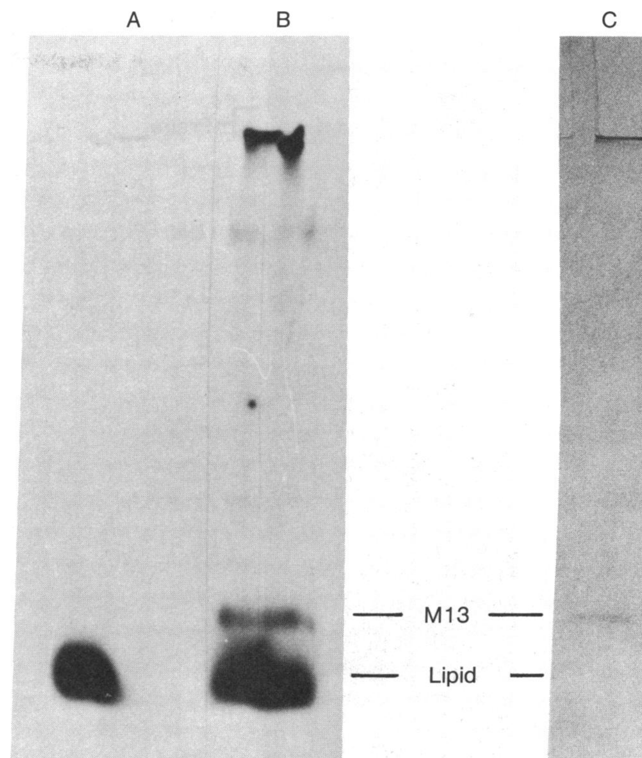

FIG. 1. Protein-stained $NaDodSO_4$/polyacrylamide gel and autoradiogram of M13 coat protein-containing vesicles before (lane A) and after (lane B) irradiation in the presence of 12-APS-GlcN. Lane C, stained M13 coat protein. Before being pelleted, each vesicle sample contained 5 μg of protein, 46 μg of lipid, and 15,000 cpm of probe. The $NaDodSO_4$/polyacrylamide gel consisted of 10% acrylamide (top half) and 15% acrylamide (bottom half).

Interestingly, the radioactivity associated with the protein is considerably higher than expected from the molar ratio of protein to lipid used in this experiment. In fact, for a model membrane containing only 0.4 mol % protein, the protein-associated ^{14}C radioactivity is about 16% of the lipid-associated radioactivity (see Table 1). This represents a 40-fold increase in efficiency on a mole-to-mole basis. Such a discrepancy between the amount of protein labeled and the amount of protein present may indicate that the reactive species of the probe is a short-lived electrophilic intermediate such as azacycloheptatetraene (11). This ring expansion product represents up to 95% of the photoproducts of phenylazide in a solid argon matrix (O. L. Chapman, personal communication). If this intermediate comprises a similar proportion of the reactive products of nitrophenylazide, we would expect a 20-fold increase in the efficiency of labeling of nucleophilic compounds in contrast to other types of compounds on a mole-to-mole basis. Our results are compatible with this expectation, and consequently we propose a reaction pathway dominated by an azacycloheptatetraene intermediate with nonspecific labeling dependent on the level of singlet-to-triplet state interconversion of photogenerated nitrenes in the membrane matrix.

To further characterize the probe's interaction with a model membrane, we undertook experiments that were designed to

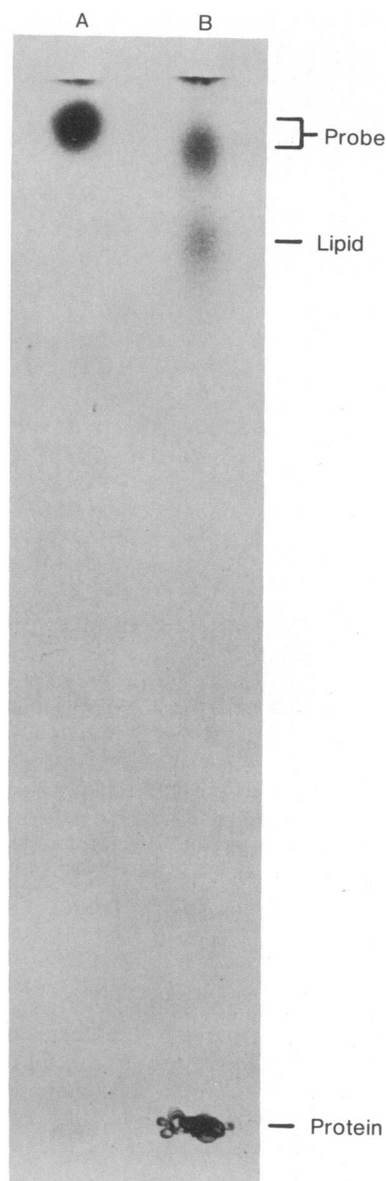

FIG. 2. Autoradiogram of a thin-layer plate demonstrating the separation of 12-APS-GlcN-labeled vesicle components: control (lane A) and irradiated (lane B) samples. Before pelleting, each vesicle sample contained 25 μg of protein, 312 μg of lipid, and 54,000 cpm of probe.

monitor the location of the photoreactive group in a vesicle suspension. We asked the following question. Can the probe label proteins or parts of proteins that are external to the hydrophobic portion of the membrane? In Fig. 3, we show the results of experiments in which M13 coat protein was either incorporated into lipid bilayer vesicles or added externally to

Table 1. 12-APS-[^{14}C]GlcN counts associated with the protein and lipid fractions of control and irradiated M13 coat protein vesicles*

Sample	Protein-associated cpm		Lipid-associated cpm	
	Control	Irradiated	Control	Irradiated
A	1380	20,361	0	107,644
B	1141	15,893	0	107,441

* Before samples were pelleted, they contained 38 μg of protein, 950 μg of lipid, and 450,000 cpm of probe. The lipid fraction was separated from free probe before radioactivity was measured (see Fig. 2).

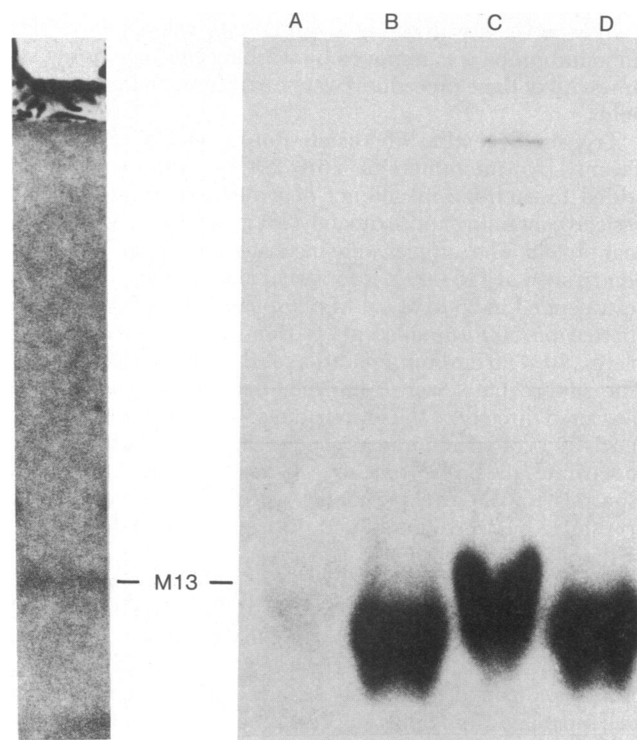

FIG. 3. Autoradiogram of a linear 10–15% gradient acrylamide gel containing: lane A, unirradiated DML vesicles with incorporated M13 coat protein; lane B, irradiated DML vesicles with external M13 coat protein; lane C, irradiated DML vesicles with incorporated M13 coat protein; and lane D, irradiated DML vesicles. The stained-gel lane on the left corresponds to sample B above. Samples A and C contained protein in a similar position. Before being pelleted, each sample contained 100 μg of lipid and 54,000 cpm of probe; samples with M13 coat protein contained 5 μg of protein.

a suspension of preformed DML vesicles. After adding the samples to tubes containing 12-APS-GlcN (dry), incubating for 15 min at 23°C, and irradiating, we found that the incorporated coat protein was labeled but the externally added protein was not. Although radiolabeled M13 coat protein in Fig. 3, lane C, was not completely resolved from labeled lipids, lanes A, B, and C contained Coomassie blue-stained protein in the position of the stained M13 coat protein shown on the left. The observation that only lane C contained radiolabel in this region proves that under conditions where discernible levels of radiolabel are achieved with M13 coat protein as an integral membrane protein, none is achieved with exogenously added M13 coat protein. This conclusion has since been confirmed by using thin-layer chromatography (see *Materials and Methods* and Fig. 2) to effect complete separation of protein and lipids (data not shown).

With respect to Figs. 1 and 3, we would like to point out that the probe usually runs ahead of the lipids in $NaDodSO_4$/polyacrylamide gel electrophoresis (3) and, therefore, the unirradiated sample shown in Fig. 3, lane A, contains only low levels of radioactivity in the lipid region. In the gel system shown in Fig. 1, the unirradiated probe did not migrate as rapidly and some can be seen in the lipid region of lane A. The probe is most likely running as $NaDodSO_4$/mixed micelles.

In our vesicle system, the M13 coat protein is oriented across the lipid bilayer in such a manner that enzymatic cleavage by trypsin or chymotrypsin removes the [^{3}H]proline label at position 6 in the sequence (5). Consequently, after vesicles containing [^{3}H]proline-labeled M13 coat protein and ^{14}C-labeled photoreactive probe were irradiated and washed, they were

Table 2. [14C]Probe- and [3H]proline-associated radioactivity in the supernatant and pellet fractions of trypsin-treated M13 coat protein vesicles*

Sample	^{3}H in total supernatant, cpm	^{3}H in vesicle protein (pellet), cpm	% ^{3}H in supernatant	^{14}C in total supernatant, cpm	^{14}C in vesicle protein (pellet), cpm	% ^{14}C in supernatant
Unirradiated + trypsin	.13,492	4,134	76.0	574	1,447	28.0
Unirradiated	104	14,370	0.7	196	1,380	12.0
Net release by trypsin			75.3			16.0
Irradiated + trypsin	11,356	8,586	57.0	2389	17,421	12.0
Irradiated	301	17,481	1.7	1764	20,361	8.0
Net release by trypsin			55.3			4.0

* The ^{14}C counts in the supernatant represent free probe as well as probe-complexed peptide because the radioactivity of the supernatant was measured directly in this experiment. The composition of the samples is the same as given in Table 1. Radioactivity in vesicle protein (pellet) refers to radiolabel associated with the protein component of the vesicles after separation from lipids by thin-layer chromatography.

treated with trypsin to determine the proportion of the radioactivity associated with the released NH_2-terminal octapeptide. The assay for the location of the photoreactive probe assumed that if the probe reacted randomly with the 50 amino acids in the sequence of M13 coat protein, 8/50 or 16% of the protein-bound probe would be released upon complete proteolysis. Table 2 shows the ^{3}H and ^{14}C levels in the total supernatant fraction and in the protein portion of the pellet (vesicle fraction). The data demonstrate that 55% proteolysis of the irradiated sample, as measured by [^{3}H]proline release, effected the net release of 4% of the ^{14}C counts into the supernatant. This value is lower than the 8.8% predicted on the basis of random labeling (0.55 × 16%). Possible sources of ^{14}C in unfractionated supernatants included labeled peptides, labeled lipids, reacted probe, or unreacted probe, depending on the treatment. The ambiguity regarding the source of the counts in the supernatant was eliminated by separating the octapeptide from lipids and probe prior to analysis. The resolution achieved by thin-layer chromatography is shown in Fig. 2. Results of this experimental approach, given in Table 3, demonstrate that only 0.1% of the ^{14}C radiolabel was associated with the released octapeptide, a value significantly lower than the 4.8% predicted on the basis of random labeling and 29.6% proteolysis (0.296 × 16%).

Our evidence clearly shows that external peptides of an *integral* membrane protein are not labeled by our photoreactive probe, supporting our contention that the probe is *membrane-restricted*. Studies with paramagnetic analogues of the glycolipid probe predict that such probes may be further restricted to the external monolayer of sealed membrane vesicles (12). We anticipate that the probe will have a range of only a few amino acid residues in the hydrophobic region of the linear transmembrane M13 coat protein and that this range may depend on the physical state of the phospholipids.

In summary, we have demonstrated that the photoreactive probe, 12-APS-GlcN, is highly effective in labeling a small integral protein in a simple model membrane. Furthermore, we have shown that the number of probe–protein complexes formed exceeds the number predicted solely on the basis of protein concentration in the membrane. These results suggest a significant amount of functional specificity in the reactivity of the probe towards proteins, at least in the model membrane system. This information is encouraging because we intend to apply these techniques to systems in which the molar ratio of protein to lipid may be even lower. With respect to the membrane restriction of the probe, we have shown that proteins added externally to phospholipid vesicles are not labeled by 12-APS-GlcN. Moreover, the extrinsic hydrophilic amino acids of an integral membrane protein are not the sites of attack by the photogenerated nitrene, implying that membrane structures external to the bilayer are not accessible to the probe. This

Table 3. [14C]Probe- and [3H]proline-associated radioactivity in trypsin-released and membrane-bound fragments of M13 coat protein*

Sample	^{3}H in supernatant peptide, cpm	^{3}H in vesicle protein (pellet), cpm	% ^{3}H in supernatant peptide	^{14}C in supernatant peptide, cpm	^{14}C in vesicle protein (pellet), cpm	% ^{14}C in supernatant peptide
Unirradiated + trypsin	5496	4,080	57.0	58	1,305	4.0
Unirradiated	50	13,953	0.4	52	1,141	4.0
Net release by trypsin			56.6			0
Irradiated + trypsin	4795	11,189	30.0	129	17,457	0.7
Irradiated	84	19,154	0.4	88	15,893	0.6
Net release by trypsin			29.6			0.1

* Free probe was separated from probe-complexed peptide in the supernatant by thin-layer chromatography. Only the peptide spot from the supernatant was assayed for ^{14}C radioactivity. Sample compositions are given in Table 1. Radioactivity in vesicle protein (pellet) refers to radiolabel associated with the protein component of the vesicles after separation from lipids by thin-layer chromatography.

feature is likewise indicated by recent probe studies of a cholera toxin–membrane system. Under physiological conditions the enzymically active subunit was radiolabeled; however, no condition resulted in labeling of the binding subunit of the toxin (ref. 4 and unpublished data). Collectively, these studies point to the wide applicability of this photoreactive molecule and reinforce our assumption that the probe–protein complexes formed upon irradiation are indeed reflecting events occurring inside the membrane rather than on its surface. Because the glycolipid probes spontaneously insert into any membrane system, the protocol we have developed for labeling membrane proteins is not restricted to use with model membrane vesicles (13) or cultured cells (13, 14).

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