PHOTOACTIVE COVALENT LABELING OF MEMBRANE COMPONENTS FROM WITHIN THE LIPID CORE

Amira Klip* and Carlos Gitler

Department of Biochemistry. Center for Research and Advanced Studies. National Polytechnic Institute.

P. O. Box 14-740, México 14, D. F.

Received August 9, 1974

Summary. Radioactive 1-Azidonaphthalene and 1-Azido-4-iodobenzene added to liposomes and sarcoplasmic reticulum membranes, are able to reach the liquid hydrocarbon like core, as demonstrated by encounter quenching of perylene fluorescence. Photoactivation converts the azides into the reactive nitrenes which label covalently the fatty acyl chains of the phospholipids. In addition, approximately half of the radioactivity is associated with membrane proteins. Only some 25% is released by exhaustive pronase treatment. Gel electrophoresis shows that the label is located in the Ca^{++}-stimulated Adenosine triphosphatase and in a high molecular weight polypeptide. These results are discussed in terms of possible labeling of biological membranes from within the lipid core.

One of the fundamental problems in understanding how biological membranes function resides in the detailed description of the manner in which proteins interact with the lipid phase. The majority of the evidence suggests that a portion of the protein molecule must be buried within the lipid bilayer. This could be an apolar end of the polypeptide chain as proteolysis experiments suggest for cytochrome b_5 of endoplasmic reticulum (1, 2) and erythrocyte glycoproteins, perhaps through an apolar α-helix of sufficient length to span the bilayer (4). Alternatively an aggregate of several proteins and lipid could be reflected by the large globular structures observed to be jutting out of the freeze-fracture faces of most membranes (4). Ionic lipid-cation-protein interactions have also been proposed to stabilize membrane proteolipids in apolar environments (5).

In order to establish which portions of the protein molecule are present within the lipid core a chemical label soluble in the membrane lipids is required which can covalently attach to the polypeptide chains present therein. Because these moieties could be mainly apolar aminoacid side chains, the reagent must be

*Fellow of the CONACYT (México).

sufficiently reactive to insert into these chemically inert groups. In addition, the labeling agent must be unreactive until it reaches the hydrophobic membrane core. These characteristics are fulfilled by nitrene-generating compounds (17).

We wish to report initial studies on two such chemical labels: 1-azidonaphthalene (AzN) and 1-azido-4-iodobenzene (AzIB). Both compounds are shown to be apolar, unreactive until photoactivated to the highly reactive nitrenes, and to label covalently the apolar components present in sarcoplasmic reticulum membranes. By the use of fluorescence quenching, additional evidence is presented which strongly supports the localization of the azides within the liquid hydrocarbon core of liposomes and membranes.

MATERIALS AND METHODS

Radioactive 1-azidonaphthalene and 1-azido-4-iodobenzene were synthesized from the corresponding amines essentially by method I of Smith and Brown (6). [^{3}H] naphthylamine was labeled by radical catalyzed tritium exchange (7). p-iodoaniline was synthesized from aniline using $^{125}I_2$ (8). The specific activities of AzN and AzIB were 2.5 Ci/mol and 10.9Ci/mol respectively. They were characterized by the presence of the infrared azide band at 4.76μ and by the UV absorption spectra which gave a maximum at 300 nm with shoulders at 313 nm and 326 nm for AzN as previously reported (9) and at 258 nm with a shoulder at 297 nm for AzIB. A single radioactive spot was obtained in thin layer chromatography in Eastman Silica gel plates using decane as solvent after removal of the free amine. Details will be published elsewhere. The partition coefficients in 1-octanol:water were 166 and 199 for AzN and AzIB, respectively.

Labeling of the membranes. Sarcoplasmic reticulum vesicles from rabbit skeletal muscle (10) were resuspended to 3.2-8.2 mg protein/ml in 10mM imidazole, 100mM KCl, pH 7.1 (imidazole-KCl). The azides (final conc. $\sim 5 \times 10^{-5}$M) dissolved in ethanol (final conc. <0.5%) were added and the suspension was incubated at 37° for 15 min. The vesicles were sedimented at 45,000g for 30 min and resuspended in imidazole-KCl. All operations where azides were present were performed in subdued light.

Because of its broad absorption spectrum, photoactivation of AzN could be performed above 320 nm. The light from three 250 W Sylvania Sunlamps was filtered by the glass of the cell and by Corning CS-052 glass sharp cut filters. The lamps were 6 cm from the cell. Since AzIB had to be irradiated in the edge of its absorption band and above

that of the membrane proteins, the light from a 500 W Xenon arc was first passed through a Bausch and Lomb monochromator at 315 nm and through a sharp cut Corning CS-053 glass filter; light below 305 nm was nill. During photoactivation the temperature of the cell was 37° and 25° for AzN and AzIB, respectively.

In order to remove free azide or non covalently bound irradiation products, the membranes were incubated with a 15 mg/ml bovine serum albumin solution in imidazole-KCl (2.5 ml/mg membrane protein) at 37°C for 15 min. The membranes were sedimented as above and the exposure to the albumin was repeated as required.

Fluorescence Quenching. Incorporation of perylene into liposomes and membranes and measurements of the fluorescence emission were performed as published (11). Quenching experiments were carried out essentially as described by Papageorgiou and Argoudelis (12) and by Pownall and Smith (13).

RESULTS AND DISCUSSION

Conditions of photoactivation. No alteration in the absorption spectrum of a serum albumin solution was observed after a 4 hr. exposure to light under the conditions described in Methods. Furthermore, a 1 hr. period of irradiation of the sarcoplasmic reticulum did not decrease the Ca^{++}-ATPase activity of the preparation. However, within 15 min more than 99% of the azides had disappeared when present in an aqueous solution. That photoreactive products were formed could be derived from the data in Fig. 1. The yield of radioactive photoproducts coeluted with the bovine serum albumin was 13% and 10% for AzN and AzIB respectively. The time course for AzN is that of Fig. 1. The label was probably bound covalently to the albumin since it was not removed by trichloroacetic acid or acetone precipitation; furthermore, when the azide was separately irradiated and then added to the serum albumin solution, no radioactivity was found in the protein peak. This was also the case in the absence of irradiation (Fig. 1 time 0). When the serum albumin was substituted by α-chymotrypsin, trypsin or ribonuclease, no detectable radioactivity appeared in the eluted protein peak.

Covalent labeling of the membranes. Addition of the azides resulted in the incorporation of >70% of the radioactivity into the vesicles. Without photoactivation, >99% of the label was removed following equilibration with a bovine serum albumin solution. However, on exposure to light, 25 to 30% of the radioactivity could not be thus removed. Repeated cold acetone precipitation, eventhough it removed some 60%

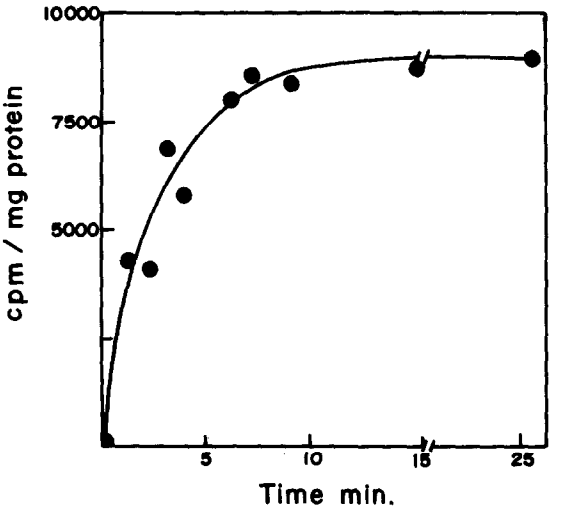

Fig. 1. Time course of insertion of AzN into Serum Albumin: 20 mg of bovine serum albumin in 5 ml of phosphate buffered saline were irradiated in the presence of 3×10^{-5} M AzN. Alicuotes were removed at different time intervals and passed in the dark through a Sephadex G-25 column equilibrated with the saline. The data refer to the exclusion peak.

---

of the membrane phospholipids, resulted in a 15% recovery of the radioactivity in the precipitate. These results together with those of the serum albumin indicate a surprisingly high attachment of the labels.

Identification of the labeled components. As is apparent from Table I, 44% (8 determinations ranging from 34% to 52%) of the total radioactivity was present in the lipid fraction. Of this, 87.1% (4 determinations, range 82.7% to 94.5%) was associated with the ether extract containing the fatty acids. This fraction contained in addition the non saponifyalbe lipids, but these are only minor components in these membranes (14). Thin layer chromatography of the methanol-chloroform or ether extracts did not show the presence of free azide, amine or azoderivatives.

The fact that the fatty acids contained the majority of the radioactivity strongly suggests that the nitrenes are either formed or migrate in the liquid hydrocarbon core.

The radioactivity not extracted with methanol-chloroform (56%, 8 determinations, range 48 to 66%) would seem to be associated mainly with the membrane proteins. The qualitative distribution of this radioactivity in the membrane proteins showed (Fig. 2) that only two main protein bands were significantly labeled. These were a

TABLE I

DISTRIBUTION OF THE LABEL IN THE MEMBRANE ELEMENTS

Azide	Label in the Lipids % of label in the membranes.	Label in the fatty acids. % of label in the lipids.
AzN	45.4	86.9
AzIB	40.7	87.6

Membranes were precipitated with 0.8 M $HClO_4$. The lipids were extracted with methanol: chloroform (2:1 υ/ν) twice. The nitrogen dried extracts were saponified by exposure to 5% (ω/υ) KOH in methanol for 30 min at room temperature. Extraction of the fatty acids with ether was performed after adjusting to pH 1.0.

polypeptide of > 150,000 d and the polypeptide associated with the Ca^{++} dependant-ATPase (∿ 100,000 d). A major fraction of the radioactivity migrated ahead of the tracking dye, and was probably associated with the membrane lipids. However it could also contain the low molecular weight polypeptide of the proteolipid described by McLennan (15).

Exhaustive proteolysis with pronase (0.5mg enzyme added per 0.5 mg of membrane protein) which removed 79% (6 determinations, range 69 to 85%) of the proteins only liberated 19.4% (5 determinations range 14 to 26%) of the radioactivity. Eventhough the interpretation of these results depends on an accurate estimate of the total protein bound radioactivity, it could be roughly calculated that some 24 to 46% of this radioactivity was unaccessible to the lytic enzyme. Our uncertainties are due to the possibility that insertion of the photoproducts might have altered the behaviour of the various fractions extracted by standard procedures, as well as their lability to the proteolytic enzyme.

It is plausible that some of the azide might have inserted in the membrane proteins to apolar crevices in contact with the aqueous phase. Such sites would have to be equivalent to those or seroalbumin, so this does not invalidate the tenet that the probe is

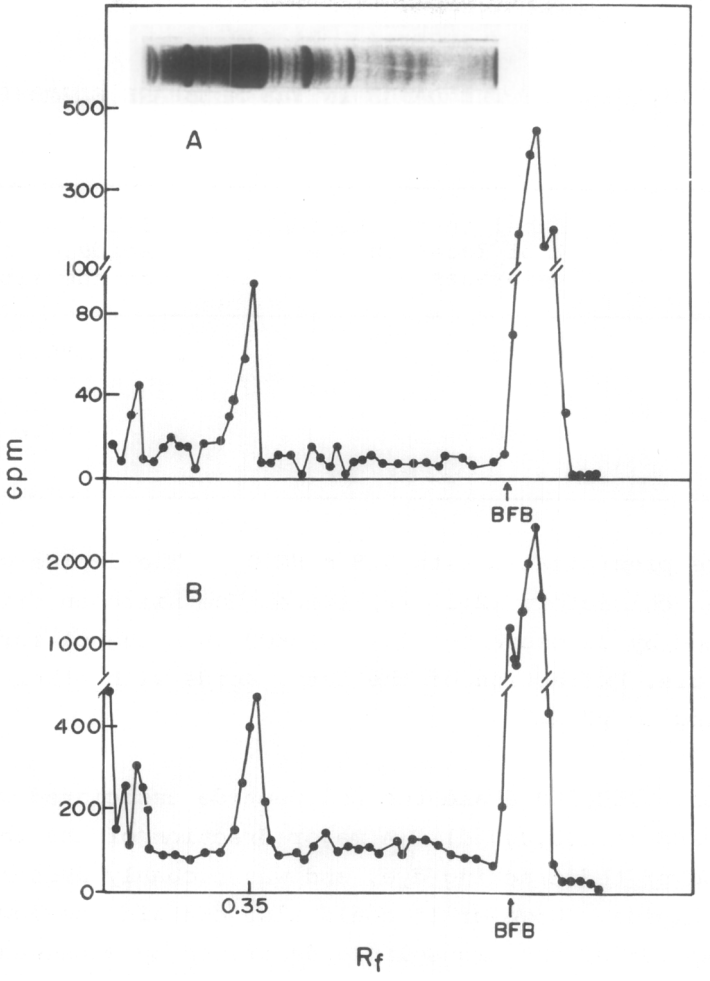

Fig. 2. Sodium dodecyl sulfate disc gel electrophoresis of labeled sarcoplasmic reticulum vesicles was performed according to Laemmli (18). Fig. 2-A: membranes labeled with AzIB. The gamma emmision was counted directly from the unfixed gel discs (see below) in 4g PPO, 0.2g POPOP, 1 L. toluene. Fig. 2-B: membranes labeled with AzN. The unfixed gels were sliced into 2 mm discs at end of run, placed in liquid scintillation vials containing 0.5ml of 10mM Brij 36-T (polyoxyethylated n=10 dodecanol ether) and left overnight at room temperature to allow diffusion of the proteins out of the gels. Ten ml of a detergent - based scintillation mixture (polyoxyethylated n=10 nonylphenol 260g, PPO 5.5g POPOP 0.0694 g, toluene 1L.) were then added, and counts determined with a 20% efficiency in a Packard Tri-Carb Liquid Scintillation Spectrometer model 3003.

directed to apolar regions. However, from the fatty acid labeling, the unaccessibility to pronase and from the quenching experiments shown below, it is clear that a significant fraction of the membrane proteins might be labeled from whithin the lipid core.

TABLE II

QUENCHING CONSTANTS FOR PERYLENE IN DIFFERENT ENVIRONMENTS

Quencher	K_Q		
	Methanol	Liposomes	Sarcoplasmic Reticulum
Sodium Iodide	17	< 1.0	< 1.0
Butilpyridinium bromide	53.8	< 1.0	< 1.0
1,3-Dinitrobenzene	149	816	816
1-Azido-4-iodobenzene	88	2,325	2,797
1-Azidonaphthalene	62	2,777	5,263

K_Q is expressed in M^{-1}

Topological location of the azides as derived from fluorescence quenching. Encounter quenching depends on either the formation of a complex between the flurophore and the quencher (Q) which decreases the fraction of the flurophore which can be excited, or on the collision between the activated fluophore and Q (16) which depopulates the excited state. Both mechanisms depend on the accesibility of Q to the fluorescent molecule, and give the following relation for the steady state: $\emptyset o/\emptyset = Io/I = 1 + K_Q [Q]$, where $\emptyset$ is the fluorescence quantum yield, I, the fluorescence emission and K_Q the encounter quenching constant. The suffix o indicates absence of quencher.

As fluorophore we used perylene which had previously been shown to lacate in the liquid hydrocarbon regions of liposomes and membranes (11). The quenching constants for perylene dissolved in methanol were essentially similar for the different quenchers (Table II). When the perylene was present in the liposomes and membranes, it became nearly unquenched by the ionic quenchers (Io/I=2 at a butylpyridinium concentration of 1.25M). On the other hand, the K_Q values increased significantly with the apolar quenchers, eventhough the viscosity was

some 200 fold grater in the membrane lipid core than in the methanol (11). The interpretation of these results leads to a conclusion similar to those of Papageorgiou and Argoudelis (12) and of Pownall and Smith (13), namely, that the perylene must be located in a region of the liposomes and membranes which can not be reached by iodide or butyl-pyridinium. This latter molecule should be able to reach the vicinity of the phosphate groups on the phospholipids because of its Van der Waals contribution and charge. Moreover the two azides must partition preferentially into the apolar core in which the perylene is located.

The present results suggest that membrane proteins can be labeled from within the lipid core. Studies are underway to label in situ other proteins such as cytochrome b_5 and rhodopsin. These should complement the present findings.

Acknowledgements: We are endebted to Dr. G. Gojon for his help in the radioactive labeling of AzN, and to Dr. L. Brener for valuable discussion. The excellent technical assitance of Miss Eunice Zavala is highly appreciated.

REFERENCES

1. Ito, A., and Sato, R. (1968) J. Biol. Chem. 243, 4922-4923.
2. Spatz, L., and Strittmatter, P. (1971) Proc. Nat. Acad. Sci. USA 68, 1042-1046.
3. Segrest, J. P., Jackson, R.L., and Marchesi, V. T. (1972) Biochem. Biophys. Res. Commun. 49, 964-969.
4. Branton, D. (1969) Ann. Rev. Plant. Physiol. 20, 209-238.
5. Gitler, C., and Montal, M. (1972) Febs Letters 28, 329-332.
6. Smith, P.A.S., and Brown, B.B. (1951) J. Amer. Chem. Soc. 73, 2438-2441.
7. Gojon, G. personal communication.
8. Vogel, A. (1956) Practical Organic Chemistry, p. 647, Longmans, Green and Co., London.
9. Reiser, A., and Wagner, H.M. (1971) in Patai, S. (Editor) The Chemistry of the Azido Group, p. 446, Interscience Publishers, London.
10. Martonosi, A. (1968) J. Biol. Chem. 243, 71-81.
11. Rudy, B., and Gitler, C. (1972) Biochem. Biophys. Acta 288, 231-236.
12. Papageorgiou, G., and Argoudelis, C. (1973) Arch. Biochem. Biophys. 156, 134-142.
13. Pownall, H. J., and Smith, L. C. (1974) Biochemistry 13, 2594-2597.
14. Inesi, G. (1972) Ann. Rev. Biophys. Bioeng. 1, 191-210.
15. MacLennan, D.H., Yip, C.C., Iles, G. H., and Seeman, P. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 469-477.
16. Stern, O., and Volmer, M. (1919) Physikal. Z. 20, 183.
17. Knowles, J. R. (1972) Accounts Chem. Res. 5, 155-160.
18. Laemmli, U.K. (1970) Nature 227, 680-685.