

Photolabelling of membrane proteins with photoactive phospholipids

Roberto BISSON and Cesare MONTECUCCO

*C.N.R. Center for the Physiology of Mitochondria and Laboratory of Biophysics and Molecular Biology,
Institute of General Pathology, University of Padua, Via Loredan 16, Padua, Italy*

(Received 11 August 1980/Accepted 3 September 1980)

Photoactive probes have been introduced recently to study the hydrophobic sector of integral membrane proteins. A simple procedure to synthesize a new series of highly radioactive aryl azido-phospholipids is presented. They effectively exchange with the boundary lipids and, on illumination, they cross-link to several membrane proteins with high efficiency. The procedure and analysis of labelling of ATPase from sarcoplasmic reticulum is reported as an example. The advantages in using these photoactive phospholipids are discussed together with some information obtained on their use.

Membrane proteins have been divided in two categories: peripheral and integral proteins (Singer, 1974). Proteins of the first group do not interact with the fatty-acid portion of phospholipids and can be purified free of lipids in soluble form. Integral proteins possess hydrophobic domains exposed to lipids. Even after their purification, which generally requires detergents, they are usually associated with lipids. In addition lipids or detergents are often required for their full activity (De Pierre & Ernster, 1977; Sandermann, 1978).

Several methods have been introduced for the study of the hydrophilic portion of membrane-bound proteins (De Pierre & Ernster, 1977). On the other hand, the lack of reactive residues, in their hydrophobic segments has made the design of probes for the study of the membrane-intercalated polypeptides more difficult.

Gitler and his co-workers (Klip & Gitler, 1974; Karlish *et al.*, 1977; Kahane & Gitler, 1978) have introduced the use of photoactive probes such as aryl azides. Due to their hydrophobicity, they dissolve in the lipid bilayer with high partition coefficients. On illumination aryl azides are converted into the corresponding nitrenes, which cross-link with neighbouring molecules with broad specificity (Reiser *et al.*, 1968; Knowles, 1972; Bayley & Knowles, 1977). However, the electrophilic character of the photogenerated nitrene and its long lifetime raise the possibility that labelling in the hydrophilic protein surface might occur (Bayley & Knowles, 1978). Moreover, the location of the aryl azides in the membrane is undefined. The possibility that these

probes can be absorbed in hydrophobic pockets present in the hydrophilic portion of a membrane protein or excluded from areas of strong lipid-protein interaction has also to be taken into account.

These limitations can be overcome by anchoring the photoactive group directly to a particular carbon atom of the phospholipid fatty-acid chain.

The synthesis of photoactive phospholipids, containing nitrene or carbene precursors, was first reported by Chakrabarti & Khorana (1975) and data about their interaction with lipids are already available (Gupta *et al.*, 1979). However, little information exists about the interaction of photoactive phospholipids with membrane proteins. Technical problems in obtaining highly radioactive phospholipids has limited the studies only to samples containing large amounts of photoactive phospholipids, which may result in perturbation of the native protein structure or the lipid-protein interaction (Stoffel & Metz, 1979; Takagaki *et al.*, 1979).

To improve this situation a simple synthesis of highly radioactive aryl azido-phosphatidylcholines is described in the present paper. Their use in labelling several membrane proteins is also reported.

Materials and methods

12-Aminododecanoic acid was purchased from EGA Chemie (Steinheim, Germany). [³H]Methyl iodide (sp. radioactivity 1.3 Ci/mmol) and [¹⁴C]methyl iodide (sp. radioactivity 59.1 mCi/mol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Carbonyldiimidazole and 2-amino-4-nitrobenzoic acid were purchased from Merck (Darmstadt, Germany). Phospholipase A₂ and phospholipase D were the product of

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

Boehringer (Mannheim, Germany). All solvents and salts were of the highest purity commercially available. 4-Fluoro-3-nitrophenyl azide was prepared as reported by Fleet *et al.* (1972). Chromatographically pure egg phosphatidylcholine was a generous gift of Dr. A. Pitotti. (1-Myristoyl)-lysolecithin and (1-palmitoyl)-lysolecithin were prepared as described by Wells & Hanahan (1969). The ATPase from sacroplasmic reticulum was prepared as reported by Warren *et al.* (1974a). Its activity measured with an NADH-coupled system ranged from 10.4 to 12.5 units and its lipid/protein ratio from 28 to 37. ($\text{Na}^+ + \text{K}^+$)-activated ATPase from pig kidney, cytochrome *c* oxidase and oligomycin-sensitive ATPase from bovine mitochondria were prepared and assayed as reported previously (Montecucco *et al.*, 1979, 1980; Bisson *et al.*, 1979a). Protein concentration was determined by the method of Lowry *et al.* (1951) and lipids either by the method of Bartlett (1959) or by g.l.c. after transmethylation of the fatty acids. Phospholipids were detected on t.l.c. plates by the Dittmer spray reagent (Dittmer & Lester, 1964) and by u.v. absorbance. All operations involving azides were performed under a red safety light.

Preparation of 12-[N-(4-azido-2-nitrophenyl)]-aminododecanoic acid

An ethanol solution of 140 mg of 4-fluoro-3-nitrophenyl azide was added to an equal volume of a suspension of 200 mg of 12-aminododecanoic acid in 1 M- Na_2CO_3 with stirring. After 16 h at 60°C, the solution was acidified with HCl and extracted three times with diethyl ether. The diethyl ether extracts were pooled and the solvent was removed under reduced pressure. The product (167 mg, 82% yield) was found as a single spot after t.l.c. (Silica gel 60 F, Merck) with, as solvents, chloroform/methanol/water (65:36:4, by vol.) (solvent A), giving an R_F value of 0.85, chloroform/methanol/7 M- NH_3 (65:35:4, by vol.) (solvent B), giving an R_F value of 0.71, chloroform/methanol/water/acetic acid (65:35:7:3, by vol.) (solvent C), giving an R_F value of 0.87. The spectral data were: i.r. (Nujol) 2121 and 2096 (N_3) cm^{-1} ; u.v. and visible (ethanol) λ_{max} 261 nm (ϵ_{mm} 18.8), λ_{max} 456 nm (ϵ_{mm} 5.9) (Found: C, 56.29; H, 6.51; N, 19.08; $\text{C}_{18}\text{H}_{28}\text{O}_4\text{N}_3$ requires C, 57.29; H, 7.16; N, 18.57 %).

Preparation of 2-azido-4-nitrobenzoic acid

NaNO_2 (1.3 g) was added to 3.64 g of 2-amino-4-nitrobenzoic acid dissolved in 25 ml of 1 M- NaOH . This mixture was dropwise added to 33 ml of H_2SO_4 (3 ml of conc. H_2SO_4 in 30 ml of water) under vigorous stirring at -10°C. After 1 h, the suspension was filtered and a saturated solution of NaN_3 (corresponding to 1.5 g of NaN_3) was added dropwise to the filtrate at -10°C in a period of 1 h.

When evolution of N_2 ended, the suspension was filtered. The pale-yellow precipitate was recrystallized from ethanol. The yield was 2.4 g (66%). T.l.c. showed a single spot in solvent A gave (R_F 0.36), in solvent B (R_F 0.62) and in solvent C (R_F 0.58). The spectral data were: i.r. (Nujol) 2148 and 2132 (N_3) cm^{-1} ; u.v. and visible (ethanol) λ_{max} 261 nm (ϵ_{mm} 8.0), λ_{max} 329 nm (ϵ_{mm} 3.2) (Found: C, 39.34; H, 1.82; N, 26.55; $\text{C}_7\text{H}_4\text{N}_4\text{O}_4$ required C, 40.38; H, 1.92; N, 26.9%).

Preparation of 2-{12-[N-(4-azido-2-nitrophenyl)]-aminododecanoyl}-1-myristoyl-sn-glycero-3-phospho[^{14}C]choline (phospholipid I) and of 2-(2-azido-4-nitrobenzoyl)-1-palmitoyl-sn-glycero-3-phospho[^3H]choline (phospholipid II)

12-[N-(4-azido-2-nitrophenyl)]aminododecanoic acid and 2-azido-4-nitrobenzoic acid were coupled respectively to (1-myristoyl)-lysolecithin and to (1-palmitoyl)-lysolecithin by using the procedure introduced by Warner & Benson (1977). The only modification was that the imidazole derivative of the azidonitrobenzoic acid was formed in dimethyl sulphoxide instead of tetrahydrofuran. The two phospholipids were purified by preparative t.l.c. on Merck silica-gel 60 F plates with solvent A as eluent. They showed single spots in all the chromatographic systems used above. Phospholipid I behaved as egg phosphatidylcholine, whereas the R_F values of phospholipid II were 0.21 in solvent A, 0.36 in solvent B and 0.24 in solvent C. The intactness of the azido group was checked by i.r. spectroscopy. The spectral data were: for phospholipid I u.v. and visible [chloroform/methanol (2:1, v/v)] λ_{max} 469 (ϵ_{mm} 5.2) and 262 nm (ϵ_{mm} 18.2); for phospholipid II, u.v. and visible λ_{max} 250 (ϵ_{mm} 18.1) and 334 nm (ϵ_{mm} 1.5).

Transphosphatidylation of azidophosphatidylcholine to azidophosphatidylethanolamine was performed as reported previously (Smith *et al.*, 1978). The two phosphatidylethanolamines were purified by preparative t.l.c. as before. 2-{12-[N-(4-azido-2-nitrophenyl)]aminododecanoyl}-1-myristoyl-sn-glycero-3-phosphoethanolamine and 2-(2-azido-4-nitrobenzoyl)-1-palmitoyl-sn-glycero-3-phosphoethanolamine were methylated respectively with [^{14}C]methyl iodide and with [^3H]methyl iodide by a slight modification of the procedure of Smith *et al.* (1978). The reaction, scaled down to use 2 mg of starting phosphatidylethanolamine, was conducted under vacuum at 35°C for 48 h. The yield of phospholipid I was 60%; mono- and di-methylphosphatidylethanolamines (10%) and unreacted phosphatidylethanolamine (29%) were also present. The preparation of phospholipid II gave a yield of 56%; mono- and di-methylphosphatidylethanolamines (7%) and unreacted material (37%) were also present. The two radioactive azido-phosphatidyl-

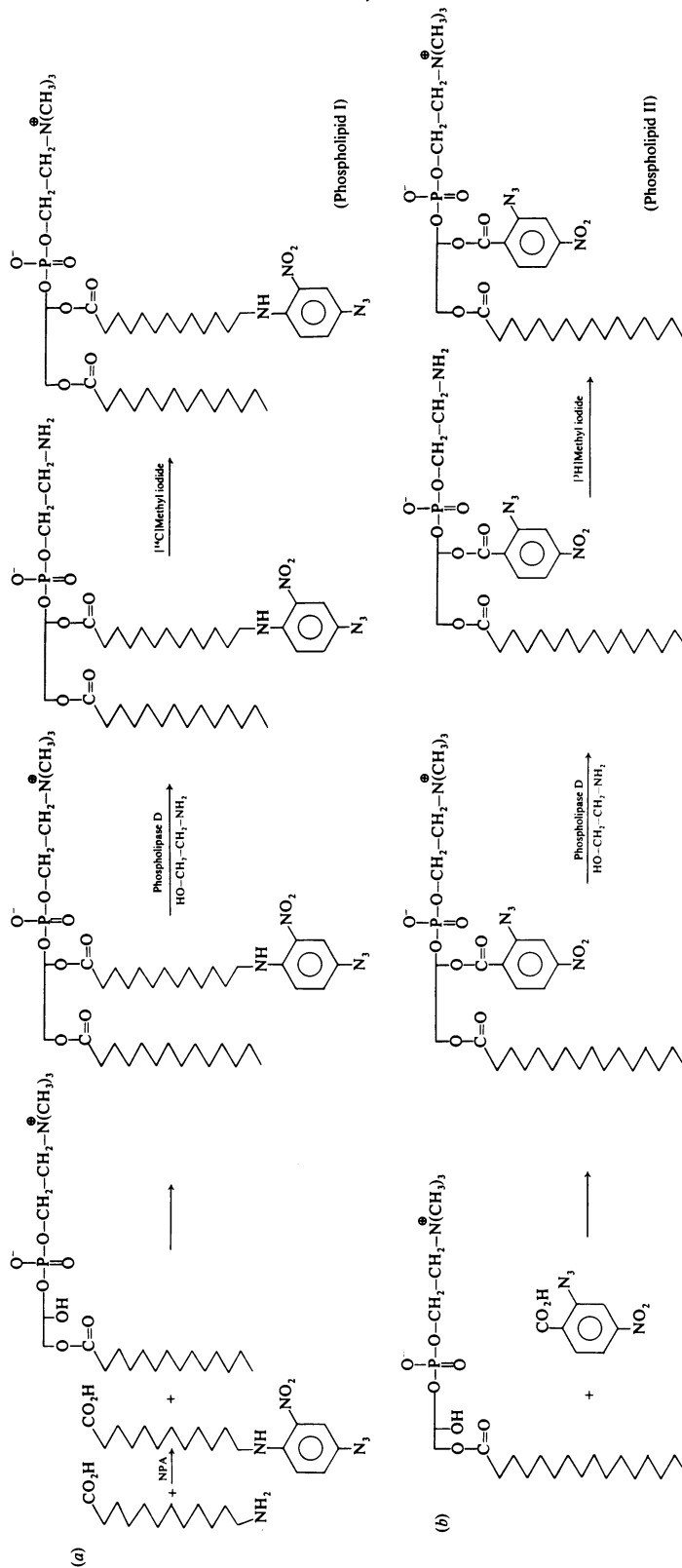


Fig. 1. Reaction scheme for the synthesis of 2-[12-[N-(4-azido-2-nitrophenyl)aminododecanoyl]-1-myristoyl-sn-glycero-3-phospholipid]choleine (a) and of 2-(2-azido-4-nitrobenzoyl)-1-palmitoyl-sn-glycero-3-phospho[^3H]choleine (b). For details see the Materials and methods section. Abbreviation used: NPA, nitrophenyl azide.

cholines were purified by preparative t.l.c. as described above. The products were stored in absolute ethanol at -80°C .

Labelling of membrane proteins with aryl azido-phosphatidylcholines

Lipid-protein complexes were formed by incubation with exogenous lipids (Eytan & Broza, 1978), by sonication (Racker, 1973) and by detergent-mediated exchange (Warren *et al.*, 1974*b*). The final content of azido-phospholipids was 0.4% (w/w) for phospholipid I and 0.1% (w/w) for phospholipid II with respect to the total lipids. The samples were illuminated at 0°C with a 100 W mineral lamp (Ultra-Violet Products, San Gabriel, CA, U.S.A.) for various periods of time between 10 and 20 min, with a glass/water filter to cut down radiation lower than 300 nm. After illumination proteins were recovered, electrophoresed and counted for radioactivity as reported previously (Bisson *et al.*, 1979*a*; Montecucco *et al.*, 1980).

Results

The reaction scheme for the synthesis of phospholipids I and II is presented in Fig. 1. The coupling of a long-chain fatty acid to lysolecithin with the production of a phosphatidylcholine containing the photoactive aryl azido group about 2 nm (20 Å) distant from the carbonyl group is shown in Fig. 1(a). Fig. 1(b) shows the preparation of a phosphatidylcholine in which the photoactive group is close to the level of the carbonyl moiety. The two phosphatidylcholines are made highly radioactive by the procedure of Smith *et al.* (1978). Phosphatidylcholine is converted into phosphatidylethanolamine by transphosphatidyl transfer catalysed by phospholipase D in the presence of excess of ethanolamine. This phospholipid is then methylated with radioactive methyl iodide to give again phosphatidylcholine. As a result all three methyl groups of choline are isotopically labelled either at the carbon or at the hydrogen atoms depending on the radioactive isotope in the methyl iodide. It was thus possible to prepare derivatives with specific radioactivities of 177 mCi/mol (phospholipid I) and 3.9 Ci/mmol (phospholipid II).

In Figs. 2(a) and 2(b) the u.v. and visible spectra of the two phospholipid derivatives are reported together with the spectra obtained after extensive exposure to u.v. light. Lipid-protein complexes, containing the photoactivatable phospholipids, were illuminated with a u.v. lamp in the absence of O_2 , with the protection of a glass/water filter. Under these conditions enzymic activity was unaffected in all membrane enzymes tested so far. If O_2 was present the loss of activity ranged from 10–15% for the ATPases to 50% for yeast cytochrome *c* oxidase.

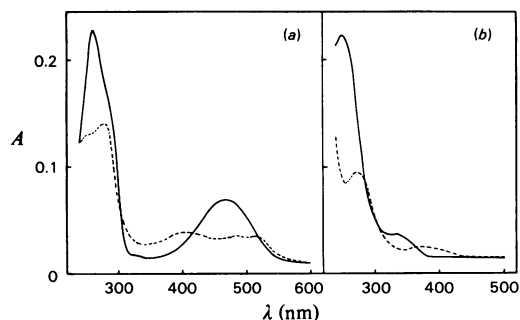


Fig. 2. Absorption spectra of phospholipid I (a) and phospholipid II (b) before (—) and after (---) u.v. irradiation

Protein-containing vesicles were separated from excess lipids by centrifugation on 10% sucrose and recovered as pellets. In Table 1, column (A), the percentage of modified phospholipids associated with the pellet with respect to the amount initially added to the protein is reported. These numbers, which include both covalently and non-covalently bound photoactivated phospholipids, roughly reflect the efficiency of interaction between protein and lipid in the different procedures used to form lipid-protein complexes. These values are not corrected for the loss of protein during manipulation, which ranged from 10 to 40%. Among the methods tested, the detergent-mediated exchange procedure was generally the most efficient in taking up added phospholipids. This was the case particularly with purified proteins containing high amounts of endogenous lipids. It also appears that the two photoactivated phospholipids, despite their different structure, participate to the same extent in the formation of lipid-protein complexes with the membrane proteins used. To detect the fraction of modified phospholipids covalently bound to the protein the pellets were analysed by SDS/polyacrylamide-gel electrophoresis. Fig. 3 shows the electrophoretic profile of sarcoplasmic-reticulum ATPase. This enzyme is made up of one unit of mol.wt. 115 000 and it is pure as judged by SDS/polyacrylamide-gel electrophoresis. In Fig. 3(b) the distribution of radioactivity of phospholipid I associated with the gel is shown. Radioactivity is associated with the protein band and with the phospholipids that run just behind the tracking dye Pyronine. No radioactivity is bound to the protein in a non-illuminated sample or when the protein is treated with pre-illuminated liposomes. Similar results are obtained with phospholipid II (result not shown).

The percentage of radioactivity covalently associated with the protein, estimated as the ratio between the radioactivity associated with the protein

Table 1. Labelling yields of phospholipids I and II with sarcoplasmic-reticulum ATPase, ($\text{Na}^+ + \text{K}^+$)-activated ATPase, oligomycin-sensitive ATPase and cytochrome *c* oxidase

(a) Incubation. Proteins were incubated with sonicated egg phosphatidylcholine vesicles (0.30 μmol of lipid/mg of protein) in the appropriate buffer at 0°C for 2h. (b) Sonication. Oligomycin-sensitive ATPase was mixed with sonicated egg phosphatidylcholine (0.30 mg of lipid/mg of protein) and sonicated for 6 min at 20°C in a sonicating bath (80W, 22–38 kHz; Mazzali I.A.E., Monza, Italy). (c) Detergent-mediated exchange. Proteins were incubated with the appropriate buffer containing 0.2% cholate and aryl azido-phospholipid in trace amounts. Samples were incubated up to 3h at 0°C and then slowly diluted 50 times with the same buffer without cholate (see the Materials and methods section). Buffers used were: 0.25 M-sucrose/150 mM-KCl/50 mM- P_i , pH 8.0 for sarcoplasmic-reticulum ATPase; 0.25 M-sucrose/30 mM-histidine/1 mM-EDTA, pH 7.4, for ($\text{Na}^+ + \text{K}^+$)-activated ATPase; 10 mM-Tris/Hepes, pH 7.5, for oligomycin-sensitive ATPase; 10 mM- P_i , pH 7.4, for cytochrome *c* oxidase. The values reported in the Table are calculated as ratios of radioactivities expressed in d.p.m.

Procedure	Number of experiments	(A) Radioactivity associated with the pellet after centrifugation (%)		(B) Radioactivity of the pellet covalently bound to the protein (%)	
		Phospholipid I	Phospholipid II	Phospholipid I	Phospholipid II
Sarcoplasmic-reticulum ATPase	(a) 6	6–18	5–20	1–5	2–4
	(c) 3	14–17	12–15	2–3	4–5
($\text{Na}^+ + \text{K}^+$)-activated ATPase	(a) 4	3–4	6–9	1–2	1–2
	(c) 6	19–30	20–27	1–3	1–3
Oligomycin-sensitive mitochondrial ATPase	(a) 6	5–6	5–6	14–18	4–8
	(b) 2	3–5	3–5	13–15	4–5
	(c) 4	10–14	8–14	7–9	2–3
Bovine heart cytochrome <i>c</i> oxidase	(a) 7	15–30	15–25	5–10	2–4
	(c) 5	65–80	45–60	20–30	10–15

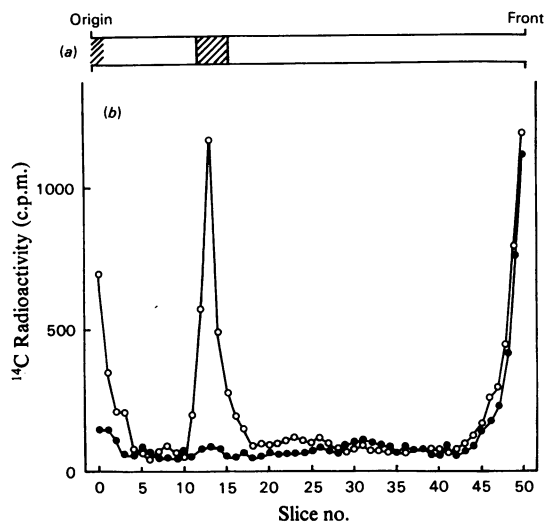


Fig. 3. Distribution of radioactivity associated with SDS/polyacrylamide gel electrophoresis of isolated sarcoplasmic-reticulum ATPase

(a) Coomassie Blue staining profile of the protein (50 μg) after SDS/polyacrylamide-gel electrophoresis on a 7.5% polyacrylamide slab gel (Weber & Osborn, 1969). (b) Distribution on the gel of ^{14}C radioactivity of phospholipid I of an illuminated (○) and of a non-illuminated (●) sample of 50 μg of protein is also shown. Gels were cut in 1.8 mm thick slices before counting for radioactivity.

on the gel and the radioactivity of the pellet, is reported in Table 1, column (B). This parameter may be taken as an estimation of the ability of these probes to cross-link with membrane proteins. The apparently low values can be explained mainly by the fact that most of the added photoactive phospholipids are surrounded by other phospholipid molecules rather than being in contact with the protein. The extent of this effect is largely controlled by the lipid/protein ratio of the reconstituted lipid-protein complex. This was tested by using the reconstitution procedure (c) (see the legend to Table 1) with cytochrome *c* oxidase preparations containing a low amount of phospholipids, not sufficient to form a single shell of boundary lipids around the protein. In this case the yield of covalent labelling rose to 15–30%. From Table 1, column (B), it also appears that different membrane proteins are labelled to different extents by the two azido-phospholipids. This result may be related to the amount of protein surface exposed to lipids, but also to its particular amino-acid composition. In fact, despite the broad reactivity of aryl nitrenes, some selectivity toward certain amino-acid residues cannot be excluded.

Discussion

Although the structure at high resolution of many soluble and peripheral membrane proteins is known,

few primary sequences and low-resolution 'maps' of integral membrane proteins are available (Henderson & Unwin, 1975; Fuller *et al.*, 1979; Steffens & Buse, 1979; Sacher *et al.*, 1979; Ovchinnikov *et al.*, 1979). In this situation it would be useful if methods could be developed to identify which subunits of a multi-subunit enzyme and which parts of the polypeptide sequence form the hydrophobic sector of a membrane protein. Several studies dealing with the use of small hydrophobic photoreactive probes appeared recently (Karlsh *et al.*, 1977; Kahane & Gitler, 1978; Bercovici & Gitler, 1978; Abu-Salah & Findlay, 1977; Cerletti & Schatz, 1979; Goldman *et al.*, 1979). In addition to the question raised about their use by Bayley & Knowles (1978) artefactual labelling of the hydrophilic domain of integral proteins may derive from their absorption into hydrophobic pockets of the water-exposed surface of the protein. This has been shown to occur with several soluble proteins and hydrophobic molecules of very different chemical structure and size (Halsey *et al.*, 1978; Ueda *et al.*, 1976; Stefanini *et al.*, 1979). Another potential fault is that these probes might be prevented from contacting the protein in regions where its interaction with lipids is stronger. These drawbacks can be overcome, at least in principle, by anchoring the photoactive group to a selected position of the fatty-acid chain of a phospholipid. Photoactive phospholipids with aromatic and aliphatic azides and carbenes have been prepared (Chakrabarti & Khorana, 1975; Gupta *et al.*, 1979; Stoffel & Metz, 1979; Moonen *et al.*, 1979). We have chosen as photoactive group the nitrophenyl azide because of its large absorbance at wavelengths greater than 300 nm, which lowers the possibility of protein damage (Fleet *et al.*, 1972; Bayley & Knowles, 1977) and because of its high efficiency in binding covalently to proteins (Fleet *et al.*, 1972). The synthetic procedure described in the present paper offers several advantages. Nitroaryl azido-fatty acids of any length can be made from the commercially available amino acids and fluoro-phenyl azide. The aryl azido-phospholipids are made radioactive only at the last step of the synthesis. The reaction occurs under mild conditions and gives high yields. Three ^{14}C atoms and nine ^3H atoms can be introduced per phosphatidylcholine molecule. The high specific radioactivity of these photoactivatable phosphatidylcholines allows their use in trace amounts so that the biological system under study is minimally perturbed by the probe. It is also noteworthy that increasing amounts of photoactive lipids in the lipid-protein complex do not proportionally increase the amount of labelling since the aryl azido group itself acts as an efficient quencher of the activated nitrene (Reiser *et al.*, 1968). The choice among the methods used to prepare lipid-protein complexes depends on the lipid content of

the purified protein sample and on its sensitivity to detergent or sonication. As shown in column (A) of Table 1, these factors affect the amount of lipids, which remain associated with the protein after centrifugation on sucrose, giving rise to the large variability found. However, this does not affect the patterns of labelling of all the multi-subunit membrane enzymes tested so far. The yield of labelling with membrane proteins is an important parameter in assessing the usefulness of these probes. The ratio between the azido-phospholipids covalently bound to the protein and those present in the lipid-protein complex after centrifugation (Table 1, column B) is only an underestimated value of the labelling efficiency. In fact only a fraction of the photoactive phospholipids present in the lipid vesicles comes into contact with the protein surface during the lifetime of the nitrene. This effect can be decreased by using lipid-deficient protein samples. With lipid-depleted cytochrome *c* oxidase it is possible to obtain a lipid-protein complex in which the azido-phospholipids are present mainly at the protein surface. In this case, the percentage of cross-linking depends largely on the effectiveness of the azido group to link covalently to the protein even if other interactions (i.e. with detergent molecules) may still occur. In such a case the yield of labelling is up to 30%. As shown in Fig. 3, SDS/polyacrylamide-gel electrophoresis provides a very effective system for removing all the radioactive photoactivated phospholipids not covalently bound to the protein. In fact no radioactivity is associated with protein samples treated with pre-illuminated aryl azido-phospholipids or when the illumination step is not performed. These results rule out the possibility that overlapping or unspecific binding of the photo-decomposed products to the protein occur when SDS/polyacrylamide-gel electrophoresis is used to monitor the labelling.

The present work shows that nitrophenyl azido-phospholipids can be used advantageously in protein-topology studies. Indeed we have already reported preliminary studies of well-characterized membrane proteins such as cytochrome *b₅* (Bisson *et al.*, 1979b), cytochrome *c* oxidase (Bisson *et al.*, 1979a) and the mitochondrial and ($\text{Na}^+ + \text{K}^+$)-activated ATPases (Montecucco *et al.*, 1979, 1980). Nevertheless some points need further investigation. For example, areas of the protein where the interaction with other classes of lipids is strong and selective may not be labelled by a photoactive phosphatidylcholine. Moreover, it is possible that only certain amino acids are able to react with nitroarylnitrene, which again will result in a lack of labelling.

A critical test for the use of these reagents concerns the possibility that they bind to protein sites outside the lipid bilayer (Bayley & Knowles,

1978). We have some experimental evidence showing that this does not happen with these probes. Cytochrome b_5 is labelled only in its hydrophobic tail, while no label is found on the water soluble trypsin-cleavable domain (Bisson *et al.*, 1979b). Both phospholipids I and II label five out of seven subunits of bovine heart cytochrome c oxidase (Bisson *et al.*, 1979a), but do not label anti-oxidase antibodies bound to the enzyme (Prochaska *et al.*, 1980). In the mitochondrial ATPase complex no labelling is found in the F_1 portion, which is believed to project out of the lipid bilayer into the aqueous medium (Montecucco *et al.*, 1980).

We thank Dr. G. A. Smith and Dr. H. Gutweniger for helpful discussions and suggestions and Dr. R. A. Capaldi and Dr. L. Prochaska for critical reading of the manuscript. We gratefully acknowledge the encouragement and support of Professor A. Azzi and Professor G. F. Azzone and the valuable technical assistance of Mr. M. Santato.

References

- Abu-Salah, K. M. & Findlay, J. B. (1977) *Biochem. J.* **161**, 223–228
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Bayley, H. & Knowles, J. R. (1977) *Methods Enzymol.* **66**, 69–114
- Bayley, H. & Knowles, J. R. (1978) *Biochemistry* **17**, 2414–2419
- Bercovici, T. & Gitler, C. (1978) *Biochemistry* **17**, 1484–1489
- Bisson, R., Montecucco, C., Gutweniger, H. & Azzi, A. (1979a) *J. Biol. Chem.* **254**, 9962–9965
- Bisson, R., Montecucco, C. & Capaldi, R. A. (1979b) *FEBS Lett.* **106**, 317–320
- Cerletti, N. & Schatz, G. (1979) *J. Biol. Chem.* **254**, 7746–7751
- Chakrabarti, P. & Khorana, H. G. (1975) *Biochemistry* **14**, 5021–5033
- De Pierre, J. W. & Ernster, L. (1977) *Annu. Rev. Biochem.* **46**, 201–262
- Dittmer, J. C. & Lester, R. L. (1964) *J. Lipid Res.* **5**, 126–127
- Eytan, G. D. & Broza, R. (1978) *J. Biol. Chem.* **253**, 3196–3202
- Fleet, G. W. J., Knowles, J. R. & Porter, R. R. (1972) *Biochem. J.* **128**, 499–508
- Fuller, S. F., Capaldi, R. A. & Henderson, R. (1979) *J. Mol. Biol.* **134**, 305–327
- Goldman, D. W., Pober, J. S., White, J. & Bayley, H. (1979) *Nature (London)* **280**, 841–843
- Gupta, C. M., Costello, C. E. & Khorana, H. G. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3139–3143
- Halsey, M. J., Brown, F. F. & Richards, R. E. (1978) in *Molecular Interactions and Activity in Proteins; CIBA Found. Symp.* **60**, 123–136
- Henderson, R. & Unwin, P. N. T. (1975) *Nature (London)* **257**, 28–31
- Kahane, I. & Gitler, C. (1978) *Science* **201**, 351–352
- Karlish, S. J. D., Jørgensen, P. L. & Gitler, C. (1977) *Nature (London)* **269**, 715–717
- Klip, A. & Gitler, C. (1974) *Biochem. Biophys. Res. Commun.* **60**, 1155–1162
- Knowles, J. R. (1972) *Acc. Chem. Res.* **5**, 155–160
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Montecucco, C., Gutweniger, H., Johanson, A. & Bisson, R. (1979) *Biochem. Soc. Trans.* **7**, 952–953
- Montecucco, C., Bisson, R., Pitotti, A., Dabbeni-Sala, F. & Gutweniger, H. (1980) *J. Biol. Chem.* in the press
- Moonen, P., Haagsman, H. P., Van Deenan, L. L. M. & Wirtz, K. W. A. (1979) *Eur. J. Biochem.* **99**, 439–445
- Ovchinnikov, Y. A., Abdulaev, N. G., Feigina, M. Yu., Kieselev, A. V. & Lobanov, N. A. (1979) *FEBS Lett.* **100**, 219–224
- Prochaska, L., Bisson, R. & Capaldi, R. A. (1980) *Biochemistry* in the press
- Racker, E. (1973) *Biochem. Biophys. Res. Commun.* **55**, 224–230
- Reiser, A., Willets, F. W., Terry, G. C., Williams, V. & Marley, R. (1968) *Trans. Faraday Soc.* **64**, 3265–3275
- Sacher, R., Steffens, G. J. & Buse, G. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1385–1392
- Sandermann, H. (1978) *Biochim. Biophys. Acta* **515**, 209–237
- Singer, S. J. (1974) *Annu. Rev. Biochem.* **43**, 805–833
- Smith, G. A., Montecucco, C. & Bennett, J. P. (1978) *Lipids* **13**, 92–94
- Stefanini, S., Finazzi-Agrò, A., Chiancone, E. & Antonini, E. (1979) *FEBS Lett.* **100**, 296–300
- Steffens, G. J. & Buse, G. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 613–619
- Stoffel, W. & Metz, P. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 197–206
- Takagaki, Y., Gupta, C. M., Nihei, K., Gerber, G. E. & Khorana, H. G. (1979) *Abstr. Int. Congr. Biochem. 11th, Toronto*, 381
- Ueda, I., Kamaya, H. & Eyring, H. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 481–485
- Warner, T. G. & Benson, A. A. (1977) *J. Lipid Res.* **18**, 548–552
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1974a) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 622–626
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1974b) *Biochemistry* **18**, 5501–5507
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Wells, M. A. & Hanahan, D. J. (1969) *Methods Enzymol.* **14**, 178–184