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Depth-dependent photolabelling of membrane hydrophobic core with 9-diazofluorene-2-butyric acid

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Hydrophobic photoactivable reagents, which readily partition into membranes, have proved very useful for studying membrane hydrophobic core. These reagents have been linked to fatty acids in order to obtain amphipathic photoactivable reagents which label membranes more effectively. By varying the length of these amphipathic reagents, an attempt to label membrane hydrophobic core at different depths can be made. We report here 9-diazofluorene-2-butyric acid as a new photoactivable reagent which labels the single bilayer vesicles prepared from egg phosphatidylcholine. The labelling site on the fatty acyl chains could be traced to be between the carbon atom 4 and 6. The new probe thus labels the membrane at a site which is proximal to what can be predicted from its length and transverse location in membranes.

The membrane hydrophobic core incorporates non-polar fatty acyl chains of lipids and membrane spanning domains of integral membrane proteins. These membrane spanning domains have a very high density of hydrophobic amino acids. A study of such a non polar milieu by chemical reagents makes it essential to use reactive intermediates capable of inserting into a non polar bond e.g. C-H bond. A photochemical approach has been developed to achieve this objective [1]. It involves the use of lipophilic photoactivable precursors of carbenes and nitrenes e.g. iodonaphthylazide [2], adamantyl diazirine [3], trifluoromethylphenyl diazirine [4] and diazofluorene [5]. These photoactivable reagents can be used as such or linked to fatty acids of variable lengths for depth-dependent probing of membrane hydrophobic core. These photoactivable fatty acids or phospholipids derived from them, on incorporation in membranes followed by photolysis give rise to crosslinked products. Analysis of crosslinking sites can provide useful information on the nature of functional groups present at different depths in the membrane, an information difficult to obtain from alternative techniques. In this report we restrict our selves to studies related to artificial membranes. Khorana and co-workers [6] have reported the use of α-diazo-β-trifluoropropionyloxy and diazirinophenoxy groups linked to the ω-position of fatty acid chains as carbene precursors for studying lipid–lipid interaction in single bilayer vesicles. These results indicated that the photoactivable group on activation crosslinks to the neighbouring fatty acyl chain at a site which is proximal to the resulting carbene. In a similar approach Breslow and co-workers [7] have reported the use of benzophenone based fatty acids as conformational probes to study micelles and bilayers wherein the insertion site is again dictated by the transverse location of the photoactivable group in the membrane.

We have recently reported the use of diazofluorene (DAF) as a new photoactivable reagent for labeling membrane hydrophobic core in artificial and natural membranes [5]. These studies indicate that fluorenylidine generated on photolysis of DAF effectively labels the membrane spanning domains of integral membrane proteins in human erythrocytes. Here we report depth-dependent photolabelling of single bilayer vesicles with 9-diazofluorene-2-butyric acid.

2-Fluorenyl-butyric acid (I) was prepared by acylation of fluorene with succinic anhydride followed by Wolff-Kishner reduction of the intermediate keto acid [8]. The 2-fluorenyl-butyric acid (I) was then oxidised to 9-ketofluorene-2-butyric acid (II) by bubbling oxygen in a solution of (I) in 40% Triton B in pyridine (Fig. 1). The keto acid (II) on treatment with *p*-toluene sulfonyl hydrazide in *t*-butanol under reflux for 3 h gave the tosyl hydrazone of (II), which on subsequent treatment with 2.5 molar excess of sodium methoxide in methanol

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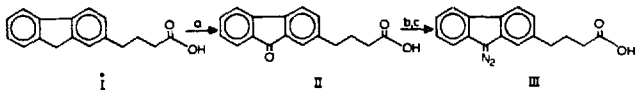

Fig. 1. Synthetic scheme for preparation of 9-diazofluorene-2-butyric acid; (a) 40% Triton B in pyridine, (b) *p*-toluene sulfonyl hydrazide in *t*-butanol, (c) sodium methoxide in methanol.

for 6 h at 50 °C gave 9-diazofluorene-2-butyric acid (III). UV-visible spectra of (III) gave absorption bands at 239, 295, 331 and 345 nm which are characteristic of diazofluorene [9] (Fig. 2). The infrared spectra gave the characteristic diazo absorption band at 2015 cm^{-1}. The final product (III) is very sensitive to light and was stored as an ethanolic solution at − 20 °C in dark. Photolysis of (III) in methanol could be easily followed by disappearance of the longer wavelength bands corresponding to diazofluorene nucleus and the resulting UV-visible spectra clearly indicated the isobestic points (Fig. 2).

Phosphatidylcholine (PC) was purified from egg yolk and single bilayer vesicles were prepared as reported earlier [5]. An alcoholic solution of 9-diazofluorene-2-butyric acid was then added to PC vesicles prepared in 100 mM NaCl, so that the final alcohol concentration was 1% v/v and the PC to probe molar ratio was 10 : 1. After incubation for 40 min in dark at 25 °C the sample was photolysed in a Rayonet minireactor using four 25 watt 3000 A lamps for 15 min. The photolysate was

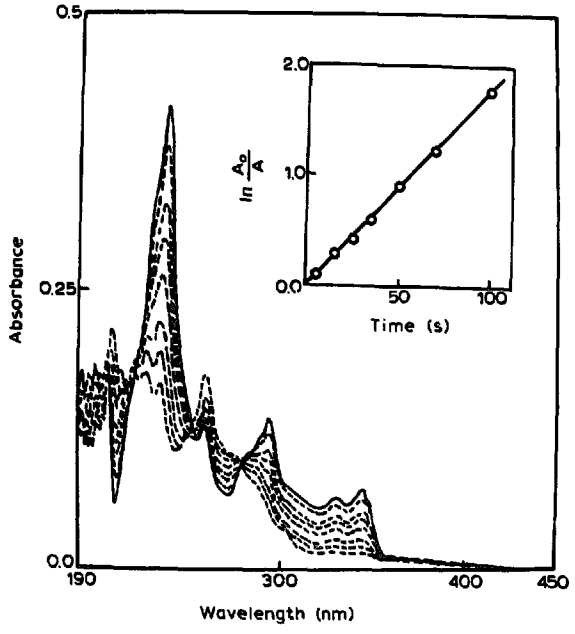

Fig. 2. UV-visible spectrum (———) of 9-diazofluorene-2-butyric acid. A 6 μM solution of the reagent (III) in methanol was photolysed for different time intervals (0, 5, 15, 25, 35, 50, 70 and 100 s) using a Rayonet RMR-500 miniphotoreactor with four 3000 Å lamps, and UV-visible spectrum (- - - - -) was recorded. The inset indicates first order kinetics $k = 0.018\ s^{-1}$ and $t_{1/2} = 38$ s for the disappearance of (III) on photolysis. A_0 and A are absorbance at 344 nm at times 0 and t, respectively.

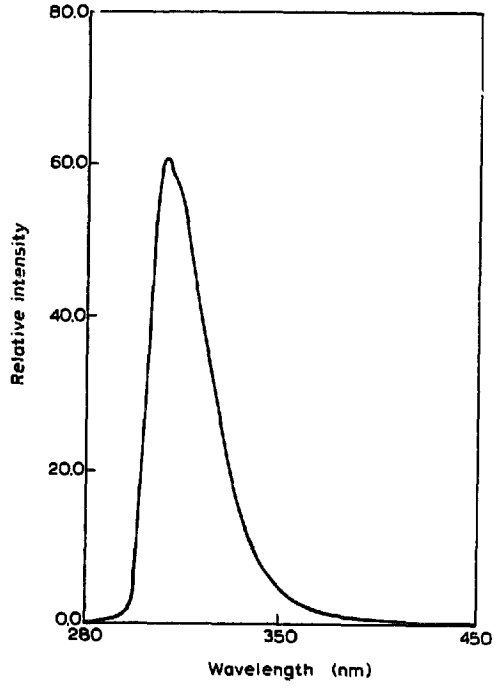

Fig. 3. Fluorescence spectrum of the insertion product formed between the photoprobe (III) and phosphatidylcholine (PC). The spectrum was recorded with excitation wavelength set at 272 nm.

then extracted with chloroform/methanol (2 : 1, v/v) and the organic extract was analysed by TLC on silica gel using chloroform/methanol/water (65 : 25 : 4, v/v) as the developing solvent system. The TLC plate gave two distinct spots with R_f values of 0.36 and 0.40 as observed by staining with phosphate sensitive spray reagent. These two spots could also be visualised by staining with iodine, but in this case an additional spot was observed on the solvent front. The material running on the solvent front was found to be the photorearrangement products of the probe (III). The R_f value of PC under these conditions was found to be 0.40 and thus the spot at R_f 0.36 was ascribed to be insertion product. The slight increase in polarity of insertion product relative to PC is justifiable in view of the fact that insertion product resulting from PC and the probe (III) would contain an additional carboxyl group. Similar results were obtained by HPLC analysis on a silica gel column using a on line UV and fluorescence detector. The polar fraction mentioned above was isolated by preparative TLC. We have reported earlier that while diazofluorene itself is nonfluorescent, on photolysis it inserts into solvents like cyclohexane and into PC in single bilayer vesicles and gives rise to fluorescent insertion products [9]. Thus fluorescence spectrum of the polar fraction mentioned above was recorded to confirm that it corresponds to the insertion product (Fig. 3). The presence of emission maxima at 320 nm indicated that the carbene generated on photolysis of (III) in PC vesicles had inserted in phosphatidylcholine.

Once it was established that the 9-diazofluorene-2-butyric acid inserts into PC single bilayer vesicles it was proposed to establish the site of insertion in the fatty acid chain. Earlier studies involving analysis of insertion sites in hydrocarbon chain using benzophenone based fatty acid [7] and phenyl carbene based fatty acid [8] have involved mass spectral analysis. In our hands this technique was not very successful as the mass spectra of insertion products was dominated by fluorenyl ion. Consequently we developed an independent method for determination of insertion site which takes advantage of the fact that the insertion product is fluorescent where as the starting materials, PC and the probe (III) are not fluorescent.

The orientation of fluorescent chromophores in membranes has been studied extensively using depth-dependent quenching of their fluorescence in membranes [10]. We have recently reported the synthesis of a series of fluorenyl fatty acids i.e. 2-fluorenyl-acetic, -butyric, -hexanoic and -octanoic acid, and determined their orientation in membranes by studying their fluorescence quenching in phosphatidylcholine vesicles using extrinsic and intrinsic quenchers [8]. These studies indicated that the fluorene chromophore in the fluorenyl fatty acids incorporated in PC vesicles is located at a depth which corresponds to the distance between the chromophore and the carboxyl group. In brief the fluorenyl fatty acids were found to be effective depth-dependent fluorescent probes for membranes. We have used this technique to determine the insertion site in the crosslinked product obtained on photochemical insertion of 9-diazofluorene-2-butyric acid (III) and PC in vesicles. The crosslinked product and two model compounds, 2-fluorenylbuytric acid and 2-fluorenylhexanoic acid were separately incorporated in PC vesicles in a molar ratio of 1 : 520 and their fluorescence quenching studied using an intrinsic quencher, 9,10-dibromostearic acid. Brominated fatty acids have recently proved very effective in determining transverse location of fluorescent chromophores in membranes [11–13].

A vesicle preparation containing 0.5 mM PC and 1.52 μM crosslinked product was taken for the quenching studies. A 5.4 mM solution of 9,10-dibromostearic acid in methanol was used for quenching studies and small aliquots (4 μl) were added successively to the vesicle preparation. After each addition of the quencher the sample was incubated for 45 min to ensure the uptake of the quencher before recording the emission spectrum. The Stern-Volmer plots for quenching of 2-fluorenyl-butyric and -hexanoic acid and the crosslinked product, in PC vesicles by 9,10-dibromostearic acid is given in Fig. 4. These data clearly indicated the following order of quenching, 2-fluorenylhexanoic acid > crosslinked product > 2-fluorenylbutyric acid. These results clearly indicated that 9-diazofluorene-2-butyric acid (III) successfully labels the fatty acyl chain of the

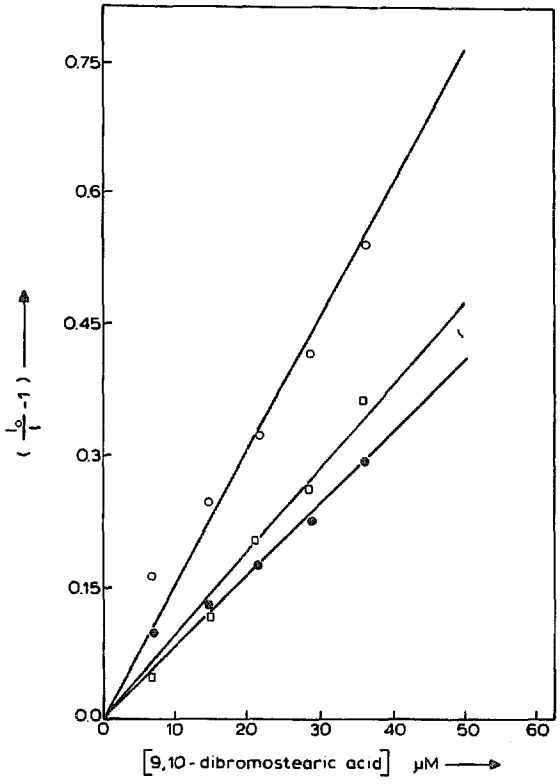

Fig. 4. Stern-Volmer plots of 9,10-dibromostearic acid quenching of, crosslinked product formed between the photoprobe (III) and phosphatidylcholine (□), 2-fluorenylbutyric acid (●) and 2-fluorenylhexanoic acid (○), incorporated in phosphatidylcholine vesicles. I_0 is the fluorescence intensity in the absence of quencher and I is the intensity in the presence of the quencher. Fluorescence spectra were recorded with excitation wavelength set at 272 nm. The excitation and emission slits were 5 and 10 nm, respectively.

phosphatidylcholine at a graded depth wherein the statistically average insertion site lies between carbon number 4 and 6.

In conclusion we will like to add that use of 9-diazofluorene-2-butyric acid (III) and related probes which are currently under preperation in our lab, are likely to prove useful for photolabelling of membrane hydrophobic core at different depths.

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