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THIOLS AND THE DIAZO GROUP IN PHOTOAFFINITY LABELS

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The photoactivable carbene precursor, 2-diazo-3,3,3-tri-fluoro-propionyloxy group, has been introduced recently for light-induced covalent crosslinking in studies of protein-phospholipid interactions in biomembranes. The diazo group in this reagent has now been shown to undergo reduction in the dark by a number of thiols (dithiothreitol, 2-mercapto-ethanol, cysteine and reduced glutathione) used commonly as protective agents for proteins. In contrast, thioglycolate did not cause significant reduction and, therefore, can be safely used as a protective agent. In vesicles formed from phospholipids containing the above photolabel in the fatty acyl chain, dithiothreitol and 2-mercaptoethanol caused reduction. However, cysteine and reduced glutathione caused insignificant reduction of the diazo group, presumably because of their non-permeant nature.

Currently, photoaffinity labeling is widely used in studies of molecular interactions in biological processes (1,2). The photosensitive groups commonly used produce, on irradiation, the highly reactive nitrene or carbene intermediates. An important requirement of the precursors to these intermediates is that they should be stable in the dark under the conditions that are subsequently used for photolysis. Reports have, however, appeared which point to considerable instability of nitrene as well as carbene precursors under certain conditions. Thus, the reduction of 8-azido-adenosine derivatives and of arylazides by thiols has been recorded (3-5). Similarly, the reduction of diazoalkanes to corresponding hydrazones by ammonium sulfide has been reported previously (6-8).

Recently, the preparation of phospholipids containing the photo-activable carbene precursor, 2-diazo-3,3,3-trifluoropropionyloxy group (9)

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Abbreviations used: DTT, dithiothreitol; GSH, reduced glutathione; TGA, thioglycolate.

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in the ω-position of sn-2 fatty acyl chains has been reported (10) (Ia, Fig. 1). Upon photolysis of the vesicles prepared from the phospholipids, extensive intermolecular crosslinking between phospholipid molecules was demonstrated (11,12). In extending this work to the study of phospholipid-protein interactions in membranes using the phospholipid, Ia, it was noted that certain thiols which are commonly added to protect proteins against inactivation, can cause reduction of the diazo group to the corresponding hydrazone. The present communication reports a study of this reaction and shows that while reagents such as dithiothreitol (DTT), 2-mercaptoethanol, cysteine and reduced glutathione (GSH) cause extensive reduction, thioglycolate (TGA) brings about only insignificant damage to the carbene precursor and is, therefore, the reagent of choice when a sulfhydryl protecting agent must be used. We also report the effect of thiols on the diazo group when the latter is present inside the phospholipid bilayer.

MATERIALS: 1-Palmitoyl-2-ω-(2-diazo-3,3,3-trifluoro-[1-^{14}C]-propionyloxy)-lauroyl-sn-phosphatidylcholine (Ia) was prepared as described elsewhere (10). Methyl-ω-(2-diazo-3,3,3-trifluoro-propionyloxy)-laurate (II) was prepared by a procedure similar to that used for the phospholipid Ia. Thiol reagents were commercial products, and their aqueous solutions were titrated with aqueous NaOH to the desired pH.

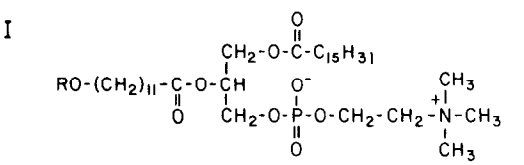
METHODS: The general conditions for treatment of phospholipid Ia with thiol reagents were as follows: 2 μg of Ia was suspended in 60 μl of a solution containing 5 μmoles of Tris-HCl, pH 8.0. It was then either sonicated by a bath type sonicator (Laboratory Supply Co.) for 1 min to form phospholipid vesicles, or converted to phospholipid-detergent micelles by the addition of 2.5 μmoles of potassium cholate, pH 8.0. After the addition of 1.5 μmoles of DTT or 3 μmoles of other thiol reagents, the reaction mixture (final volume 100 μl) was kept at room temperature in the dark.

TLC was performed on fluorescent silica gel plates (E. Merck, Cat. No. 5775) using chloroform:methanol:water (65:25:4, v/v/v) (Solvent I) for compound Ia, and diethyl ether:chloroform (1:9, v/v) (Solvent II) for compound II.

UV measurements were performed on a Cary 15 spectrophotometer, and IR spectra were recorded with Beckman IR4210. NMR spectra were recorded on a Varian T-60 NMR instrument using tetrametylsilane as the internal standard. Resonances (δ) in the NMR spectra are given in parts per million downfield from tetramethylsilane.

RESULTS

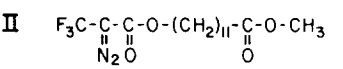
The Reaction of Phospholipid Ia (Fig. 1) Containing 2-diazo-3,3,3-trifluoropropionyloxy Group in the Presence of Thiol Reagents: Treatment of an aqueous phospholipid (Ia)-detergent complex with DTT resulted in the formation of one major product which moved slower on TLC in Solvent I (B, Fig. 2, lane 2) than the starting material (A, Fig. 2). The product (B) showed very strong UV absorption in contrast to the weak UV absorption of the phospholipid Ia (see below). Similar treatments of Ia with 2-mercaptoethanol and Cys (lanes 3 and 4, respectively in Fig. 2) or with thioglycerol (data not shown) all gave the same major product (B, Fig. 2) as judged by the identical mobility on TLC. GSH also produced the same product but with a slower rate (Fig. 2, lane 6 and Table I). In contrast, TGA caused only a barely detectable formation of B (Fig. 2, lane 5 and Table I). The phospholipids, 1-,2-dipalmitoyl-sn-phosphatidylcholine (Ib



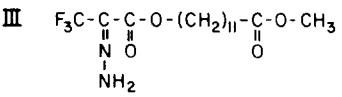
I

Ia : R = $F_3C-\underset{\underset{N_2}{\|}}{C}-\underset{\underset{O}{\|}}{C}-$

Ib : R = H



II



III

FIGURE 1: Structures of Compounds Used.
Ia: 1-palmitoyl-2-ω-(2-3,3,3-trifluoropropionyloxy)-lauroyl-sn-glycero-3-phosphatidylcholine. Ib: 1-palmitoyl-2-ω-hydroxylauroyl-sn-glycero-3-phosphatidylcholine. II: ω-(2-diazo-3,3,3-trifluoro-propionyloxy)laurate methyl ester. III: ω-(2-hydrazono-3,3,3-trifluoropropionyloxy)laurate methyl ester.

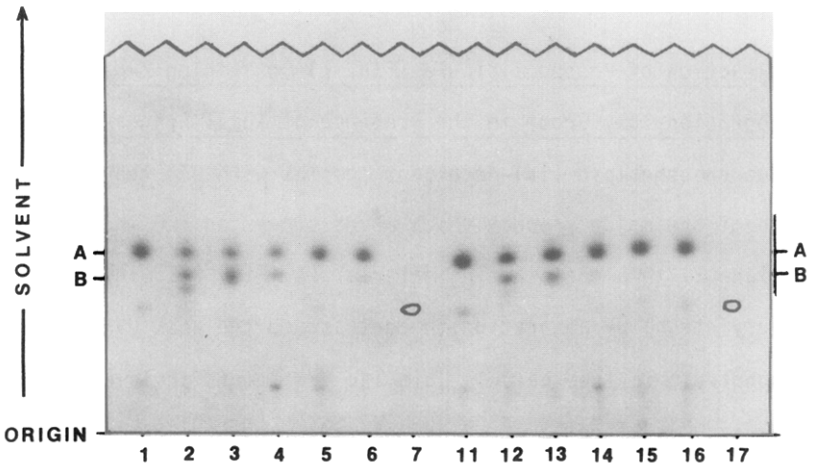


FIGURE 2: Action of Various Thiol Reagents on Compound Ia. ^{14}C-labeled Ia (2 µg) was incubated for 3 hr at room temperature with different thiol reagents as in Methods. After extraction of reaction mixtures by the method of Bligh and Dyer (13), in the cold, the organic phase was evaporated, the residue as a solution in methanol was applied on a silica gel plate and the latter developed in Solvent I. The autoradiogram is shown: lanes 1 to 7 are phospholipid-cholate micelles and lanes 11 to 17 are sonicated phospholipid vesicles. 1, 11, no thiol; 2, 12, DTT; 3, 13, 2-mercaptoethanol; 4, 14, Cys; 5, 15, TGA; 6, 16 GSH; 7, 17, compound Ib visualized by phospholipid spray (14). A shows the position of compound Ia, which has a weak UV absorption, and B shows the reduced product of Ia, which has a strong UV absorption.

in Fig. 1) as well as the photosensitive analog of the phospholipid Ia containing the diazirinophenoxy group (10) remained completely unchanged on identical treatment with DTT.

TABLE I

Formation of Product B (Fig. 2) from Ia by Different Thiol Reagents

Thiol Reagent	Reaction Time (hr)	Product B (%)	
		Phospholipid-cholate micelles	Phospholipid vesicles
DTT	3	28	24
Cys	3	24	3
TGA	3	0	3
TGA	12	4	6
GSH	3	6	2
GSH	12	29	5

Reactions were performed as in Fig. 2. The radioactive spots on TLC were cut out and their radioactivity was measured.

The formation of product B (Fig. 2) was also observed when sonicated vesicles of phospholipid Ia were treated with DTT (lane 12) or 2-mercaptoethanol (lane 13). In contrast, the use of thiols with charged groups such as Cys (lane 14) or GSH (lane 16) did not give any significant amounts of the product B (see also Table I). These results are presumably due to the permeant or non-permeant nature of the thiol reagents in the bilayer.

It is noted that additional products were sometimes noticable, as in lanes 2, 3 and 4 of Fig. 2. These products, however, were minor and often were not detected. Therefore, only product B was investigated further.

Characterization of Product B (Fig. 2): Since it was clear that product B was formed only from the Phospholipid Ia containing the diazotrifluoropropionyl group, further experiments to characterize the reaction leading to B were carried out using the more readily accessible fatty acid ester, II (Fig. 1). The compound (100 mg) was treated with 1 equiv. of $NaOH^{\dagger}$ and 10 equiv. of DTT, and after incubation at 45°C for 1 hr, the mixture was neutralized by the addition of HCl and lyophilyzed. The residue was resuspended in water and extracted with ether. The ether extract was purified by gel filtration through a Sephadex LH-20 column (2.5 x 80 cm) using chloroform:methanol (1:1, v/v) as the solvent. The product (60 mg) obtained was crystallized from petroleum ether. The product showed a single spot on TLC in solvent II (R_f 0.46: original compound II, R_f 0.60) and was ninhydrin-positive (pink). The melting point was 56°C. UV spectrum in ethanol is shown in Fig. 3 (ϵ_{260}, 9414). As is seen, a very large increase (~500-fold) in UV absorbance at 260 nm was observed relative to the starting compound. IR spectrum (Nujol) showed peaks at 3440 and 3300 cm^{-1} (NH_2), 1730 and 1690 cm^{-1} (C=O): NMR ($CDCl_3$); s 4.2 (t, J=7HZ, 2H, -O-CH_2-), 3.6 (s, 3H, -O-CH_3), 2.2 (t, J=7HZ, 2H, -CH_2-C(=O)-), 1.9-1.0 (m, 18H). High resolution mass spectral analysis showed M^+ at m/e 368.1928,

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†A kinetic experiment showed that the reaction rate was higher in more alkali conditions.

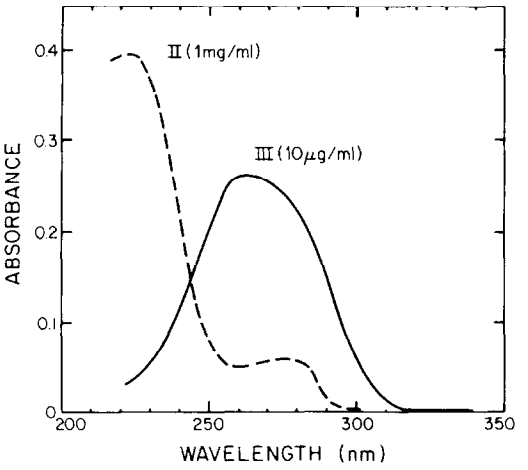

FIGURE 3: UV Absorption Spectra of Compounds II and III. -----: compound II (1 mg/ml) in ethanol; ——: compound III (10 mg/ml) in ethanol. Note that compound II is x100 concentrated than III.

calculated for $C_{16}H_{27}N_2O_4F_3$ = m/e 368.1923. Other fragment ions present in the spectrum are consistent with the structure III for the compound. Thus, the data conclusively showed the reduction of the diazo group in compound II to the corresponding hydrazone derivative III (Fig. 1).

Crosslinking Reactions Using Phospholipid Ia in the Presence of TGA: Phospholipid vesicles were formed from Ia by the cholate dialysis procedure (15) at 4°C with 50 mM Tris-HCl, pH 8.0,/0.3 M sucrose/20 mM histidine/2 mM ATP containing either 5 mM TGA or DTT. The two preparations were photolyzed as described previously (11). With TGA, phospholipid-phospholipid crosslinking was observed in an amount comparable to that obtained previously in the absence of thiol reagents. In contrast, with DTT, no crosslinked products were observed.

CONCLUDING REMARKS

Photosensitive carbene precursors containing the diazo group have been used in a variety of biochemical studies. The present work has demonstrated the vulnerability of these compounds to thiol reagents. The process

involves the reduction of the diazo group to the corresponding hydrazone. From a comparative study of a variety of thiol reagents (including mercaptosuccinic acid and 3-mercaptopropionic acid, which are not reported here), TGA has been found to be essentially safe and is recommended for work with soluble and membrane proteins which require the protection of sulfhydryl groups. In this laboratory TGA is used routinely in place of DTT in the delipidation and reconstitution studies of sarcoplasmic reticulum Ca^{2+}/Mg^{2+}-ATPase.

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REFERENCES

1. Jori, G. and Spikes, J.D. (1978) Photochem. Photobiol. Rev. 3, 193-275.
2. Chowdhry, V. and Westheimer, F.H. (1979) Ann. Rev. Biochem. 48, 293-325.
3. Cartwright, I.L., Hutchinson, D.W. and Armstrong, V.W. (1976) Nucleic Acids Res. 3, 2331-2339.
4. Staros, J.V., Bayley, H., Standring, D.N. and Knowles, J.R. (1978) Biochem. Biophys. Res. Comm. 80, 568-572.
5. Bayley, H., Standring, D.N. and Knowles, J.R. (1978) Tetrahedron Lett. 39, 3633-3634.
6. Dimroth, O. (1910) Ann Chem. 373, 336-370.
7. Wolff, L. (1912) Ann. Chem. 394, 23-59.
8. Wolfrom, M.L. and Miller, J.B. (1958) J. Amer. Chem. Soc. 80, 1678-1680.
9. Chowdhry, V., Vaughan, R. and Westheimer, F.H. (1976) Proc. Natl. Acad. Sci. USA 73, 1406-1408.
10. Gupta, C.M., Radhakrishnan, R. and Khorana, H.G. (1977) Proc. Natl. Acad. Sci. USA 74, 4315-4319.
11. Gupta, C.M., Radhakrishnan, R., Gerber, G.E., Olsen, W.L., Quay, S.C. and Khorana, H.G. (1979) Proc. Natl. Acad. Sci. USA 76, 2595-2599.
12. Gupta, C.M., Costello, C.E. and Khorana, H.G. (1979) Proc. Natl. Acad. Sci. USA 76, 3139-3143.
13. Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
14. Goswami, S.K. and Frey, C.F. (1971) J. Lipid Res. 12, 509-510.
15. Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477-5487.