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A new radioactive diazofluorene based heterobifunctional reagent

ANIL K. LALA*, H. F. BATLIWALA and R. M. MOGRE
Department of Chemistry, Indian Institute of Technology, Powai, Bombay 400 076, India.

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Abstract. Synthesis of a radioactive photoactivable heterobifunctional reagent, N-oxysuccinimide ester of 2-[^{14}C]glycyl carboxy-9-diazofluorene is described. This reagent on photolysis gives rise to a reactive carbene which rapidly inserts into solvents like methanol. The probe can be easily linked to aldolase which on photolysis gives rise to aldolase dimer, trimer and tetramer depending on the density of linked probe. This probe has also been linked to concanavalin A. The radioactive concanavalin A so obtained was incubated with erythrocyte ghosts and photolysed. The membrane protein analysis by gel electrophoresis indicated that concanavalin A has been covalently crosslinked to band 3.

Keywords. Photoactivable; heterobifunctional reagent; carbene; diazofluorene; aldolase; concanavalin A-band 3.

Introduction

Photoaffinity labelling has proved to be a powerful technique for studying biomolecular interactions (Bayley, 1984). Its use in identification of several receptor proteins has been well documented (Eberle and DeGran, 1985). This application involves the use of photoactivable heterobifunctional reagents, which incorporate a chemically reactive and a photochemically reactive site. The photoactivable site is usually a nitrene precursor *e.g.* aryl azides and several reagents belonging to this category have been reported (Ji, 1983). Despite the documented advantages of carbene precursors as photoactivable reagents (Bayley and Knowles, 1978; Brunner and Semenza, 1981) and their extensive use *e.g.* in labelling membrane bound proteins, very few carbene based heterobifunctional reagents have been reported (Nassal, 1983). We have recently reported N-oxysuccinimide ester of 2-carboxy-9-diazofluorene (NOH-CAB-DAF) as one such reagent (Mogre *et al.*, 1987). We report here a radioactive analogue of NOH-CAB-DAF which provides a sensitive method of monitoring and gives rise to aldolase crosslinking data which is similar to that obtained with NOH-CAB-DAF. Further, concanavalin A (ConA) was modified with this new reagent and used to study its crosslinking with band 3.

Materials and methods

All chemicals and solvents were commercial grades of highest purity and were further purified if required according to Perrin *et al.* (1980). UV-visible spectra were

*To whom all the correspondence should be addressed.
Abbreviations used: NOH-CAB-DAF, N-Oxysuccinimide ester of 2-carboxy-9-diazofluorene; ConA, concanavalin A; HPLC, high performance liquid chromatography; DAF, diazofluorene; NOH-[^{14}C]GLY-DAF, N-oxysuccinimide ester of 2-[^{14}C]glycylcarboxy-9-diazofluorene; R_t, retention time; SDS, sodium dodecyl sulphate; PAGE, Polyacrylamide gel electrophoresis; M_r molecular weight; PPO, diphenyl oxazole; POPOP, 1,4-bis-(5-phenyl oxazol-2-yl) benzene.

recorded on Shimadzu UV-260 spectrophotometer. High performance liquid chromatography (HPLC) was carried out on a Shimadzu LC-4A liquid Chromatograph using Shimpak CLC ODS column (6 × 150 mm) and methanol: water (80:20 v/v) as a mobile phase at a flow rate of 1 ml/min. All HPLC analyses were carried out under these conditions unless specified otherwise. Eluent was usually monitored at 230 nm where all fluorenyl compounds could be detected while monitoring at 350 nm detected only the diazo compounds as diazofluorene (DAF) and its analogues reported here absorb strongly around 350 nm. NOH-CAB-DAF was prepared from 2-carboxy-9-DAF as reported earlier (Mogre *et al*., 1987). [^{14}C]Glycine of specific activity 21·4 mCi/mmol was purchased from Bhabha Atomic Research Centre, Bombay. Radioactivity was determined on a LKB 1217 Rackbeta liquid scintillation counter using a toluene based scintillant (0·4% PPO, 0·025% dimethyl-POPOP). Photolysis was performed on Rayonet miniphotochemical reactor RMR-500 fitted with four 3500 Å lamps. Care was exercised in handling unphotolysed material and all operations were carried out using safe brown light. Samples were purged with a stream of nitrogen prior to photolysis. Aldolase type IV and ConA were purchased from Sigma Chemical Co., Missouri, USA. Crystalline suspension of rabbit muscle aldolase in 2·5 M $(NH_4)_2SO_4$ was extensively dialysed against 5 mM sodium phosphate buffer pH 7·4. The final concentration of aldolase was determined by absorbance at 280 nm, $E^{1\%}_{280}$ =9.38 (Donavan, 1964). Erythrocyte ghosts were prepared according to Steck and Kant (1974). Ghosts protein concentration was determined by modified Lowry procedure (Markwell *et al*., 1981). Electrophoresis was performed according to Fairbanks system (Fairbanks *et al*., 1971). Samples were applied to a 4% Polyacrylamide gel rods and run at a constant current of 3 mA/gel. After electrophoresis the radioactive gels were cut into 2 mm slices. The gel slices were then solubilized in 10 μl of water and 200 μl of Luma Solve (Lumac Systems) for 12 h and counted in toluene based scintillation fluid.

NOH-[^{14}C] GL Y-DAF

NOH-CAB-DAF (2·1 mg, 6 μmol) was dissolved in dry dioxan (0·5 ml) and aqueous sodium bicarbonate (2 μl of a 1·2 M solution) was added. It was then stirred with [^{14}C]glycine (25 μCi, 1·17 μmol) for 5 h at room temperature, poured into ice-cold water (10 ml) and carefully acidified with 0·05 N acetic acid (100 μl) at 0°C. It was then extracted with CH_2C1_2. The solvent was removed with a stream of nitrogen and the residue was taken in dry dioxan (0·5 ml). The UV-visible spectrum showed bands at 242, 305 and 372 nm, as observed in the case of authentic 2-glycylcarboxy-9-DAF. The radioisotope HPLC analysis was carried out at this stage and 1 ml fractions were collected. These fractions were counted on a liquid scintillation counter using toluene based scintillation fluid. The radioactivity elution profile derived from the counts in each fraction indicated that more than 80% of [^{14}C] glycylcarboxy-9-DAF is formed based on the retention time (R_t) of 2-glycylcarboxy-9-DAF(R_t = 5·1min).,

To the above dioxan solution (0·5 ml), N-hydroxy succinimide (0·8 mg, 6 μmol) and dicyclohexylcarbodiimide (1·2 mg, 6 μmol) were added. It was then stirred for 20 h at room temperature. The radio HPLC analysis was again carried out at this stage as before. The radioactivity elution profile derived from the counts in each

fraction indicated that more than 60% of the parent [^{14}C]glycine condensation product has been converted to the N-oxysuccinimide ester based on the R_t of authentic N-oxysuccinimide ester of 2-glycylcarboxy-9-DAF (R_t=10·6 min). The UV-visible spectrum showed bands at 250, 301 and 372 nm as observed in N-oxysuccinimide ester of 2-glycylcarboxy-9-DAF. This material could be easily purified further by HPLC and the specific activity of the material was found to be 21·1 mCi/mmol. The crosslinking experiments could also be carried out without purification by HPLC as at this stage the final reagents purity is 60% and on incubation with aldolase only the N-oxysuccinimide ester *i.e.* NOH-[^{14}C]-GLY-DAF is linked whereas the residual unreacted [^{14}C]GLY-DAF is removed during gel permeation chromatography on Sephadex G-25. Similar crosslinking of aldolase was observed even with 60% pure reagent.

Crosslinking of aldolase using N0H-[^{14}C] GLY-DAF

Aldolase (0·04 nmol, 0·133 μM) in 5 mM phosphate buffer pH 7·4 containing 0·1 M $NaHCO_3$, was treated in dark with 7·5 (7·4 nCi) and 37·5 (31·8 nCi) fold molar excess of NOH-[^{14}C]GLY-DAF. The probe was added as a dioxan solution, 1 and 5 μl respectively, to 0·3 ml of aldolase solution. The samples were then incubated for 1 h at room temperature and then applied to a Sephadex G-25 column and eluted with 50 mM phosphate buffer pH 7·4. Fractions appearing in the void volume were collected and photolysed for 3 min and analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Chemical modification of ConA with N0H-[^{14}C] GLY-DAF and its crosslinking to erythrocyte ghosts

A aliquot (1 μCi) from dioxan solution of the probe was added to 10·2 μg (0·4 nmol) of ConA in 5 mM phosphate buffer, pH 7·4 containing 0·1 M $NaHCO_3$ so that the final pH was 8·6. The probe to protein molar ratio was 1:7·5 and final dioxan concentration was 3·3% v/v. After incubation for 2h the whole solution was subjected to chromatography on a Sephadex G-25 column. Erythrocyte ghosts were equilibrated with 50 mM phosphate buffer, pH 7·4. The material appearing in the void volume, which corresponds to probe linked ConA, was added to erythrocyte ghosts (0·2 mg protein). After incubation for 30 min the whole preparation was photolysed for 3 min. It was then centrifuged at 15000 *g* for 30 min, washed with 5 mM phosphate buffer, pH 7·4 and subjected to SDS-PAGE(4%).

Results and discussion

The radioactive reagent, NOH-[^{14}C] GLY-DAF was prepared from NOH-CAB-DAF. The reagent was analysed by HPLC and was found to be identical to authentic NOH-GLY-DAF. The detailed synthesis and characterization of NOH-GLY-DAF will be reported elsewhere. The final reagent could be purified by HPLC and had a specific activity of 21·1 mCi/mmol. The UV-visible spectrum of NOH-[^{14}C]GLY-DAF is given in figure 1. NOH-[^{14}C]GLY-DAF is highly sensitive to light and heat. It should be handled carefully in a dark room and stored at – 20°C

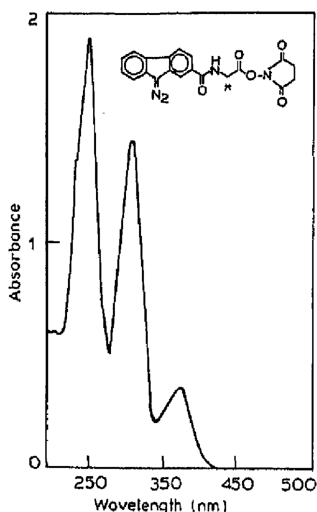

Figure 1. UV-visible spectrum of NOH-[14C]GLY-DAF.

in dry dioxan. Interestingly the HPLC analysis indicated that it is not always the photochemical degradation, but the chemical degradation of probes which predominates. Thus the N-oxysuccinimide ester group in both NOH-CAB-DAF (R_t= 11·03min) and NOH-GLY-DAF (R_t= 10·6 min) hydrolyse in methanol solution at 25°C to the parent acids, 2-carboxy-9-DAF (R_t=5·4 min) and 2-glycylcarboxy-9-DAF (R_t = 5·1 min). This hydrolysis in methanol takes place only if methanol contains traces of water. The hydrolysis is a slow reaction and approximately 10% hydrolysis per day was observed in a sample stored at 4°C in dark. The diazo group did not undergo decomposition under these conditions as estabilished by monitoring the eluant at 350 nm. The normal HPLC eluent is monitored at 230 nm but monitoring at 350 nm gives peaks only if the parent DAF moiety is intact In short, solutions of NOH-[^{14}C]GLY-DAF in dioxan or methanol should be prepared in anhydrous solvents taking care that moisture is not absorbed during preparation of the solution.

Crosslinking of aldolase using N0H-[^{14}C]GLY-DAF

The NOH-[^{14}C]GLY-DAF was linked to aldolase at molar ratio of 1:7·5 and 1:37·5. The unlinked probe was removed by gel permeation chromatography. The modified aldolase was then photolysed and analysed by SDS-PAGE (figure 2). At the lower molar ratio one observes largely the monomer along with small amounts of dimer, trimer and tetramer (figure 2A), whereas at higher molar ratio tetramer appears as the major band (figure 2B). Due to lower resolution of tetramer and higher oligomers of aldolase on SDS-PAGE(4%), Coomassie blue staining of aldolase crosslinked with NOH-CAB-DAF gives rise to a blur on top of the gel (Mogre *et al.*, 1987). The use of [^{14}C] labelled probe, NOH-[^{14}C]GLY-DAF permits a more clearer analysis indicating that higher oligomers are formed but the

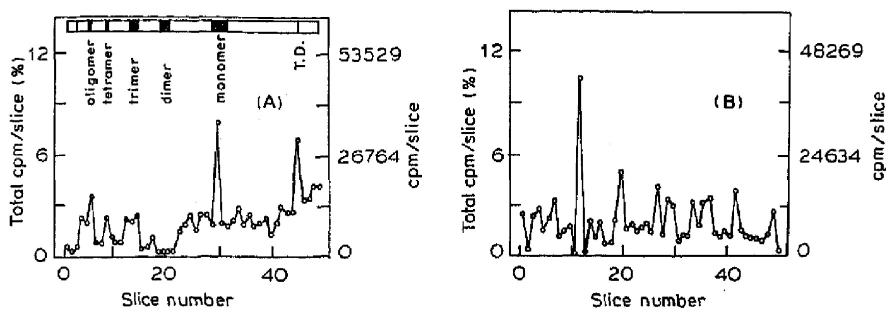

Figure 2. Photochemical crosslinking of aldolase modified with NOH-[^{14}C]GLY-DAF. Aldolase was linked to this probe by adding 7·5 and 37·5 molar excess of NOH-[^{14}C]GLY-DAF, the unlinked probe being removed by chromatography on Sephadex G-25. The material appearing in the void volume was photolysed and subjected to SDS-PAGE (4%). These gels were then cut into 2 mm slices and counted. (A), Lower probe concentration gel. (B), Higher probe concentration gel. The gel shown on top of (A) is a schematic of parallel gel run with crosslinked aldolase and stained with Coomassie blue.

tetramer is the major band, as expected from the native tetrameric state of rabbit muscle aldolase.

Rabbit muscle aldolase has been used in the past as a model protein for studying crosslinking. It normally exists as a tetramer comprising of 4 subunits with a M_r of 40000. Recently thiol specific fluorogenic heterobifunctional reagents based on azido coumarins have been used for crosslinking of aldolase (Ueno *et el.*, 1984). Using this reagent one observes largely the formation of aldolase dimer and barely observable trimer or tetramer. The use of NOH-CAB-DAF reported by us earlier (Mogre *et al.*, 1987) and the radioactive analogue reported here, NOH-[^{14}C]GLY-DAF, gives rise to a much higher degree of crosslinking possibly as a result of higher reactivity of carbenes.

Crosslinking studies of NOH-[^{14}C]GLY-DAF modified ConA with erythrocyte ghosts

ConA was modified with NOH-[^{14}C]GLY-DAF at 1:7·5 molar ratio and subjected to gel permeation chromatography to remove the unlinked probe. The modified ConA was incubated with erythrocyte ghosts and photolysed. The SDS-PAGE (figure 3) showed both ConA monomer and dimer with the latter appearing as the major peak. In addition one observes two small peaks corresponding to M_r 120,000 and 142,000 Da and some high M_r material running at the top of the gel. The 120,000 and 142,000 Da bands correspond to expected M_r of ConA-band 3 and ConA dimer-band 3 crosslinked products.

The lectin ConA is known to bind to erythrocytes and we chose this as a model system to assess the potential of our probe as this system has been investigated earlier using phenyl azide based heterobifunctional reagents *i.e.* 4-azidobenzoimidate (Ji, 1977) and N-oxysuccinimide ester of 4-azidosalicylic acid (Ji and Ji 1982). In both these cases a band corresponding to ConA dimer and a band which barely moved in the SDS-polyacrylamide gel was refered to as ConA crosslinked to its receptor, possibly band 3. Using NOH-[^{14}C]GLY-DAF modified ConA we also observed the ConA dimer observed by Ji (1977) and Ji and Ji (1982). In addition

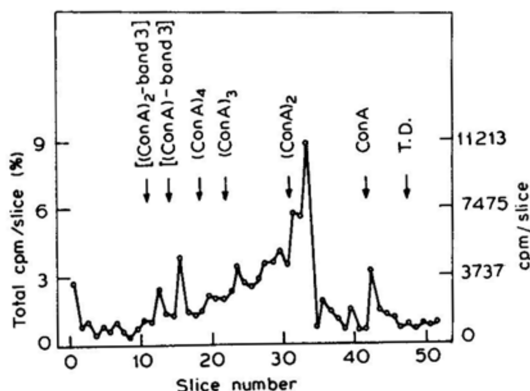

Figure 3. Photochemical crosslinking of ConA modified with NOH-[^{14}C]GLY-DAF to erythrocyte ghosts. The expected position of bands correspond to M_r of ConA monomer, dimer-(ConA)$_2$, trimer-(ConA)$_3$, tetramer-(ConA)$_4$, ConA crosslinked to band 3-[ConA-band 3] are marked on top. These R_f values were derived on the basis of semilog plot of standard M_r marker proteins run on a parallel gel and taking ConA monomer M_r as 25,500 and band 3 as 97,000.

two bands corresponding to M_r of crosslinked ConA-band 3 and ConA dimer-band 3 were observed besides some material which barely enters the gel (figure 3). These results clearly indicate that ConA is crosslinked to band 3 in erythrocytes. Further studies can be carried out to comment further on the nature of ConA-band 3 interactions in erythrocytes.

We will finally like to conclude that high reactivity of this reagent and the availability of the radioactive preparation should make this reagent very useful in protein crosslinking studies.

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