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A new carbene based heterobifunctional reagent

Photochemical crosslinking of aldolase

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The synthesis of a new photoactivatable heterobifunctional crosslinking reagent, the *N*-oxysuccinimide ester of 2-carboxy-9-diazofluorene, is described. The ability of the parent chromophore 2-carbomethoxy-9-diazofluorene to insert into cyclohexane and methanol has been established. The reagent has been linked to aldolase and the stoichiometry determined. Photolysis of the probe-linked aldolase indicated that photolysis was very rapid and that the photolysed product was constituted of crosslinked dimer, trimer and tetramer. Increase in concentration of probe linked to aldolase followed by photolysis gave rise to largely tetramer and higher oligomers of aldolase. The use of this carbene-based reagent vis a vis arylazide-based reagent for studying protein crosslinking is discussed.

Photoactivable group; Heterobifunctional reagent; Carbene; Diazofluorene; Aldolase crosslinking

1. INTRODUCTION

Chemical crosslinking of proteins using homobifunctional reagents has been a useful tool for studying various biomolecular systems [1]. However, the nonspecific nature of crosslinking of these reagents has of late led to a greater use of photoactivatable heterobifunctional reagents [2–5]. These reagents consist of a chemically reactive site and a photochemically reactive site. The protein of interest is first linked via the chemically reactive group to usually a lysine, arginine or a cysteine side chain. The modified protein now contains a photoactivatable group and thus can be used as a conventional photoaffinity labeling reagent. Various hormone receptors have been identified using this approach [6–8]. The efficiency of crosslinking is very important in such studies and therefore the choice of photoactivatable group is critical. Interestingly, the photoactivatable group used in heterobifunctional reagents is invariably a nitrene precursor like arylazides and only one carbene precursor based on trifluoromethylphenyldiazirine has been reported [9,10]. This is surprising in view of the fact that several advantages of the use of carbenes over nitrenes in photoaffinity labeling studies have been pointed out for quite some time [11,12].

We have reported the use of a carbene precursor, diazofluorene (DAF), as a photoactivatable reagent for labeling the membrane hydrophobic core [13,14]. Here we report a new heterobifunctional reagent, the *N*-oxysuccinimide ester of 2-carboxy-9-diazofluorene (NOH-CAB-DAF). This carbene-based reagent is conveniently synthesised and is photochemically highly reactive. Crosslinking of aldolase using this reagent has been demonstrated.

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2. MATERIALS AND METHODS

All chemicals and solvents were commercial grades of the highest purity and were further purified if required according to Perrin et al. [15]. UV-visible spectra were recorded on a Shimadzu UV-260 and IR spectra on a Perkin-Elmer 681 spectrometer. NMR spectra were recorded in $CDCl_3$ on a Hitachi R-600 or Varian XL-100 A spectrometer. HPLC was carried out on a Shimadzu LC-4A liquid chromatograph using a 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 DAF and all DAF analogs reported here absorb strongly around 350 nm. Aldolase type IV was purchased from Sigma. A 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, $E^{1\%}_{280} = 9.38$ [16].

2.1. *2-Carbomethoxy-9-diazofluorene*

2-Carbomethoxy-9-fluorenone was prepared from fluorene according to Rieveschi and Ray [17] with an overall yield of 50%, m.p. 180–181°C (lit. 181°C). This was converted into the hydrazone, m.p. 138–139°C and then oxidized with yellow HgO essentially according to Warren [18] to obtain 2-carbomethoxy-9-diazofluorene in 91% yield. HPLC R_t = 10.6 min, m.p. 112–113°C (lit. 112–113.5°C). UV (methanol): λ_{max} 247 nm (ϵ 49472), 304 (34400), 372 (10036). IR: 2070 cm^{-1} (diazo), 1730 cm^{-1} (ester carbonyl). NMR: δ 3.98 (s, 3-H, $-OCH_3$), 7.25–8.25 (7-H, aromatic).

2.2. *Photolysis of 2-carbomethoxy-9-diazofluorene in cyclohexane*

2-Carbomethoxy-9-diazofluorene (250 mg, 1 mmol) in dry, deoxygenated cyclohexane (25 ml) was photolysed with a pyrex filter in an annular photorector (Applied Photophysics) with a medium-pressure mercury lamp (400 W) till the UV-visible spectrum showed no absorption characteristic of diazo compound. The solvent was removed under reduced pressure to obtain 260 mg of crude product. This was separated by silica gel column chromatography. Elution with 50% benzene in petroleum ether gave 64.5 mg 2-carbomethoxy-9-cyclohexylfluorene in 28% yield, m.p. 151–152°C. UV (methanol): λ_{max} 290 nm (ϵ 19140), 301 (17760), 312 (19140). IR: 1720 cm^{-1} (ester carbonyl). NMR: δ 0.95–1.82 (m, 11-H, cyclohexyl -CH), 3.92 (d, 1-H, C-9 fluorene, J = 4 Hz), 3.96 (s, 3-H, $COOCH_3$), 7.21–8.31 (m, 7-H, aromatic). The mass spectrum indicated the molecular ion at m/z 306 and base peak at m/z 223 corresponding to the loss of cyclohexyl fragment. Elution with 75% benzene in petroleum ether gave 22 mg 2-carbomethoxyfluorenone.

2.3. *2-Carboxy-9-diazofluorene*

2-Carbomethoxy-9-diazofluorene (70 mg, 280 μmol) was dissolved in methanol (30 ml) with slight warming (around 40°C). It was then stirred with 3% methanolic KOH (2.5 ml) for 4.5 h at 45–50°C, poured into ice-cold water (50 ml) and filtered to remove any unhydrolysed ester. The filtrate was cooled to 0°C and carefully acidified with 0.05 N acetic acid to pH 4 maintaining the temperature at 0°C. The precipitated solid was filtered, washed with excess water and dried to obtain 67 mg of crude acid which was crystallized from methanol to obtain 54 mg 2-carboxy-9-diazofluorene in 82% yield. HPLC R_t = 5.4 min, m.p. 225–228°C. UV (methanol): λ_{max} 243 nm (ϵ 30096), 301 (18290), 361 (4918). IR: 2070 cm^{-1} (diazo), 1700 cm^{-1} (acid carbonyl). The acidification step should be carried out carefully, i.e. the temperature of the solution during acidification should not rise above 5°C and the strength of the acetic acid used should be no more than 0.05 N. If this step is not carried out carefully, the diazo group is displaced by acetic acid to give the 9-acetoxy-2-carbomethoxyfluorene which has an R_t of 6.8 min in the system used for HPLC analysis.

2.4. N-*Oxysuccinimide ester of 2-carboxy-9-diazofluorene (NOH-CAB-DAF)*

2-Carboxy-9-diazofluorene (25 mg, 106 μmol) was dissolved in dry dioxane (5 ml) and *N*-hydroxysuccinimide (13 mg, 110 μmol) was added. It was stirred with dicyclohexylcarbodiimide (22 mg, 106 μmol) for 20 h at room temperature.

The precipitated dicyclohexyl urea was filtered and the filtrate was evaporated to dryness with nitrogen. The residue was dissolved in CH_2Cl_2 (3 ml), filtered again and the whole process was repeated to remove all the dicyclohexylurea. Evaporation of the filtrate gave 30 mg of the crude *N*-oxysuccinimide ester. HPLC analysis of this material gave two peaks at 5.4 and 11.03 min corresponding to the starting acid and the corresponding ester. Attempts to purify the crude ester by basic alumina chromatography led to partial hydrolysis of the *N*-oxysuccinimide ester along with decomposition of the diazo compound as analysed by HPLC. The purification of the crude ester could be easily carried out by reverse-phase HPLC, R_t = 11.03 min, m.p. 163–65°C. UV (methanol): λ_{max} 250 nm (ϵ 37412), 305 (31322), 384 (5800). IR: 2080 cm^{-1} (diazo), 1780 cm^{-1} (imido carbonyl), 1750 cm^{-1} (ester carbonyl). NMR: δ 2.91 (s, 4-H, succinimidyl -CH_2-), 7.2–8.2 (7-H, aromatic).

2.5. *Crosslinking of aldolase using NOH-CAB-DAF*

Aldolase (1.4 mg/ml in 5 mM phosphate buffer, pH 7.4, containing 0.1 M $NaHCO_3$; final pH after addition of $NaHCO_3$, 8.4) was reacted in the dark with 20-, 60- and 100-fold molar excess of NOH-CAB-DAF. Thus 15, 47 and 78 μl of a 7.92 μM solution of the probe in dioxane was added to 0.7 ml (1 mg) of the above-mentioned aldolase solution and incubated in the dark for 2 h at room temperature. A control aldolase was treated in a similar way except that the probe was omitted. The samples after incubation were applied to a Sephadex G-25 column and the column was eluted with 50 mM phosphate buffer, pH 7.4. Fractions appearing in the void volume were used to quantitate the number of moles of reagent bound per mole of aldolase. The samples were then photolysed for 3 min and analysed by SDS-polyacrylamide gel electrophoresis [19].

3. RESULTS

2-Carbomethoxy-9-diazofluorene, easily prepared from the parent ketone 2-carbomethoxy-9-fluorenone, on hydrolysis gives 2-carboxy-9-diazofluorene. Reaction of this acid with *N*-hydroxysuccinimide gives the *N*-oxysuccinimide ester of 2-carboxy-9-diazofluorene (NOH-CAB-DAF). The UV-visible spectrum of NOH-CAB-DAF gives the characteristic diazofluorene absorption [13] beyond 300 nm, an important prerequisite for photoactivatable reagents.

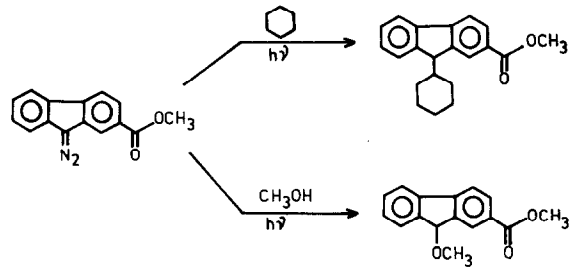

Fig.1. Photolysis of 2-carbomethoxy-9-diazofluorene in cyclohexane and methanol giving rise to the C-H and O-H insertion product, respectively.

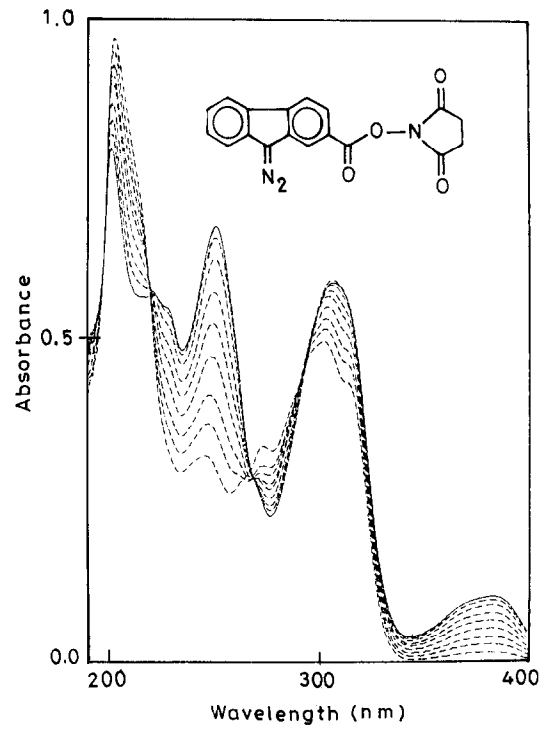

Fig.2. Time course for photolysis of NOH-CAB-DAF in methanol. A 75 μM solution in a pyrex tube was photolysed for different time intervals (0, 5, 15, 30, 50, 75, 105, 150, 210 s) using four very low flux 3500 A lamps in a Rayonet RMR-500 photoreactor and the UV-visible spectra recorded.

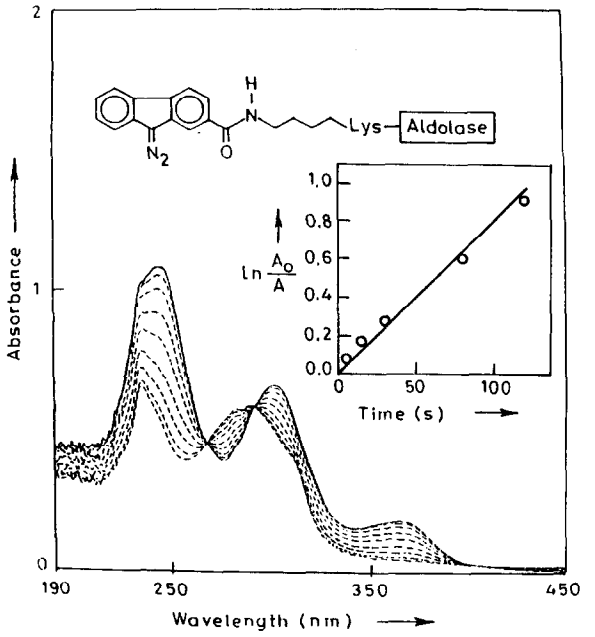

Fig.3. Time course for photolysis of aldolase linked to NOH-CAB-DAF in 50 mM phosphate buffer, pH 7.4. Aldolase was modified with NOH-CAB-DAF at a 1:20 molar ratio, the unlinked probe being removed by gel-permeation chromatography. The probe-linked aldolase was photolysed exactly as described in the legend to fig.2, for different time intervals (0, 5, 15, 30, 50, 80, 120, 170, 230 s), and the UV-visible spectra recorded and subtracted from the aldolase spectrum. (Inset) Kinetic analysis of the disappearance of the absorptions at time 0 and t. A schematic representation of the aldolase modified with the reagent is shown above.

In order to ensure the ability of this probe to generate a carbene on photolysis and give rise to an insertion product as in the case of diazofluorene [20–22], we decided to photolyse the parent chromophore 2-carbomethoxy-9-diazofluorene in cyclohexane. This photolysis gave rise to the C-H insertion product, 2-carbomethoxy-9-cyclohexyl-fluorene (fig.1). Similar photolysis in methanol gave the corresponding O-H insertion product, 2-carbomethoxy-9-methoxyfluorene. The NMR spectrum clearly indicated the C_9 methoxy protons as a three-proton singlet at 3.11 ppm besides the 2-carbomethoxy protons at 3.92 ppm and the C_9 proton at 5.61 ppm.

Photolysis of NOH-CAB-DAF in methanol was followed by UV-visible spectroscopy which indicated the disappearance of the 372 nm band (fig.2) and clearly showed the isosbestic points. Kinetic analysis indicated first-order kinetics with a $t_{1/2}$ of 84 s. NOH-CAB-DAF is highly sensitive to light, acids and heat and should be handled very carefully in a dark room and stored at −20°C.

The NOH-CAB-DAF could be easily linked to rabbit muscle aldolase by addition of a dioxane solution of the reagent to aldolase followed by gel-permeation chromatography to remove the unlinked probe. The modified aldolase appearing in the void volume was then analysed by UV-visible absorption spectroscopy. This spectrum clearly indicated characteristic absorption bands of carboxydiazofluorenene in the visible region, in which aldolase does not absorb. These results indicated that NOH-CAB-DAF has been linked to aldolase. The photolysis of this modified aldolase preparation could be followed by UV-visible spectroscopy. The absorption bands in the visible region disappeared on photolysis giving rise to first-order kinetics with a $t_{1/2}$ of 80.6 s (fig.3). This half-life value is very similar to that of 2-carbomethoxy-9-diazofluorene photolysis in methanol mentioned above. The stoichiometry of

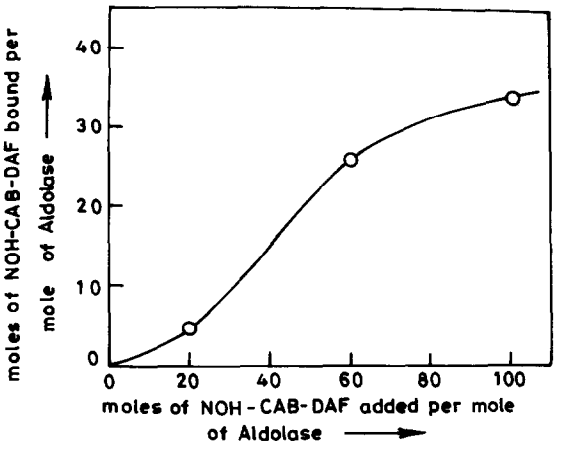

Fig.4. Concentration-dependent crosslinking of NOH-CAB-DAF to aldolase tetramer determined from UV-visible spectra of aldolase modified with 20-, 60- and 100-fold molar excess of probe, the unlinked probe being removed by gel-permeation chromatography. For calculation of stoichiometry it was assumed that the extinction coefficient of the probe at longer wavelengths is not changed on binding to aldolase. The degree of probe linking should therefore be viewed as relative and not absolute.

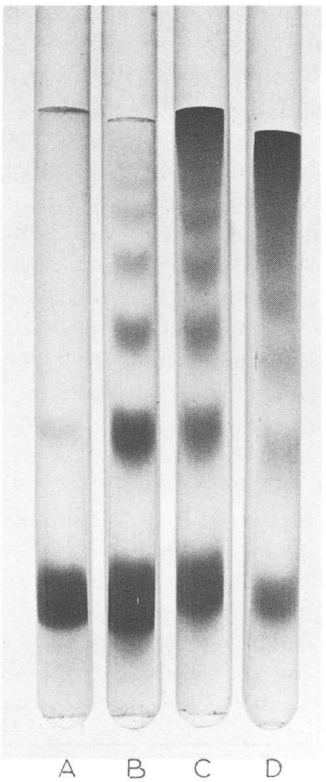

Fig.5. Photochemical crosslinking of aldolase modified with NOH-CAB-DAF by addition of 20-, 60- and 100-molar excess of the probe, the unlinked probe being removed by gel-permeation chromatography. The material appearing in the void volume was photolysed and subjected to SDS-polyacrylamide (4%) gel electrophoresis according to Fairbanks et al. [19]. (A) Control aldolase, no probe added; (B–D) gels for 20-, 60- and 100-molar excess probe added, respectively.

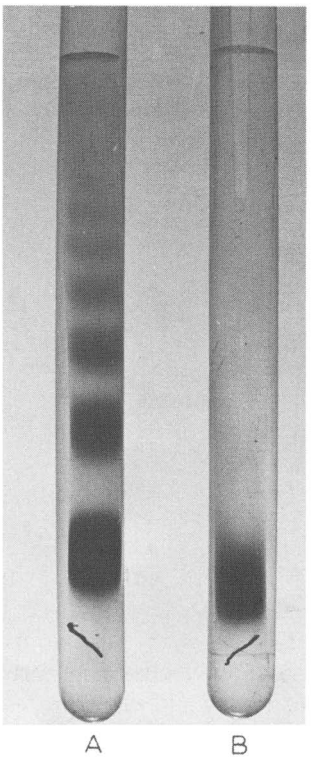

Fig.6. SDS-polyacrylamide (4%) gel electrophoresis of (A) unphotolysed and (B) photolysed sample of aldolase modified with a 20-molar excess of NOH-CAB-DAF as given in the legend to fig.5.

covalent attachment of carboxydiazofluorene to aldolase was determined by following the increase in intensity of visible region band at 364 nm in probe-linked aldolase. A concentration-dependent incorporation of NOH-CAB-DAF was observed (fig.4). At an aldolase to probe ratio of 1:20, 5 mol carboxydiazofluorene were linked per mol aldolase tetramer. Beyond 1:100 aldolase to probe ratio, the probe tends to precipitate out.

The NOH-CAB-DAF-modified aldolase was photolysed and analysed by SDS-polyacrylamide gel electrophoresis which indicated the formation of dimer (80 kDa), trimer (120 kDa), tetramer (160 kDa) and possibly higher oligomers of aldolase (fig.5B). As the number of moles of NOH-CAB-DAF linked to aldolase was increased, one could see an increase in generation of higher oligomers (fig.5C,D). The control aldolase sample did not show any crosslinking (fig.5A). The detection of absorption bands characteristic of carboxydiazofluorene in NOH-CAB-DAF-modified aldolase (fig.3) and the formation of aldolase oligomers on photolysis (fig.5) do indicate that NOH-CAB-DAF acts as an effective heterobifunctional photoactivatable reagent. On the basis of these results alone we cannot discard the possibility that a part of the crosslinking reaction could be chemical in nature, i.e. nucleophilic displacement of the diazo group by acidic residues like aspartic or glutamic acid in proteins. In order to investigate this point an independent experiment using aldolase modified with a 20-fold excess of NOH-CAB-DAF was carried out. The modified aldolase

was photolysed and a control sample was kept in the dark. Due to the highly photosensitive nature of probe-linked aldolase, these samples were analysed by SDS-polyacrylamide gel electrophoresis in the dark. The results (fig.6) clearly indicated that the unphotolysed sample was not crosslinked and thus that the formation of aldolase oligomers results from photochemical generation of a carbene.

4. DISCUSSION

In this paper we have described the synthesis and application of a new photoactivatable heterobifunctional crosslinking reagent, NOH-CAB-DAF. As a result of the successful labeling of the membrane hydrophobic core by diazofluorene (DAF) [13,14], we felt that suitable chemical modification of this molecule could lead to a useful carbene-based heterobifunctional reagent. Consequently, the probe mentioned above was synthesised by introducing a carboxyl group at C in DAF. Further the introduction of the carboxyl group into the DAF nucleus does not interfere with the C-H and O-H insertion properties of the resulting carbene as established by identifying the insertion products in cyclohexane and methanol. This probe can also be very rapidly photolysed, an important prerequisite in crosslinking studies with membrane receptors. Rapid photolysis with this reagent requires no sophisticated photolysis apparatus [23,24] and using an ordinary 400 W medium-pressure mercury lamp the time for photolysis can be reduced to less than 1 s. Reduced photolysis periods would help in surmounting both the diffusion problem and degradation of the biological systems being investigated [3,25].

Rabbit muscle aldolase was used as a model protein for studying crosslinking as it normally exists as a tetramer comprising four 40 kDa subunits. The degree of crosslinking could be varied depending on the number of probe molecules (NOH-CAB-DAF) linked to aldolase with higher oligomer appearing at high probe density on the proteins. Aldolase crosslinking has been reported using a bifunctional iodoacetamide analog [26] and various imido esters [27,28]. Our results are consistent with these studies. Recently thiol-specific fluorogenic heterobifunctional reagents based on azidocoumarins have been used for crosslinking of aldolase [29]. Using these reagents one observes largely the formation of aldolase dimer and barely observable trimer or tetramer. The use of NOH-CAB-DAF gives rise to a much higher degree of crosslinking when compared with the above-mentioned reagents. Despite the low reactivity of nitrenes relative to carbenes several azide-based reagents have been reported and successfully used [3,4]. The use of a more reactive carbene-based reagent like NOH-CAB-DAF is likely to give rise to higher protein crosslinking yields in much shorter time domains.

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