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2-[8-^{14}C]NAPHTHYL 2-DIAZO-3,3,3-TRIFLUOROPROPIONATE, A NEW CARBENE GENERATING REAGENT FOR PROBING HYDROPHOBIC MEMBRANE DOMAINS

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A new precursor of a lipophilic photolabel, 2-[8-^{14}C]naphthyl 2-diazo-3,3,3-trifluoropropionate (NADIT) has been synthesized. The suitability of the reagent for labeling the hydrophobic core of membranes is demonstrated by studying its reactivity in chromatophores of *Rhodospirillum rubrum* G-9$^+$. The label binds preferentially to the phospholipids and intrinsic membrane proteins. In isolated reaction centers treated with NADIT the hydrophobic subunits M and L are more labeled than the H subunit. The high reactivity, dark stability and ease of synthesis favors this very lipophilic reagent to identify the intrinsic hydrophobic sections of membrane proteins.

Segments of intrinsic membrane proteins, containing hydrophobic and therefore chemically inert amino acid side chains which are in close association with the lipid phase may be identified by labeling them either with group specific [1] or with photogenerated unspecific hydrophobic reagents [2–4]. The latter approach has several advantages. First, inert precursors are activated only by irradiation after their partition within the lipid bilayer. These reagents therefore react only with the membrane components that are located in the bilayer. Second, the use of photogenerated reagents allows time-dependent studies. Furthermore, some unspecific photolabels are capable of reacting with chemically inert polypeptide side chains [2]. Carbenes are most suitable for this purpose, because they have been shown to insert into carbon-hydrogen bonds [5,6]. Besides, they are more reactive than nitrenes and their insertion products are more stable than those formed from nitrenes [7,8].

Synthesis of most of the radioactive labeled carbene-generating reagents is time consuming and difficult to achieve (see, for example Refs. 9 and 10). However, we now succeeded to synthesize a new precursor of a hydrophobic photolabel, 2-[8-^{14}C]naphthyl 2-diazo-3,3,3-trifluoropropionate (NADIT), in a one step reaction. Proceeding from 2-diazo-3,3,3-trifluoropropionylchloride [11], preparation and purification of NADIT is achieved in one day. The reagent is stable and undergoes photolysis with very low Wolff rearrangement [12,13]. All chemicals and solvents used for the synthesis of NADIT were commercial grades of highest purity. Operations involving diazocompounds were performed under dim red light. 20 μmol (3.4 μl) of 2-diazo-3,3,3-trifluoropropionylchloride (Pierce Chemicals) were esterified with 15 μmol 2-naphthol (0.5 mol/l in pyridine; Fig. 1) containing between 7 and 10 μCi 2-[8-^{14}C]naphthol (CEA; spec. act. 10–15 Ci/mol). After keeping the reaction mixture 15 h in the dark at room temperature, pyridine was evaporated. The colorless residue containing crude

Abbreviations: NADIT, 2-[8-^{14}C]naphthyl 2-diazo-3,3,3-trifluoropropionate; TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; INA, 5-[^{125}I]iodonaphthyl-1-azide; SDS, sodium dodecyl sulphate.

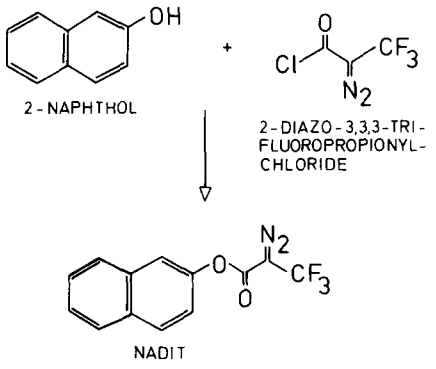

Fig. 1. Reaction scheme for the synthesis of 2-[8-^{14}C]naphthyl 2-diazo-3,3,3-trifluoropropionate.

NADIT was dissolved in ethanol and the label purified by TLC (Merck, Silica gel 60, layer thickness 0.2 mm, with fluorescent indicator; methylenchloride/pentane, 1 : 1, v/v). The position of the reagent on the plate was localized by its characteristic fluorescence quenching (R_f 0.75). The spot was scraped off, NADIT extracted twice with ethanol and dried under a gentle stream of nitrogen. The ultraviolet, infrared and mass spectra of the compound were entirely consistent with the assigned structure.

Mass spectrum: *m/e* 280 (M^+, $C_{13}H_7O_2N_2F_3^+$), *m/e* 252 ($C_{13}H_7O_2F_3^+$), *m/e* 127 ($C_{10}H_7^+$), infrared spectrum: 3060 cm^{-1} (aryl–C–H), 2145 cm^{-1} (CO–CN_2), 1730 cm^{-1} (aryl ester).

Synthesis yield determined either spectroscopically or by liquid scintillation counting was between 45 and 60%, specific radioactivity of the reagent between 0.45 and 0.55 Ci/mol. Its stability was tested periodically by TLC and subsequent thin-layer scanning (Berthold LB 2723 II). When stored at −20°C, the label is stable for months (self decomposition during 4 weeks is less than 5%). Fig. 2 shows the absorption spectrum of NADIT before and after photolysis at 254 nm. The decrease in absorbance at about 235 nm during illumination for 5 min demonstrates the rapid photolysis of the diazogroup. Hydrophobicity of NADIT was tested in the two-phase system water/cyclohexane (1 : 1, v/v). Label (6 μmol, 2.7 μCi) was dissolved in 1 ml of cyclohexane before adding 1 ml of water. After extensive shaking for 4 h only 0.5% of NADIT was found in the water phase.

Chromatophores and reaction centers of the carotinoidless mutant of *Rhodospirillum rubrum* G-9$^+$ were prepared according to Ref. 14. Chromatophores (20–28 mg · ml^{-1}) or isolated reaction centers (1 mg · ml^{-1}) were suspended either in 10 mM Tris-HCl buffer (pH 8) or 10 mM potassium phosphate buffer (pH 7). NADIT was added in ethanol/dimethylsulfoxide (1 : 1, v/v) (final concentration of ethanol and dimethylsulfoxide less than 3% each, concentration of NADIT between 3 and 5 mM). After being transferred into a quartz cuvette (0.2 mm path length), the suspensions were stirred at 20°C for 30 min in the dark. Irradiation was done at the same temperature with a low pressure mercury lamp (Hanau TNN 15/32 without filter; distance between sample and light source 5 cm) at 254 nm for 20 or 30 min. The completeness of photolysis was routinely checked by TLC with the organic extract of an aliquot of the reaction suspension. After photolysis the chromatophores were washed with bovine serum albumin buffer to remove not covalently bound reagent, the labeled reaction centers were dialyzed extensively [15].

Distribution of the label in the various components of the chromatophore membrane was determined. Polypeptides were separated on SDS-polyacrylamide gel electrophoresis [16]. Lipids, quinones and pigments were isolated from pro-

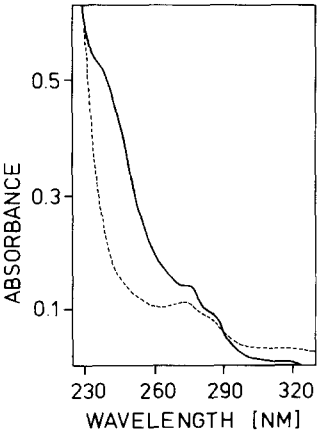

Fig. 2. Absorption spectrum of a 0.3 mM NADIT solution (in ethanol) before (solid line) and after illumination for 5 min (dashed line) recorded with a Kontron Uvikon 810 spectrophotometer.

TABLE I

DISTRIBUTION OF NADIT, TID AND INA BETWEEN MEMBRANES AND AQUEOUS BUFFER AT 20°C

Labeled and washed chromatophores were treated with a 10-fold volume of acetone/methanol (7:2, v/v) and the radioactivity was determined by liquid scintillation counting. The activity (%) is the activity found in membranes after removing unbound label (in % of total activity in the photolysis sample).

Reagent	Buffer	Activity (%)
NADIT	10 mM potassium phosphate (pH 7)	45.9
NADIT	10 mM Tris-HCl (pH 8)	48.8
TID	10 mM potassium phosphate (pH 7)	42.5
INA	10 mM potassium phosphate (pH 7)	> 80.0 [a]

[a] From Odermatt [17].

teins by organic extraction and further purified by TLC as described in ref. 14. Radioactivity on the chromatogram was determined in a thin-layer scanner, in the isolated proteins after washing them twice in cold organic solvent [14] and subsequent solubilisation by liquid scintillation counting.

Table I shows the labeling results in chromatophores of *Rhodospirillum rubrum* G-9$^+$. NADIT, and for comparison the well known TID [9] seem to have a similar affinity to the hydrophobic membrane core, whereas INA is more lipophilic [15,17]. Optimized experiments showed that the extent of labeling depends on membrane and label concentration in the photolysis sample and the duration as well as the intensity of illumination (not shown). In contrast, buffer composition and pH have little influence on labeling efficiency. Phospholipids are labeled up to 40%, whereas the protein fraction contains between 5 and 8% of the initial activity added.

Fig. 3 shows the distribution of radioactivity among the polypeptides of labeled reaction centers after separation by electrophoresis. The M and L subunit of the reaction center are clearly more labeled than the H subunit, confirming their hydrophobic nature and proposed localization in the membrane as shown with other lipophilic labels [15,18,19]. Although reaction centers contain highly photosensitive components, protein denaturation, aggregation or pigment changes have not been found either resulting in spectral shifts or in a changed SDS-gel pattern.

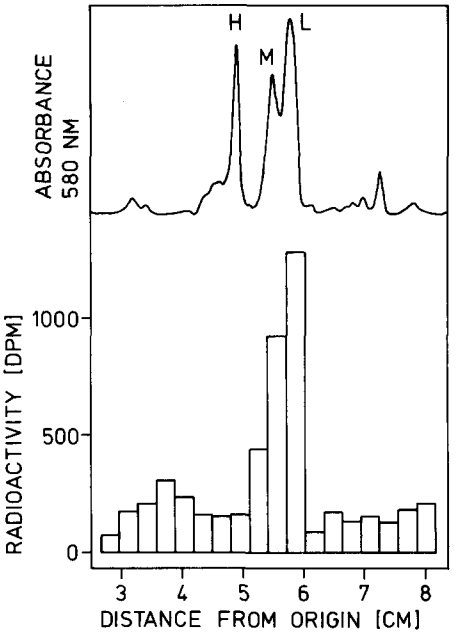

Fig. 3. Binding of NADIT to the polypeptides of reaction centers. After electrophoresis of labeled isolated reaction centers gels were stained with Coomassie blue, then one or two tracks cut out and sliced into 3 mm pieces. The radioactivity in each sample was determined after Protosol treatment at 45°C by liquid scintillation counting.

Besides the incorporation of the label into proteins, TLC of organic extracts from labeled chromatophores and reaction centers showed highly labeled phospholipids. Bacteriochlorophyll *a*, ubiquinone and rhodoquinone carried also radioactivity. Photolabeling of extracted and purified pigments and quinones in organic solution (acetone/light petroleum, b.p. 60°C (1:2, v/v)) was also successfully achieved (not shown).

We suggest that the new reagent is an excellent tool to identify domains of intrinsic proteins that are in contact with the lipid phase. Peptide mapping and determination of the radiolabeled amino acids within their sequence will give more insight into the structure and assembly of such membrane proteins in the hydrophobic core.

Acknowledgements

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