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Topography of Rhodopsin in Retinal Rod Outer Segment Disk Membranes. Photochemical Labeling with 1-Azidopyrene†

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ABSTRACT: 1-Azido[^{3}H]pyrene ([^{3}H]AP) has been synthesized with high specific radioactivity (3 Ci/mmol) and used to photochemically label retinal rod outer segment disk membranes. The reagent reacts with rhodopsin and a $M_r \approx 240000$ protein as well as with membrane lipids. When [^{3}H]AP–rhodopsin is digested with thermolysin in the disk membrane, both membrane-bound fragments of rhodopsin, F1 and F2, are found to contain [^{3}H]AP. Reaction of the reagent appears to be restricted to the lipophilic surface of rhodopsin inasmuch as the presence of the nitrene scavenger glutathione in the aqueous medium does not significantly reduce ^{3}H incorporation into rhodopsin. Labeled F1 and F2 were prepared, their cyanogen bromide peptides partially separated, and specific radioactivities determined. A factor of 4.4-fold in specific radioactivities of peptide pools was found, which suggests that some specificity has been shown in the reaction of [^{3}H]AP toward different surfaces of rhodopsin.

Rod cells of the retina, which are responsible for dim light and black and white vision, contain disk membranes which contain the photoreceptor protein rhodopsin. Rhodopsin comprises more than 95% of the intrinsic membrane protein of this highly specialized membrane (Smith et al., 1975; Krebs & Kühn, 1977). It has been proposed that rhodopsin may fulfill its function as a visual transducer by serving as a transmembrane calcium channel (Hagins, 1972) or by interacting at its hydrophilic surface with soluble rod cell enzymes (Hubbell & Bownds, 1979). A knowledge of rhodopsin's organization and topography in the membrane may help us in understanding its mode of action. We have previously employed the hydrophilic nitrene precursor *N*-(4-azido-2-nitrophenyl)-2-aminoethane[^{35}S]sulfonate as a probe for those regions of rhodopsin which are present at an aqueous surface (Mas et al., 1980). In the present study, we report the synthesis and application of 1-azido[^{3}H]pyrene as a probe for the hydrophobic topography of rhodopsin in the rod cell disk membrane.

A variety of hydrophobic nitrene or carbene precursors has been used in attempts to label hydrophobic regions of membrane proteins [reviewed in Chowdhry & Westheimer (1979); Jori & Spikes, 1979)]. Klip & Gitler (1974) have employed 1-azido[^{3}H]naphthalene and 1-azido-4-[^{125}I]iodobenzene, and Bercovici & Gitler (1978) have introduced 5-[^{125}I]iodonaphthyl azide. Limitations of the use of many lipophilic labeling reagents have been noted by Bayley & Knowles (1978). 1-Azidopyrene has been prepared and its photolysis studied (Yamaoka et al., 1972; Sumitani et al., 1976). To our knowledge, its synthesis and purification have not been described in detail, and it has not been previously prepared in radioactive form. 1-Azidopyrene has been previously employed in a study of *Escherichia coli* membranes (Nieva-Gomez & Gennis, 1977). A similar reagent, pyrenesulfonyl azide, has been used to study protein subunits of the acetylcholine receptor (Šator et al., 1979).

A preliminary report has appeared describing the use of the reagent 1-azido[^{3}H]naphthalene to label rhodopsin in disk membranes (Klip et al., 1976). In the study reported in this paper, we have used azido[^{3}H]pyrene of high specific radioactivity to label disk membranes, and we have prepared defined proteolytic fragments of rhodopsin. Distribution of label within peptides of these fragments varies severalfold, is consistent with the specificity of labeling expected by using a lipophilic probe, and complements results previously obtained with a hydrophilic probe (Mas et al., 1980).

Experimental Section

Materials

The following were obtained from Sigma Chemical Co.: β-MSH,[1] DTE, DTT, glutathione, concanavalin A–Sepharose

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4B (Con A–Sepharose), Sephadex LH20-100, and Sephadex LH60-120. Octyl-β-D-glucopyranoside (octyl glucoside) and thermolysin were purchased from Calbiochem-Behring Corp. Other chemicals and their suppliers were the following: cyanogen bromide and ethylenimine, Pierce Chemical Co.; [^{14}C]formaldehyde (42 Ci/mol, diluted to 104 mCi/mol), New England Nuclear; absolute ethanol, U.S. Industrial Chemical Co.; 1-aminopyrene, Aldrich; hydroxylapatite (DNA grade Bio-Gel HTP), Bio-Rad. All other chemicals used were the highest chemical purity available.

Sodium dodecyl sulfate (Sipon WD from Alcolac, Inc., Baltimore, MD) was recrystallized from 80% ethanol. Acrylamide (Eastman) was recrystallized from chloroform. TrTAB was synthesized from tridecyl bromide (Columbia Organics, Columbia, SC) and trimethylamine (Eastman) according to the procedure of Hong & Hubbell (1972). Sodium cyanoborohydride (Aldrich) was recrystallized from acetonitrile and methylene chloride (Jentoft & Dearborn, 1979). The white powder was stored in a desiccator over P_2O_5. 11-*cis*-Retinal was the gift of Hoffmann-LaRoche. The solid was dissolved in ethanol and its concentration determined spectrophotometrically by using $E^{1\%,1cm}_{380nm}$ = 878 (Hubbard et al., 1971).

Methods

All operations involving the use of 1-azido[^{3}H]pyrene, rod outer segment disk members, or rhodopsin were performed under dim red light at 4 °C unless otherwise specified.

Preparation of Disk Membranes. Bovine eyes were collected at a local slaughterhouse and kept in the dark on ice for transport to the laboratory. Retinas were dissected, frozen by dropping into liquid nitrogen, and stored until needed in tightly wrapped vials at −80 °C. Rod outer segment disk membranes were prepared as described by Smith et al. (1975). Membranes were used immediately or were stored at −20 °C under argon.

Preparation of Rhodopsin. Rod outer segment fragments were prepared from commercially available bovine retinas (American Stores, Inc., Omaha, NE) by using the method of Papermaster & Dreyer (1974) as modified by McDowell & Kühn (1977). Rhodopsin was purified by chromatography on hydroxylapatite by using the detergent TrTAB (Hong & Hubbell, 1973).

Reductive Methylation of Disk Membranes. Reductive methylation was based on the procedure of Jentoft & Dearborn (1979). A disk membrane suspension containing 1 mg of rhodopsin in 0.5 mL of Hepes buffer (0.1 M, pH 7.5) was combined in the dark with 0.5 mL of 5 M sodium cyanoborohydride and let stand for 5–10 min at room temperature. The reaction was initiated by addition of 5 μL of [^{14}C]formaldehyde and allowed to proceed for 2.5 h with occasional agitation by hand. Disk membranes were pelleted by centrifugation (36400*g*, 20 min) and washed 3 times by suspension in buffer A followed by centrifugation. Disk membranes were then incubated with [^{3}H]AP as described below.

Reaction of Disk Membranes with 1-Azido[^{3}H]pyrene. Aliquots of the [^{3}H]AP solution in benzene–ethanol (9:1 v/v) were transferred to a 30-mL beaker, and the solvent was removed under a stream of argon. The [^{3}H]AP was redissolved in a minimal volume of ethanol and disks in buffer A were added. Rhodopsin concentration was 0.6 mg/mL, and the percent of ethanol did not exceed 2%. The molar ratio of [^{3}H]AP to rhodopsin was 1:7.8. The suspension was stirred gently for 15 min under a blanket of argon. At the end of this incubation, the suspension was centrifuged (34800*g*, 20 min), the supernatant discarded, and the pellet resuspended in buffer A at 0.6 mg/mL rhodopsin.

For the photolysis procedure, the beaker containing the disk membranes with [^{3}H]AP was placed in a water bath at 4 °C. It was purged with argon and overlaid with a Corning glass filter (CS No. 7380, % T < 0.5% at 334 nm). Photolysis was carried out with a GE 275-W sunlamp placed 10 cm above the sample. The disk suspension was gently stirred. Samples were removed at timed intervals during photolysis and centrifuged (36400*g*, 20 min) to recover the membrane pellet.

Polyacrylamide Gel Electrophoresis. Membrane samples were dissolved in a $NaDodSO_4$–βMSH solution (Papermaster & Dreyer, 1974) and submitted to polyacrylamide gel electrophoresis by the procedure of Fairbanks et al. (1971). Each sample was run in duplicate; one gel was stained with Coomassie blue and the other was frozen and sliced into 1-mm pieces (Mickel gel slicer, Brinkmann, Inc.). Gel slices were dissolved by incubating with 0.5 mL of 30% H_2O_2 in glass vials with plastic-lined caps at 60 °C for 4 h. A detergent–xylene scintillation cocktail was added (Anderson & McClure, 1973), and radioactivity was determined on a Searle Mark III or Beckman LS7000 liquid scintillation counter.

Incorporation of [^{3}H]AP into Lipid. For investigation of whether lipids were labeled by [^{3}H]AP, disk membranes containing 6 mg of rhodopsin were labeled as described above. Following photolysis, the membranes were extracted under dim red light with CH_3OH–$CHCl_3$–H_2O (10:5:4, 15 mL) as described by Holtzman & Gillette (1968), and the organic phase was sampled for radioactive counting and an aliquot examined by thin-layer chromatography on silica gel G in $CHCl_3$–CH_3OH–HAc–H_2O (25:15:4:2) according to the method of Parker & Peterson (1965). Lipids were visualized by staining with iodine vapor. Each channel was scraped from the thin-layer plate, in 1-cm portions, and mixed with scintillation fluid, and the radioactivity was determined. In addition to the experimental sample, the following controls were used: (1) membranes containing [^{3}H]AP which were not photolyzed, and (2) [^{3}H]AP and buffer photolyzed in the absence of membranes. The products were extracted and added to disk membranes.

Digestion with Thermolysin (Analytical Scale). Both unreacted disks and [^{3}H]AP-labeled disks (containing ~200 μg of rhodopsin) were suspended in Tris–acetate buffer (0.1 M, pH 7.5) and pelleted by centrifugation (36400*g*, 15 min). The procedure was repeated, and the pellets were resuspended at ~2 mg/mL rhodopsin in Tris–acetate buffer (0.1 M, pH 7.5, 2 mM $CaCl_2$). Thermolysin was added (2% w/w), and the digestion mixture was incubated overnight in the dark at room temperature. Digestion was terminated by the addition of 10 μL of 0.2 M Na_2EDTA. The digested membranes were pelleted and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis as described above.

Large-Scale Preparation of [^{3}H]AP-F1 and -F2 Fragments of Rhodopsin. Disk membranes containing 20 mg of rhodopsin were labeled with [^{3}H]AP as described above, using a 2-min photolysis time.

(1) Regeneration of [^{3}H]AP Rhodopsin. Labeled disks were suspended and pelleted 3 times in potassium phosphate buffer

[1] Abbreviations used: OAc, acetate; AP, 1-azidopyrene; buffer A, 67 mM potassium phosphate buffer, pH 7.0, and 0.1 mM in Na_2EDTA; buffer B, 50 mM Tris–acetate, pH 6.9, and 1 mM each in $CaCl_2$, $MgCl_2$, and $MnCl_2$; β-MSH, β-mercaptoethanol; DTE, dithioerythritol, DTT, dithiothreitol; Na_2EDTA, disodium ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; TrTAB, tridecyltrimethylammonium bromide; Tris, tris(hydroxymethyl)aminomethane.

(pH 7.0, 1 mM in $MgCl_2$, Na_2EDTA, and DTT). After the final wash, the membranes were suspended in the same buffer at 2 mg/mL rhodopsin and to this was added a 3 × molar excess of 11-*cis*-retinal (in 100 μL of ethanol). Regeneration was allowed to proceed for 3 h in the dark at room temperature. Labeled disks were mixed with an equal amount (20 mg) of unlabeled disks.

(*2*) *Thermolysin Digestion* (*Preparative Scale*). Digestion with thermolysin was performed as described above, but thermolysin was added at 8% w/w rhodopsin.

(*3*) *Chromatographic Purification of* [^3H]*AP F1–F2 Complex*. Labeled F1–F2 complex (20 mg) was purified by chromatography on Con A–Sepharose. A 0.9 × 20 cm column of Con A–Sepharose was prepared in buffer B (50 mM Tris–acetate buffer, pH 6.9, and 1 mM in $CaCl_2$, $MgCl_2$, and $MnCl_2$). Regenerated disk membranes were washed in buffer B and suspended in it at a rhodopsin concentration of 1 mg/mL. Solid octyl glucoside was added to a final concentration of 60 mM. Chromatography was performed as previously described (Mas et al., 1980). Pooled fractions containing [^3H]AP F1–F2 complex were dialyzed against several changes of cold deionized water. The precipitated F1–F2 complex was harvested by centrifugation.

(*4*) *Separation of* [^3H]*AP-F1 and -F2 Fragments*. The F1–F2 complex was bleached, reduced, and aminoethylated as previously described (Hargrave, 1977). The lyophylized material was dissolved in a small volume of 88% formic acid, diluted with 2 volumes of ethanol, and centrifuged to clarify. Chromatography was performed on a column of Sephadex LH-60 (1.5 × 100 cm), equilibrated, and eluted with 88% formic acid–100% ethanol (30:70).

(*5*) *Cyanogen Bromide Peptides of* [^3H]*AP-F1 and -F2*. Pooled column fractions containing the separated F1 and F2 were individually rotary evaporated, dissolved in 100% formic acid, and then diluted to 70% formic acid to yield protein concentrations of approximately 10 mg/mL. Cyanogen bromide was added, 3× the weight of protein, and the reaction was performed in a sealed container protected from light at room temperature for 24 h. The reaction mixture was then degassed carefully under high vacuum and applied directly to the Sephadex LH-60 column described above. Pool IV (Figure 6B) from the chromatography of F2 cyanogen bromide peptides was examined further. The pool was rotary evaporated, dissolved in a small volume of 88% formic acid, diluted with 2 volumes of ethanol, and chromatographed on a 1.5 × 100 cm column of Sephadex LH-20 in 88% formic acid:100% ethanol (30:70).

Preparation of 1-Azidopyrene (*General Methods*). Melting points were taken on a Fisher–Johns hot stage apparatus and are uncorrected. Infrared data were obtained on a Beckman IR 12 spectrometer. Mass spectral data and exact mass determinations were obtained by using Varian MAT CH-5 and 731 spectrometers. Microanalysis was performed by the microanalytical service of the University of Illinois.

Radiochemical purity was determined by thin-layer chromatography on Eastman chromatosheets (No. 6061). The labeled material was spotted on top of unlabeled carrier. After development, the carrier spot was visualized (iodine vapor or UV light), and the chromatogram was cut into 1-cm strips and placed in minivials for determination of radioactivity.

Preparation of Nonradioactive 1-Azidopyrene. 1-Aminopyrene (0.5 g, 2.3 mmol) was dissolved in a solution of 10 mL of water, 10 mL of acetone, and 0.2 mL of 12 N hydrochloric acid, cooled in an ice–water bath, and diazotized by slow addition of sodium nitrite (0.16 g, 2.4 mmol). The diazonium salt solution was stirred for 1 h at 0–5 °C and then decomposed by addition of a solution of sodium azide (1.6 g, 24 mmol) in 5 mL of water. After being stirred an additional 2 h at room temperature, the aqueous solution was extracted with benzene. The benzene layers were combined, washed with water and saturated aqueous sodium bicarbonate, and dried ($MgSO_4$), and the solvent was removed under reduced pressure to yield 0.48 g (86%) of 1-azidopyrene: mp 105 °C dec; IR (KBr) 2165 (s, N_3), 2135 (s, N_3), 1295 (m), 833, 710 cm^{-1}; UV max (methanol) 357 nm (ε 49 000), 340 (38 000), 283 (40 000), 246 (54 000); mass spectrum (10 eV), *m/e* (relative intensity) 243 (39, M^+), 216 (19), 215 (100, $M^+ - N_2$), 214 (22). Anal. Calcd for $C_{16}H_9N_3$: N, 17.27. Found: N, 17.07.

Preparation of 1-Azido[^3H]*pyrene*. 1-Aminopyrene was N-acetylated prior to bromination to yield dibromo-*N*-acetyl-1-aminopyrene. This compound was subjected to catalytic hydrogenation with $^3H_2$ (New England Nuclear) to yield *N*-acetyl-1-amino[^{3}H]pyrene. Deacetylation followed by diazotization and treatment with azide yielded 1-azido-[^{3}H]pyrene.

(*1*) *N-Acetyl-1-aminopyrene*. To a solution of 1-aminopyrene (0.5 g, 2.3 mmol) in 25 mL of benzene was added dropwise a solution of acetic anhydride (0.26 g, 2.5 mmol) in 2 mL of benzene. After 4 h, the light green precipitate was collected, washed with benzene, and dried to yield 0.52 g (88%) of *N*-acetyl-1-aminopyrene: mp 260–261 °C; IR (KBr) 3500 (NH), 1650 (C=O) cm^{-1}. Anal. Calcd for $C_{18}H_{13}NO$: C, 83.38; H, 5.05; N, 5.40. Found: C, 83.60; H, 4.68; N, 5.48.

(*2*) *Dibromo-N-acetyl-1-aminopyrene*. *N*-Acetyl-1-aminopyrene (0.2 g, 0.7 mmol) was suspended in a solution of 10 mL of chloroform and 30 mL of acetic acid, and a solution of bromine (0.26 g, 1.62 mmol) in 5 mL of chloroform was added dropwise, with rapid stirring, over a 5-h period. Water was added and the tan slurry extracted with ethyl acetate. The organic layer was washed with brine, dried ($MgSO_4$), and evaporated under reduced pressure to yield 0.29 g (90%) of dibromo-*N*-acetyl-1-aminopyrene as a tan solid. Recrystallization from chloroform gave tan crystals: mp 294 °C; IR (KBr) 3550 (NH), 1655 (C=O) cm^{-1}; mass spectrum (70 eV) *m/e* (relative intensity) 419 (30), 417 (64, M^+), 415 (32), 377 (50), 375 (100), 373 (50), 339 (13), 337 (13). Anal. Calcd (exact mass determination) for $C_{18}H_{11}ONBr_2$: 416.9018. Found: 416.9210.

(*3*) *N-Acetyl-1-amino*[^3H]*pyrene*. Dibromo-*N*-acetyl-1-aminopyrene (41.7 mg, 0.1 mmol) was dissolved in 12 mL of tetrahydrofuran and 4 mL of methanol, 15 mg of 5% palladium on carbon was added, and the mixture was hydrogenated at room temperature under 1 atm of tritium gas for 2 days. Labile tritium was removed under vacuum by using methanol as solvent. Methanol was added, and the catalyst was removed by filtration. The solution was evaporated under reduced pressure and the product taken up in 10 mL of methanol. The yield was 0.407 Ci of *N*-acetyl-1-amino[^{3}H]pyrene (76% radiochemical purity; dichloromethane, two developments, R_f 0.27).

(*4*) *1-Amino*[^3H]*pyrene*. To a solution of *N*-acetyl-1-amino[^{3}H]pyrene (80 mCi, 5.05 mg, 19 μmol) in 1 mL of methanol was added potassium hydroxide (8 mg, 0.14 mmol), and the solution was refluxed under a nitrogen atmosphere for 5 days. Water was added and the aqueous solution extracted with diethyl ether. The ether was evaporated with a stream of nitrogen and the residue dissolved in 1 mL of methanol. Column chromatography (silica gel, dichloromethane) gave 21 mCi of 1-amino[^{3}H]pyrene in 70% radiochemical purity (dichloromethane, one development, R_f 0.53).

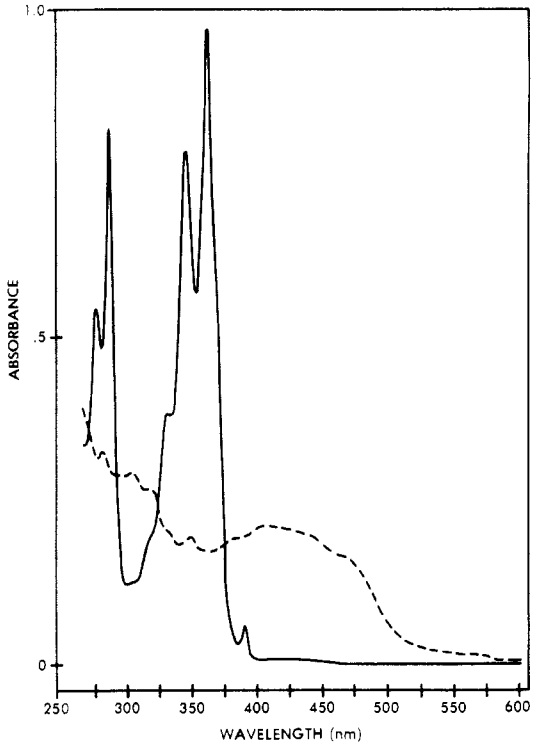

FIGURE 1: Absorption spectrum of 1-azidopyrene before and after photolysis. The absorption spectrum of 1-AP, 2×10^{-5} M in chloroform, was determined before photolysis (—) and following 3-min exposure to a 275-W sunlamp (---).

The product was free of higher R_f radiochemical impurities and was used without further purification.

(5) *1-Azido[^{3}H]pyrene.* The entire procedure was performed under reduced illumination. A benzene–ethanol solution of 1-amino[^{3}H]pyrene (21 mCi, 1.06 mg, 4 μmol) was dried under a stream of nitrogen. The residue was dissolved in 1 mL of acetone and 2 mL of 0.3 N hydrochloric acid, cooled to 0–5 °C, and diazotized by slow addition of 1 mL of a 0.22 M solution of sodium nitrite. The diazonium salt solution was stirred for 20 min and then decomposed by addition of 2 mL of a 0.21 M solution of sodium azide. After 1 h at 0–5 °C, the aqueous solution was extracted with diethyl ether, the ether evaporated with a stream of nitrogen, and the residue dissolved in 1 mL of methanol. Purification by column chromatography (dichloromethane, silica gel) yielded 1.02 mCi of 1-azido[^{3}H]pyrene in 97% radiochemical purity (dichloromethane, one development, R_f 0.76). Further elution of the column gave an additional 15 mCi of tritiated 1-azidopyrene in 78% radiochemical purity.

Results

Properties of 1-Azidopyrene. The absorption spectrum of AP and its photolysis products is presented in Figure 1. Azidopyrene possesses a 283-nm absorption band characteristic of many azido compounds (Treinin, 1971), which is nearly absent following 3-min photolysis. Azidopyrene is very soluble in nonpolar organic solvents such as cyclohexane, benzene, and chloroform in which our spectra were determined. Although AP is only sparingly soluble in ethanol, the limited amounts of AP which we needed to add to membrane suspensions could be dissolved in a small volume of ethanol.

Time Course of Incorporation of 1-Azidopyrene into Rhodopsin. Disk membranes were reductively methylated by using [^{14}C]formaldehyde. This introduced ^{14}C into rhodopsin so that the time course of incorporation of [^{3}H]AP could be followed by measuring the isotope ratio (Figure 2). Maximal

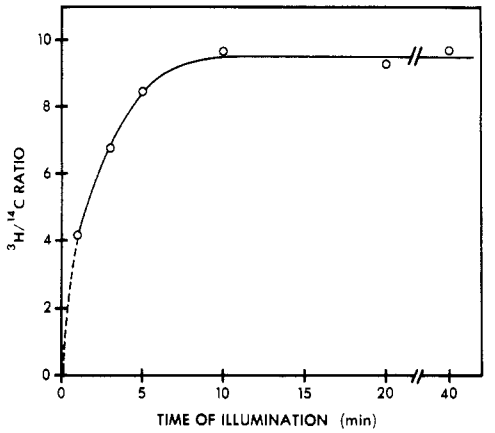

FIGURE 2: Time course of incorporation of 1-azido[^{3}H]pyrene into [^{14}C]rhodopsin in disk membranes. Disk membranes, reductively methylated by using [^{14}C]formaldehyde, were incubated with 1-azido[^{3}H]pyrene and photolyzed for various times. Membranes were solubilized in $NaDodSO_4$ and subjected to polyacrylamide gel electrophoresis, and the gels were stained with Coomassie blue to visualize the rhodopsin band. The corresponding rhodopsin-containing region was excised from unstained duplicate gels and its ^{3}H and ^{14}C content determined.

incorporation of AP into rhodopsin is reached after 10-min photolysis, with half-maximal incorporation by 2 min. Since long-term illumination is damaging to rhodopsin (Mas et al., 1980), all of our subsequent photolysis experiments were performed for 2 min.

Incorporation of 1-Azidopyrene into Disk Membranes. Disk membranes were labeled with AP by using standard conditions (2-min photolysis). Labeled membranes were examined by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3A). The bulk of the radioactivity present in the membranes (76%) migrates with the tracking dye and presumably includes products of AP photolysis and AP reaction with lipids. Fully 12% of the radioactivity recovered from the gel is found in rhodopsin and its dimer and trimer. (In control experiments in which the photolysis step was omitted or in which prephotolyzed reagent was added to membranes, negligible amounts of label were incorporated into protein.) Label was also incorporated into the $M_r \approx 240\,000$ disk membrane protein (Papermaster et al., 1976). This suggests that both rhodopsin and the high molecular weight protein are intrinsic membrane proteins embedded in the lipid bilayer. Under the conditions of this experiment, there is very little high molecular weight aggregated material containing label at the top of the gel (Figure 3A, slices 1 and 2). The amount of this material increases when larger quantities of AP are used to label the same quantity of membranes (D. Smith, unpublished results). From the experiment shown in Figure 3A, we calculate that approximately one rhodopsin molecule in 210 has been labeled by AP.

Incorporation of 1-Azidopyrene into Lipid. For determination of whether AP reacts with lipids in the disk membrane, reacted disk membranes were extracted with chloroform/methanol, and the extract was examined by thin-layer chromatography. In control experiments, photolysis was omitted or the reagent was photolyzed prior to adding to membranes. Extracts from both control samples showed that greater than 98% of the radioactivity migrated at the solvent front of the thin-layer plate. When [^{3}H]AP was photolyzed with membranes, fully 16% of the extracted label migrated below the solvent front in regions where phospholipids chromatograph (Figure 4). The chemically small amounts of products formed and their heterogeneity preclude further characterization, but it seems reasonable to conclude that they represent reaction

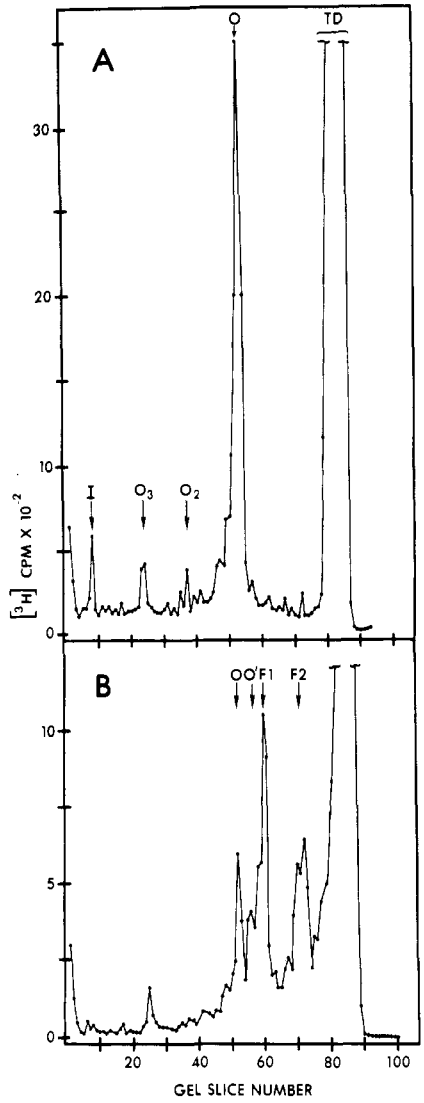

FIGURE 3: (A) $NaDodSO_4$–polyacrylamide gel electrophoresis profile of disk membranes labeled with 1-azido[^{3}H]pyrene. Disk membranes were reacted with 1-azido[^{3}H]pyrene, and an aliquot containing 50 μg of rhodopsin was solubilized and submitted to electrophoresis. Unfixed, unstained gels were cut into 1-mm slices and solubilized for determination of radioactivity. Gel top is slice 1, TD = tracking dye, O = opsin, O_2 = opsin dimer, O_3 = opsin trimer, I = the $M_r \approx 240000$ protein (positions of proteins determined from stained gels). (B) $NaDodSO_4$–polyacrylamide gel electrophoresis of disk membranes labeled with [^{3}H]AP and digested with thermolysin. Unstained gels were cut into 1-mm slices and solubilized for radioactive counting. The gel top is slice 1; tracking dye was located in gel slices 79–87. O = opsin; O', F1, and F2 are membrane-bound proteolytic fragments of opsin. The positions of polypeptide chains were determined from duplicate stained gels.

products of [^{3}H]AP with phospholipids, and not reagent or its nonlipid photolysis products.

Is Labeling by AP Confined to within the Lipid Bilayer? If the photolyzed AP were to expose its nitrene moiety at the surface of the lipid bilayer, some portions of the external aqueous-exposed surface of rhodopsin might become labeled. If any appreciable portion of label incorporation into rhodopsin occurred at its aqueous surface, the presence of a scavenger molecule in the aqueous medium should reduce label incorporation into rhodopsin. We chose glutathione as an appropriate scavenger molecule which has been reported to react with both nitrenes and azides (Bayley & Knowles, 1978; Staros et al., 1978). Under conditions in which AP was incorporated into rhodopsin to the extent of 2.8×10^5 cpm/mg, the presence of 2 mM glutathione in the reaction mixture only reduced AP

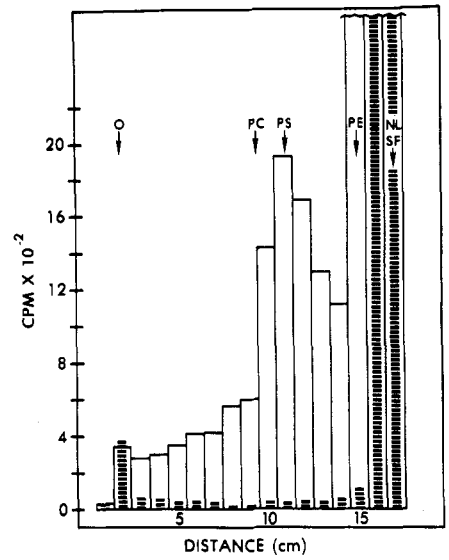

FIGURE 4: Radioactivity profile of thin-layer chromatogram of chloroform/methanol extract of disk membranes reacted with 1-azido[^{3}H]pyrene: O = origin, SF = solvent front; PC, PS, PE, and NL are the positions of phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and neutral lipids, respectively. Portions ($\sim$1 cm^2) of the silica gel were removed for radioactivity determination. Open bars represent the experimental sample, and striped bars are for a sample of [^{3}H]AP photolyzed in the absence of disk membranes (see Experimental Section).

incorporation into rhodopsin by 4.3%. This suggests that very little AP labeling of rhodopsin occurs at the external aqueous membrane surface and that AP is confined in its reaction to the lipid bilayer.

Both Membrane-Bound Fragments of Thermolysin-Digested Rhodopsin Are Labeled by AP. For further characterization of the nature of [^{3}H]AP incorporation into rhodopsin, [^{3}H]AP-labeled disk membranes were subjected to limited proteolytic digestion by thermolysin. The membrane-bound digestion products F1 and F2 were formed as well as an intermediate, O', observed previously (Hargrave & Fong, 1977). All of these membrane-bound digestion products were found to contain the ^{3}H label (Figure 3B). This suggests that the proteolysis products O', F1, and F2 all contain regions which are embedded in the lipid bilayer.

Distribution of [^{3}H]AP among Rhodopsin's Peptides. In order to further characterize the distribution of [^{3}H]AP incorporation into rhodopsin, we first sought to prepare the labeled F1 and F2 fragments in quantity. Rhodopsin in labeled membranes was regenerated by addition of 11-*cis*-retinal, and the membrane-bound rhodopsin was exhaustively digested by thermolysin in order to produce the two large proteolytic fragments. Membranes were dissolved in a solution of octyl glucoside and the noncovalent F1–F2 complex was purified by chromatography on Con A–Sepharose (data not shown). Sulfhydryl groups of the polypeptides were modified, and the F1 and F2 fragments were separated by column chromatography in formic acid/ethanol (Figure 5). F1 is found in pool I and F2 in pool II; they are characterized by their position of migration by $NaDodSO_4$–polyacrylamide gel electrophoresis and by their characteristic amino acid compositions (Pober & Stryer, 1975; E. Juszczak, personal communication). Each of the fragments was then reacted with cyanogen bromide, and the peptides produced were partially separated by column chromatography in formic acid/ethanol (Figure 6). Inspection of the profiles of peptide material and radioactivity show that the extent of label incorporation is not uniform for all peptides; thus there has been some selectivity in incorporation of AP. For better quantification of the extent of labeling, fractions

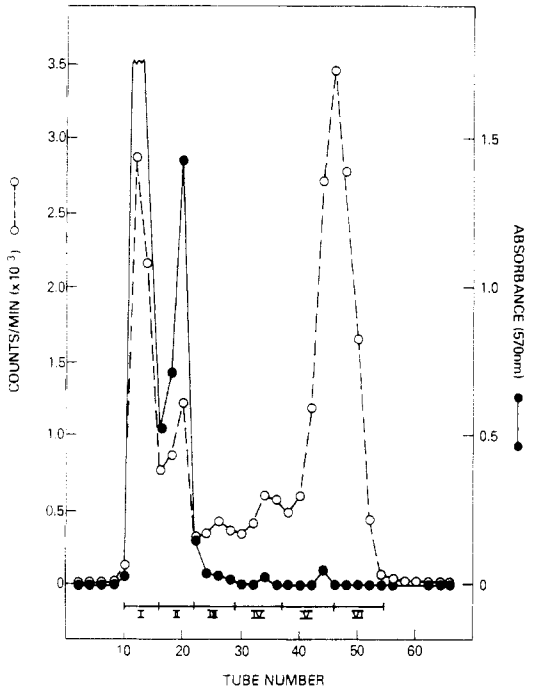

FIGURE 5: Sephadex LH-60 column chromatography of [^{3}H]AP-labeled F1 and F2 fragments of rhodopsin. Disk membranes labeled with [^{3}H]AP were digested with thermolysin, and the F1–F2 complex was purified on Con A–Sepharose. The F1–F2 complex was aminoethylated and applied in 2.5 mL to a 1.5 × 100 cm column of Sephadex LH-60 equilibrated in 88% formic acid:100% ethanol (30:70). Approximately 3-mL fractions were collected by drop counting. Aliquots (25 μL) of alternate tubes were taken for acid hydrolysis and ninhydrin analysis (570 nm). Six pools (I–VI) were made as shown.

Table I: Specific Radioactivities of Rhodopsin Peptides

pool	fragment: CNBr F1[b] (cpm/μg amino acid)	fragment: CNBr F2[c] (cpm/μg amino acid)
uncleaved thermolytic fragment[a]	114	130
I	116	112
II	120	105
III	63	104
IV	87	172[d]
V	74	99
VI[e]	(1270)	(1080)

[a] F1 and F2 peptides from Figure 5. [b] Peptides from Figure 6A. [c] Peptides from Figure 6B. [d] This pool, upon rechromatography, yielded a peak fraction whose specific radioactivity was 278 cpm/μg. [e] These pools represent the V_i for the column and contain very little peptide material.

were pooled as indicated in Figure 6, and the specific radioactivity of each pool was determined (Table I). Least incorporation was observed in pool III of the cyanogen bromide peptides of F1. This pool includes the amino-terminal glycopeptide of rhodopsin as determined by both the column profile, showing its absorbance at 280 nm, and by its glucosamine content (data not shown). The *highest* specific radioactivity was observed for pool IV of the cyanogen bromide peptides of F2. Rechromatography of this pool yielded a peak fraction which has a specific radioactivity that is 4.4 × that of peak III from the cyanogen bromide cleavage of F1 (see footnote Table I). This difference in specific radioactivities of these peptide mixtures suggests that a degree of selectivity has been obtained in the modification of portions of rhodopsin by 1-azidopyrene.

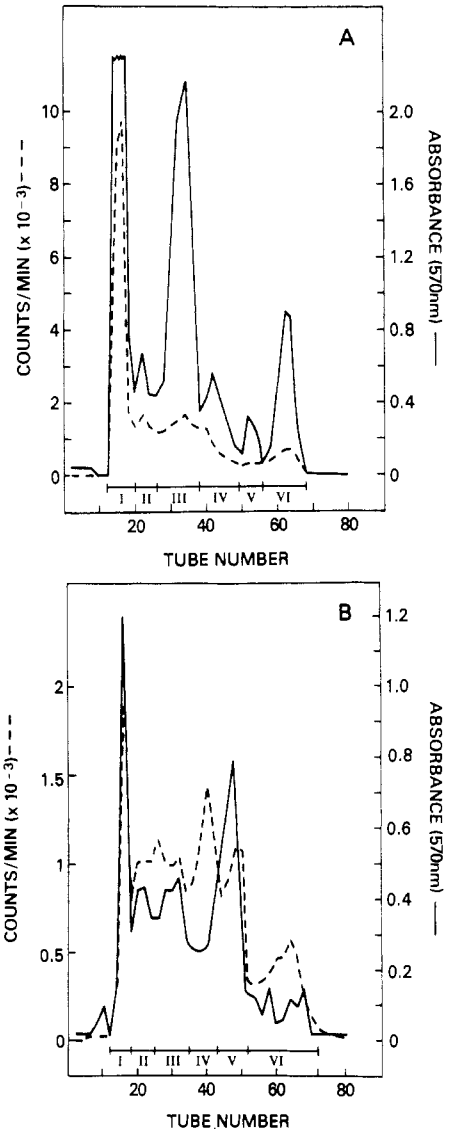

FIGURE 6: Sephadex LH-60 column chromatography of the cyanogen bromide peptides of [^{3}H]AP-F1 and -F2. (A) Separation of the cyanogen bromide peptides of F1, and (B) separation of the cyanogen bromide peptides of F2. In each experiment, the peptide mixture (~5 mL) was applied to a 1.5 × 100 cm column of Sephadex LH-60 and eluted with 88% formic acid:100% ethanol (30:70). Approximately 3-mL fractions were collected by drop counting; 200-μL aliquots of alternate tubes were taken for determination of radioactivity, and 200 μL for acid hydrolysis and ninhydrin analysis. Six column pools were made as indicated.

Discussion

We have prepared 1-azido[^{3}H]pyrene of high specific radioactivity and used it to label rhodopsin in disk membranes. Conditions for performing the reaction were conveniently assessed by following the incorporation of ^{3}H into [^{14}C]-rhodopsin (labeled by reductive methylation by using [^{14}C] formaldehyde). It was found necessary to limit the amount of reagent added to approximately 1 mol of reagent per 8 mol of rhodopsin in order to minimize rhodopsin aggregation. Short reaction times (2 min) were chosen which yielded no detectable photolytic damage to rhodopsin yet allowed half of the maximal incorporation of reagent to occur. The high specific radioactivity of the reagent (~3 Ci/mmol) allowed good incorporation of radioactivity into rhodopsin at a low level of substitution (approximately 1 mole of reagent incorporated per 210 mol of rhodopsin). It was also possible to detect incorporation of [^{3}H]AP into the $M_r \simeq 240\,000$ protein, a disk protein present in only $^1/_{300}$–$^1/_{900}$ the molar amount of rho-

dopsin (Papermaster et al., 1976). This high molecular weight protein, therefore, is an intrinsic disk membrane protein and is the only other such disk protein labeled in addition to rhodopsin. Phospholipids are also labeled when [^{3}H]AP is photolyzed in disk membranes.

Azidopyrene is a nonpolar molecule which is nicely soluble in organic solvents. Upon addition to a membrane, it would be expected to dissolve in the lipid bilayer and, upon photolysis, to react with neighboring molecules within the hydrophobic domain of the bilayer. However, Bayley & Knowles (1978) in their study of the reaction of phenyl azide with phospholipid in vesicles have reported that a hydrophobic nitrene can label proteins and other nucleophiles present in the aqueous solution surrounding the membrane. Diffusion of the nitrene to the membrane surface prior to its reaction and the electrophilicity of nitrenes make it possible that the presumed labeling of hydrophobic surfaces by a lipophilic nitrene may be far less selective than generally supposed. Bayley & Knowles (1978) report that the presence of glutathione in the aqueous phase reduced the extent of phenylnitrene labeling of lipid in liposomes by 8-fold. In our studies, it would appear, however, that the labeling of rhodopsin by AP occurs predominantly on lipophilic surfaces since the presence of glutathione in the external aqueous medium reduces labeling of rhodopsin by only 4%.

In preliminary experiments designed to map the incorporation of the hydrophobic probe [^{3}H]AP into rhodopsin, we prepared [^{3}H]AP–rhodopsin, digested it with cyanogen bromide, and attempted to separate the peptides in 20% formic acid on Sephadex G-50 (D. Smith and P. Hargrave, unpublished results). Although such a procedure had proven successful in evaluation of the peptides of rhodopsin reacted with *N*-(4-azido-2-nitrophenyl)-2-aminoethanesulfonate (Mas et al., 1980), there was poor recovery of the [^{3}H]AP peptides from the column, and the majority of recovered radioactivity emerged as aggregated material in the void volume. In order to deal with the hydrophobic AP–peptides, we evaluated chromatography in the solvent formic acid/ethanol by using the lipophilic resin Sephadex LH-60 (Gerber et al., 1979). Chromatography of the cyanogen bromide peptides of [^{3}H]-AP–rhodopsin in this system showed a much better recovery and more uniform chromatographic elution of radioactivity with peptide material (Smith, 1980). In order to assure ourselves that peptides containing AP would cochromatograph with their unlabeled counterparts in the formic acid/ethanol system, we sought to prepare a model peptide containing pyrene. We prepared the cyanogen bromide peptides of cytochrome *c*, lactonized the carboxyl-terminal homoserines with trifluoroacetic acid, and reacted the peptides with aminopyrene. We found that the 1850 molecular weight peptide became partially modified, and we were able to demonstrate that the modified and unmodified peptide did, in fact, cochromatograph on Sephadex LH60 in formic acid/ethanol (Smith, 1980). We subsequently employed this chromatographic system successfully for both the separation of AP-F1 and -F2 and chromatography of their cyanogen bromide peptides.

[^{3}H]AP was found to label both thermolytic fragments of rhodopsin, F1 and F2, as might be expected for strongly membrane-associated polypeptides. Neither F1 nor F2 was appreciably more highly labeled than the other, yet when smaller peptides were produced by cyanogen bromide cleavage, labeling differences of severalfold became evident. A peptide pool containing rhodopsin's glycopeptide contained the lowest specific radioactivity, as might be expected for a peptide which has been shown to be located at the membrane surface (Adams et al., 1978; Mas et al., 1980). The peptide most highly labeled by AP is located in F2 (pool IV, Table I). This peptide mixture contains lysine and alanine in its composition (D. Smith and P. Hargrave, unpublished results), as does the retinal binding site which is located in F2 (Hargrave et al., 1980). The nature of this most highly labeled peptide is currently being investigated.

The quantification of label into the various peptides of rhodopsin must be viewed as only approximate since it has been observed that peptides modified by nitrenes lose label during subsequent purification steps (Galardy et al., 1974; Mas et al., 1980). This is also true for the [^{3}H]AP-labeled rhodopsin since much of the label present in the chromatographically purified F1–F2 complex appears in the peak of the small molecules during separation of F1 and F2 (Figure 5) and continues to be lost from the peptides following cyanogen bromide cleavage (Figure 6).

The 1-azido[^{3}H]pyrene which we have synthesized appears to have many advantages compared to other lipophilic probes. It has a shorter lifetime than the similar pyrenesulfonyl azide (which should improve its specificity for reaction in a hydrophobic environment), and it has been synthesized with specific radioactivity 375 × greater than that of the pyrenesulfonyl azide (Šator et al., 1979). It appears to be better restricted to the membrane bilayer than phenylnitrene (Bayley & Knowles, 1978). In addition, it does not contain iodine, as does 5-[^{125}I]iodonaphthyl azide (Bercovici & Gitler, 1978); iodine may become photolytically dissociated during photolysis (Levy et al., 1973). The absorption maximum of 1-azidopyrene is in the visible region (357 nm) and can be easily photolyzed with visible light under conditions which do not damage membrane components. In contrast, 4-[^{125}I]iodobenzene 1-azide (Klip & Gitler, 1974) has an absorption maximum of 258 nm and must be excited (with low efficiency) at the edge of its absorption band in order not to photolytically damage the membrane.

In summary, 1-azido[^{3}H]pyrene of high specific radioactivity appears to be a useful lipophilic photoreactive probe for modification of rhodopsin in the disk membrane and should be a valuable reagent for the study of intrinsic proteins in other membranes.

Acknowledgments

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