

Selective labeling of proteins in biological systems by photosensitization of 5-iodonaphthalene-1-azide

(adenylate cyclase/membrane proteins/rhodopsin)

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ABSTRACT The apolar azide of 5-iodonaphthalene-1-azide (Ina) partitions into the lipid bilayer of biological membranes. Upon photolysis at 314 nm, it is rapidly converted into the reactive nitrene, which efficiently attaches covalently to lipid-embedded domains of proteins and, to a lesser extent, to membrane phospholipids. Above 370 nm, Ina absorption is negligible and photolysis at these wavelengths does not occur. However, on addition of the photosensitizing molecule 3-aminopyrene, trifluoperazine, or 8-anilino-naphthalene-1-sulfonate, followed by irradiation at 380 nm, efficient conversion of Ina to reactive species was observed, as measured by [¹²⁵I]Ina-labeling of membrane proteins and inactivation of the hormonal response of adenylate cyclase. Irradiation at 480 nm in the presence of a fluorescein derivative of *n*-undecylamine also resulted in a pattern of [¹²⁵I]Ina-labeled membrane proteins and hormone uncoupling indistinguishable from that obtained following direct photolysis at 314 nm. Photosensitization of the azide molecules is confined to the vicinity of the photosensitizer chromophore. This allowed selective labeling of chromophore-bearing proteins in solution or in membranes. Bovine serum albumin–fluorescein conjugate, in the presence of nonderivatized soluble proteins, was exclusively labeled by [¹²⁵I]Ina when irradiated at 480 nm, but random labeling occurred on photolysis at 314 nm. Likewise, rhodopsin in rod outer segment membranes from frog retina was exclusively labeled by [¹²⁵I]Ina upon photosensitization at 380 nm. Random labeling again occurred on direct irradiation at 314 nm. The results suggest that selective labeling in complex biological systems may be achieved by photosensitized activation of azides.

Klip and Gitler (1, 2) introduced the use of highly lipid-soluble azides for the labeling of proteins in membranes. Various reagents of this type have been developed and their properties have been reviewed (3–7). 5-Iodonaphthalene-1-azide (Ina) was used frequently and proved to be efficient in labeling hydrophobic domains of membrane proteins (8–12). Ina is highly hydrophobic (partition coefficient >10⁵) (11) and partitions efficiently into the lipid bilayer of various cellular membranes. Upon photolysis ($\lambda_{\max} = 310$ nm, $\epsilon = 21,400$ M⁻¹·cm⁻¹) with the 314-nm line of a mercury lamp, it is rapidly and efficiently converted into the reactive nitrene, which inserts covalently into intrinsic membrane proteins that contain lipid-embedded domains and, to a lesser extent, into phospholipids. When photoactivated in the plasma membrane, Ina blocks transduction of hormonal stimulation of adenylate cyclase by uncoupling of the hormone receptor from the enzyme (13).

It was of interest to determine whether energy-transfer processes could be used to photoactivate a discrete fraction of the azide molecules present in a membrane or cell.

Resonance energy transfer could not be used because Ina absorbs light down to 260 nm and any donor molecules chosen for its photoactivation would have to have absorption maxima below this wavelength, this clearly being impractical. Aromatic azides, however, are capable of photosensitized activation by donors that absorb at longer wavelengths than those absorbed by the azides themselves (14, 15). Photosensitization occurs by various mechanisms (16, 17) in a process that requires the approach of the photosensitizer and acceptor pair to within the collisional range in order for intermolecular exchange of excitation energy to take place (18). The nature of the reactive intermediates generated by photosensitization of aryl azides in different solvents is not completely understood. However, they seem to be reactive in hydrogen-abstraction, dimerization, and insertion reactions (19, 20). Since many sensitizers are aromatic apolar molecules, it is likely that they will parallel the distribution of Ina into lipophilic regions of the membrane. This should increase their local concentration and greatly enhance the efficiency of the energy-transfer process (1, 21, 22).

In this paper we examine whether Ina can be effectively activated to form reactive species by photosensitization using (i) donor chromophores that partition into the lipid bilayer, (ii) donor chromophores such as fluorescein isothiocyanate (FluNCS) that can be covalently attached to proteins, and (iii) intrinsic donor chromophores such as the retinal of rhodopsin. To establish whether the reactive species formed following photosensitization of Ina were equivalent to those generated by direct photolysis of Ina, we determined (a) the pattern of incorporation of [¹²⁵I]Ina-derived radioactivity into proteins in solution and in membranes and (b) the inactivation of the hormone-mediated activation of adenylate cyclase (13). The results show that photosensitized activation of Ina results in a protein-labeling pattern indistinguishable from that observed following direct photoactivation. Since photosensitization is limited in range, [¹²⁵I]Ina excitation is restricted to the vicinity of the photosensitizer chromophore. Thus, selective labeling of chromophore-bearing proteins in solution and in membranes could be demonstrated. The photosensitized labeling approach offers a method for site-directed labeling in biological systems.

MATERIALS AND METHODS

3-Aminopyrene, dansyl chloride (DnsCl), fluorescein 5-isothiocyanate (FluNCS), trifluoperazine dihydrochloride, bovine serum albumin (BSA), and individual protein molecular weight markers were from Sigma. 8-Anilino-naphthalene-1-sulfonate (Ans) sodium salt was from Aldrich, 5-amino-

Abbreviations: Ans, 8-anilino-naphthalene-1-sulfonate; BSA, bovine serum albumin; Dns, dansyl; FluNCS, fluorescein 5-isothiocyanate; FluNCS-NHC₁₁, *N*-(fluorescein 5-thiocarbonyl)-*n*-undecylamine; hCG, human chorionic gonadotropin; Ina, 5-iodonaphthalene-1-azide; ROS, rod outer segment.

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naphthalene-1-azide from Fluka, and Na¹²⁵I from New England Nuclear. Human chorionic gonadotropin (hCG) CR-123 and [α -³²P]ATP were as described (13). The protein molecular weight marker mixture was from Pharmacia. Monoclonal anti-rhodopsin antibodies were kindly provided by H. E. Hamm (23). Highly purified testicular calmodulin was kindly provided by J. Shiloach and C. Klee (National Institutes of Health, Bethesda, MD). All other reagents were of analytical grade.

Preparation of Membranes. Ovarian plasma membranes (24) and mouse S49 lymphoma plasma membranes (25) were prepared as described. Rod outer segment (ROS) membranes were prepared from frog (*Rana ridibunda*) eyes (26).

Adenylate Cyclase Assay. Conditions were as described (27). One unit of enzyme activity was defined as the amount catalyzing the formation of 1 pmol of cAMP per min at 30°C.

BSA-Chromophore Conjugates. Dns-BSA was prepared by adding DnsCl (0.8 μ mol in 20 μ l of acetone) to BSA (0.2 μ mol in 1 ml of 0.1 M Na₂HPO₄, pH 9.0) and incubating the mixture at room temperature for 1 hr. Dns-BSA was separated from free DnsCl by gel filtration on a Sephadex G-25 column. Fluorescein 5-thiocarbamoyl-BSA (FluNCS-BSA) and anthranoyl-BSA were prepared essentially as described for the preparation of Dns-BSA, except that FluNCS (0.4 μ mol) or *N*-hydroxysuccinimidyl anthranilate (28) (2 μ mol) in 20 μ l of acetone was used.

***N*-(Fluorescein 5-thiocarbamoyl)-*n*-Undecylamine (FluNCS-NHC₁₁).** FluNCS (40 mg in 400 μ l of tetrahydrofuran) was mixed with *n*-undecylamine (45 μ l) and incubated for 60 min at room temperature. Glacial acetic acid (400 μ l) was added, and the mixture was incubated for 120 min and then centrifuged (800 \times *g*, 10 min). The lower phase was removed and 20 ml of anhydrous benzene was added. The mixture was dried with anhydrous MgSO₄ and evaporated completely. The residue was dissolved in 2 ml of dry ethyl ether, filtered, evaporated again, and dissolved in tetrahydrofuran for further use.

Synthesis of Ina and General Conditions for Its Use. Ina and [¹²⁵I]Ina (2–10 Ci/mmol; 1 Ci = 37 GBq) were synthesized from 5-aminonaphthalene-1-azide as described (8). All operations in which Ina was used, including NaDodSO₄/PAGE, were performed under subdued light (λ > 600 nm). Ina and sensitizers were added as ethanol or tetrahydrofuran solutions; final solvent concentration was <1%.

Labeling of Plasma Membrane Proteins by Direct Photoactivation of [¹²⁵I]Ina. Membranes (1 mg/ml) in phosphate-buffered saline (PBS: 137 mM NaCl/2.7 mM KCl/1.5 mM KH₂PO₄/7.6 mM Na₂HPO₄/0.9 mM CaCl₂/0.5 mM MgCl₂, pH 7.3) were incubated for 5 min with [¹²⁵I]Ina (5 \times 10⁷ cpm/mg of membrane protein). Reduced glutathione was then added to 20 mM and the suspension was irradiated for 90 sec with a 200-W mercury lamp under conditions that permitted irradiation with the 314-nm line only. These irradiation conditions are referred to hereafter as irradiation at 314 nm (8).

Inactivation of the Hormonal Response of Adenylate Cyclase by Direct Photoactivation of Ina. Inactivation by photoproducts of Ina of the response of adenylate cyclase to hormonal stimulation was carried out as described (13). In brief, rat ovarian plasma membranes (0.1 mg of protein per ml) were mixed in the dark with unlabeled Ina (15 μ M). After 5 min of incubation, the suspension was irradiated as described for the individual experiments. Subsequently, residual adenylate cyclase activity (in response to stimulation by hCG) was determined. Under these conditions, no changes were observed in hormone binding to the receptor and in the response of adenylate cyclase to NaF (13).

Photosensitization of Ina in Plasma Membranes. Photosensitizer was added (20–50 μ M) to 50–500 μ l of a membrane suspension (0.5–1 mg of protein per ml in PBS), and the

suspension was incubated 10 min at 4°C in the dark. Pre-treatment with FluNCS was in 100 mM sodium bicarbonate buffer (pH 8) at 4°C for 1 hr. The membranes were then sedimented once (100,000 \times *g*, 10 min) and resuspended in PBS to the original volume. Subsequently membranes were treated with Ina or [¹²⁵I]Ina as described above. The membrane suspension (in a Kimble 6 \times 50-mm test tube) was immersed in a glass-jacketed chamber cooled with circulating ice water and was irradiated for the time indicated. The light source (a slide projector, Liesegang Fantimat, F.R.G.), equipped with a 300-W tungsten lamp, was placed 5 cm from the test tube. Use of the filter combination (Corning 7-54, 0-52, and 3-75) that permitted transmittance at 370–400 nm (λ _{max} 380 nm) was designated as irradiation at 380 nm. Use of the filter combination (Jena GG-436 and Corning 4-72) that permitted transmittance at 450–580 nm (λ _{max} 480 nm) was designated as irradiation at 480 nm. Pretreated membranes were subsequently assayed for residual adenylate cyclase activity or prepared for NaDodSO₄/PAGE.

Photosensitized [¹²⁵I]Ina Labeling of BSA-Chromophore Conjugates. BSA-chromophore conjugates (0.5 mg/0.5 ml) were incubated with [¹²⁵I]Ina (5 \times 10⁶ cpm) for 5 min in the dark. The solution was then irradiated at the appropriate wavelength (see above) for the time indicated. [¹²⁵I]Ina-labeled protein was precipitated with 9 volumes of ice-cold acetone, and radioactivity incorporated into the pellet was determined. Nonirradiated controls were treated similarly.

Electrophoresis. NaDodSO₄/PAGE (29) was performed under subdued light, using 10% gels. The proteins in sample buffer were not boiled, to avoid thermal activation of residual Ina.

RESULTS

Labeling of Membrane Proteins by Photosensitization of [¹²⁵I]Ina. To determine whether insertion of Ina into proteins could be induced by photosensitized irradiation, we screened compounds that (i) can be excited at wavelengths that do not excite Ina (λ > 370 nm) and (ii) can partition into the lipid bilayer of the membrane to allow for efficient interaction with Ina. Photosensitization of Ina was determined by measuring the incorporation of [¹²⁵I]Ina into membrane proteins. Fig. 1 describes an experiment in which Ina, in combination with each one of the indicated photosensitizers, was permitted to partition into a preparation of mouse S49 lymphoma cell membranes in the dark. The membrane samples were then irradiated at the appropriate wavelength to excite the respective photosensitizer and subjected to NaDodSO₄/PAGE. Upon inclusion of FluNCS-NHC₁₁ (lane 1), trifluoperazine (lane 2), Ans (lane 3), or 3-aminopyrene (lane 4) and [¹²⁵I]Ina, energy transfer was evident from the radioactive labeling of multiple membrane protein bands. Incorporation of [¹²⁵I]Ina into membrane proteins upon direct photoactivation showed an identical pattern of protein labeling (lane 7). Control preparations from which the photosensitizer was omitted were irradiated at 480 nm (lane 5) or 380 nm (lane 6) and showed essentially no protein labeling.

Uncoupling of Hormone-Sensitive Adenylate Cyclase by Photosensitization of Ina. It was of interest to further support the finding that the reactive species formed following photosensitized activation of Ina were equivalent to those obtained by direct photolysis. We thus studied selective Ina-dependent photoinduced uncoupling of the response of rat ovarian adenylate cyclase to stimulation by hCG. In the experiments described in Table 1, ovarian plasma membranes were treated with photosensitizers and Ina (in the dark) essentially as described for the previous experiments (Fig. 1). After irradiation at the appropriate wavelength, residual hormone-stimulated adenylate cyclase activity was measured. The maximal effect of Ina was determined on

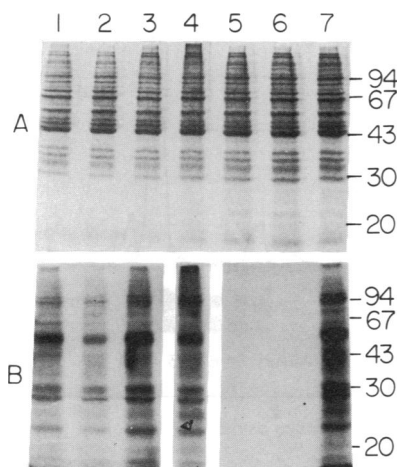


FIG. 1. Labeling of S49 lymphoma cell membranes by photosensitization of [¹²⁵I]Ina. Seven samples (30 μg of protein) of S49 lymphoma plasma membranes each were suspended in 50 μl of PBS in a 6 × 50-mm glass tube. Five microliters of sensitizing chromophore or PBS (control) was added to each test tube. The final concentration of each chromophore was 50 μM, except for FluNCS-NHC₁₁ (20 μM). [¹²⁵I]Ina (5 × 10⁶ cpm) was then added and the samples were irradiated (wavelengths and times as follows). Lane 1: FluNCS-NHC₁₁; 480 nm, 10 min. Lanes 2–4: trifluoperazine, Ans, and 3-aminopyrene, respectively; 380 nm, 5 min. Lane 5: [¹²⁵I]Ina-treated control (no photosensitizer); 480 nm, 10 min. Lane 6: control as in lane 5 but irradiated at 380 nm for 5 min. Lane 7: control as in lane 5 but irradiated at 314 nm for 90 sec. Subsequently, the samples were subjected to NaDodSO₄/PAGE. For the purpose of demonstrating relative intensity, lanes 1–3 and 5–6 were autoradiographed for 5 days, and lanes 4 and 7 for 36 hr only. (A) Coomassie blue staining pattern. (B) [¹²⁵I]Ina labeling pattern of the same lanes as shown by autoradiography. Positions of protein molecular weight markers (*M_r* × 10⁻³) run in parallel are shown at right.

samples in which Ina was directly photoactivated at 314 nm. Control values for adenylate cyclase activity were obtained from samples that were not irradiated (dark). As seen in Table 1, and as previously reported (13), direct photolysis of Ina abolished >88% of the response of adenylate cyclase to

Table 1. Uncoupling of hormone-sensitive adenylate cyclase by photosensitization of Ina

Photosensitizer	Adenylate cyclase, units/mg of protein			
	314 nm (90 sec)	380 nm (15 min)	480 nm (30 min)	Dark
Experiment 1				
None	0	193 ± 14	—	—
3-Aminopyrene	—	2.1 ± 2	—	194 ± 13
Experiment 2				
None	3.5 ± 3	163 ± 5	—	—
Trifluoperazine	—	23 ± 2	—	155 ± 12
Experiment 3				
None	12 ± 2	92 ± 5	—	—
Ans	—	23 ± 5	—	101 ± 11
Experiment 4				
None	0	—	194 ± 20	—
FluNCS	—	209 ± 12	39 ± 11	216 ± 9

Purified ovarian plasma membranes were suspended in PBS to a final concentration of 0.2 mg of protein/ml and treated with photosensitizer (concentrations: 3-aminopyrene, 20 μM; trifluoperazine, 30 μM; Ans, 40 μM; FluNCS, 20 μM). Ina treatment, irradiation at the indicated wavelengths, and inactivation of the hormonal response of adenylate cyclase by photoactivation were conducted in separate experiments for each photosensitizer. Variations in the dark control values represent innate variations in the specific activity of adenylate cyclase among different membrane preparations.

hormonal stimulation within 90 sec. Irradiation at 380 nm of membranes suspensions treated with 3-aminopyrene, trifluoperazine, or Ans or at 480 nm of membranes treated with FluNCS led to a similar reduction in the response of the enzyme to hormonal stimulation. However, this effect was only seen in membrane suspensions that contained both photosensitizer and Ina. No effect was detected (values equal to dark control) in the absence of photosensitizer. Likewise, irradiation of membranes in the presence of photosensitizer but in the absence of Ina was also without effect (data not shown). The concentrations of photosensitizers were selected so that a maximal effect on the hormonal response of adenylate cyclase was obtained within the indicated time of irradiation. These conditions did not affect the response of adenylate cyclase to stimulation by 10 mM NaF.

Photosensitized [¹²⁵I]Ina Labeling of Chromophore-BSA Conjugates in Solution. After incubation (10 min) with [¹²⁵I]Ina in the dark, Dns-BSA and anthranoyl-BSA were irradiated at 380 nm, and FluNCS-BSA at 480 nm. Samples were taken at various times and the radioactivity incorporated into protein was measured (Fig. 2). Maximal [¹²⁵I]Ina incorporation (100%) was obtained following irradiation of native BSA at 314 nm (2 min). Since irradiation of anthranoyl- and Dns-BSA was performed at the edge of their absorption band (where Ina does not absorb), the protein-labeling efficiencies obtained do not reflect quantum yield as sensitizers. In the case of FluNCS-BSA, irradiation could be performed at the absorption maximum and labeling was as efficient as that obtained by direct photolysis, albeit a longer time of irradiation was required. When native BSA was irradiated at 480 nm or 380 nm, incorporation of [¹²⁵I]Ina was negligible.

Selective Photosensitized [¹²⁵I]Ina Labeling of FluNCS-BSA in a Mixture of Soluble Proteins. The photosensitization process is limited in distance to the immediate vicinity of the chromophore. We wondered whether this property could be exploited to selectively label chromophore-protein conjugates in the presence of other proteins. A mixture of soluble proteins was incubated with [¹²⁵I]Ina and irradiated at 314 nm (Fig. 3, lane 1), resulting in varying degrees of labeling of all proteins. Calmodulin was labeled but was not resolved from the dye front. The same protein mixture but with FluNCS-BSA substituted for native BSA was irradiated at 480 nm. The results showed exclusive labeling of the chromophore-

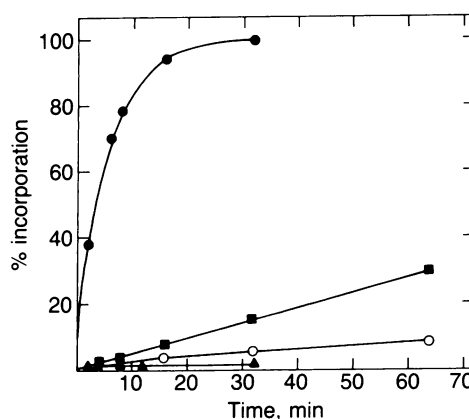


FIG. 2. Photosensitized [¹²⁵I]Ina labeling of chromophore-BSA conjugates in solution. FluNCS-BSA (●), Dns-BSA (■), anthranoyl-BSA (○), and native BSA (▲) (1 mg/ml) were incubated in the dark with [¹²⁵I]Ina for 10 min. Subsequently, the samples were irradiated as follows: FluNCS-BSA at 480 nm; Dns-BSA and anthranoyl-BSA at 380 nm; native BSA at 480 nm. At the indicated times, samples were removed and protein was precipitated in acetone for assessment of the radioactivity incorporated. Results are expressed as percentage of [¹²⁵I] incorporated into BSA following irradiation at 314 nm for 120 sec (this amounts to 50% of the total radioactivity added).

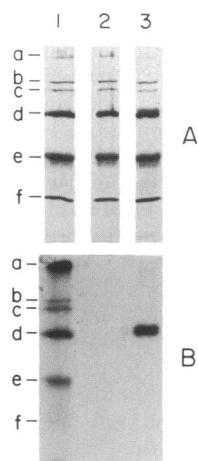


FIG. 3. Selective photosensitized [^{125}I]Ina labeling of FluNCS-BSA in a mixture of soluble proteins. Three samples of a protein mixture were prepared. Each sample contained six proteins: myosin (heavy chain, 205 kDa, 5.6 μg per lane, band a), β -galactosidase (116 kDa, 2.2 μg , band b), phosphorylase *b* (97.4 kDa, 2.2 μg , band c), BSA (FluNCS-BSA for lane 3; 66 kDa, 3.2 μg , band d), ovalbumin (45 kDa, 5.0 μg , band e), and carbonic anhydrase (29 kDa, 2.2 μg , band f). The following ingredients were included in a final volume of 60 μl : 20 mM Tris-HCl (pH 7.5), protein mixture, 10 μg of purified testicular calmodulin (17 kDa), 20 mM reduced glutathione, 1 mM CaCl_2 , 1 mM MnCl_2 , and 5×10^6 cpm of [^{125}I]Ina. The samples were then irradiated at 314 nm for 90 sec (lanes 1) or at 480 nm for 10 min (lanes 2 and 3) and then subjected to NaDodSO₄/PAGE followed by autoradiography. (A) Coomassie blue staining pattern. (B) [^{125}I]Ina labeling pattern as revealed by autoradiography.

containing BSA (Fig. 3, lane 3). In contrast, no labeling of BSA or any of the other proteins was observed upon irradiation at 480 nm (Fig. 3, lane 2). Reduced glutathione, a scavenger of long-lived excited-state photoproducts of Ina, was present to prevent diffusion of these species from their site of formation (chromophore-BSA) to the other proteins. The presence of calmodulin was found also to decrease background labeling.

Selective Labeling of Rhodopsin in ROS Membranes by Photosensitization of [^{125}I]Ina. It seemed likely that certain intrinsic chromophore-containing membrane proteins might become selectively labeled due to photosensitization of [^{125}I]Ina. Frog retinal ROS were incubated with [^{125}I]Ina and irradiated at 380 or 314 nm. As expected, upon irradiation at 314 nm, many intrinsic membrane proteins became labeled (Fig. 4 *Upper*). In contrast, irradiation at 380 nm (light absorbed by rhodopsin) led to the nearly exclusive labeling of a major protein band at 35–38 kDa (Fig. 4, *Lower*). The labeled protein was identified as rhodopsin (data not shown) by heat-induced polymerization (30) and by immunoblotting with monoclonal antibodies to frog rhodopsin (23).

DISCUSSION

Sensitized photoactivation of aryl azides has been reported (19, 20) to yield intermediates that react by hydrogen abstraction, by dimerization, and, to a lesser extent, by insertion. It was surprising, therefore, that the present findings indicated that photosensitized activation of Ina with sensitizers such as 3-aminopyrene, trifluoperazine, Ans, and FluNCS-NHC₁₁ resulted in photoproducts that could insert into membrane components in a manner equivalent to that obtained by direct irradiation of Ina. Close analysis of proteins labeled by [^{125}I]Ina revealed complete identity between the labeling pattern obtained by direct activation (Fig. 1, lane 7) and by photosensitized activation of Ina (Fig. 1, lanes 1–4). We reported previously (13) that low concentrations (<10 μM) of Ina added to rat ovarian plasma mem-

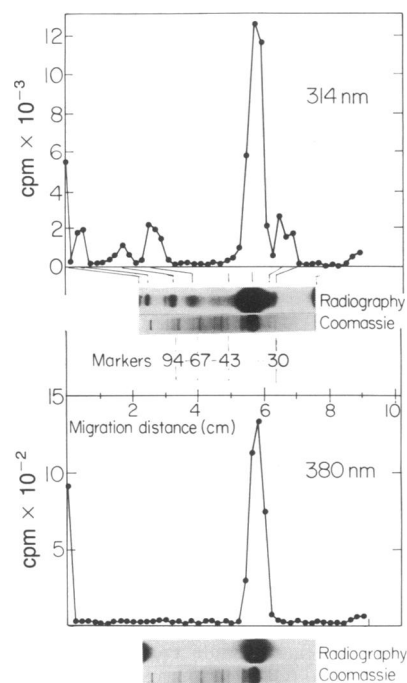


FIG. 4. Selective labeling of rhodopsin in frog ROS membranes by photosensitization of [^{125}I]Ina. ROS membranes were treated with [^{125}I]Ina, irradiated at 380 or 314 nm, and subjected to NaDodSO₄/PAGE. Gels were stained with Coomassie brilliant blue, autoradiographed, and cut into 2-mm slices (45 per lane) for measurement of radioactivity by scintillation counting. The radioactivity of the slices, after subtraction of background radioactivity (100 cpm per slice) as calculated from equivalent gel material adjacent to the protein lanes, was plotted against migration distance. Positions of molecular weight markers ($M_r \times 10^{-3}$) are indicated.

branes induced, on photoactivation, a selective and complete loss of the response of the adenylate cyclase to stimulation by hCG or luteinizing hormone. This treatment did not affect hormone binding to the receptor or stimulation of the adenylate cyclase by NaF (13). By use of this specific functional assay, it was also found that sensitized photoactivation of Ina resulted in selective uncoupling of the hormone-binding step from the activation of adenylate cyclase in a manner equivalent to that observed following direct photolysis of Ina (Table 1). In the absence of photosensitizer, neither protein labeling nor loss of hormone-sensitive adenylate cyclase activity was observed after irradiation of Ina at $\lambda > 380$ nm (Fig. 1, lanes 5 and 6, and Table 1). FluNCS, which reacted covalently with membrane components, gave results equivalent to those obtained with 3-aminopyrene, trifluoperazine, and Ans, which were associated with the membrane by simple partition. This suggests that some of the FluNCS molecules attach to amino groups of proteins or lipids at the lipid-water interface where, like Ans, they can sensitize Ina.

Even when the sensitizer was attached covalently to a protein, as was the case with FluNCS-BSA, effective energy transfer occurred leading to the formation of an Ina-derived reactive species that was incorporated covalently into BSA with high efficiency. Direct irradiation (2 min) and sensitized photoactivation (30 min), using FluNCS-BSA, resulted in the same level of radioactivity incorporated into protein (Fig. 2). This suggests that the sensitizer and the apolar azide collide on the surface of the BSA. It may be that the FluNCS is attached to lysine-223, which is the site of covalent binding of pyridoxal phosphate and which lies next to the bilirubin and fatty acid binding sites (31).

Sensitized photoactivation of Ina is restricted to the vicinity of the donor chromophore because of the short effective

range of this energy-transfer process (molecular overlap). Hence, labeling can be exclusively targeted to sites or compartments defined by the distribution of the photosensitizer. This is demonstrated by the finding that chromophore-containing proteins were selectively labeled in a mixture with other proteins (Fig. 3) or when natively present in biological membranes as in the case of retinal-rhodopsin (Fig. 4). The selective labeling of rhodopsin by photosensitization of Ina strongly suggests that retinal is present in rhodopsin in a disposition that allows its collision with Ina located in the lipid phase of the membrane, and that it is solely this fraction of the total Ina that labels the rhodopsin molecule. It is clear, however, that the reactive photoproducts of Ina generated by photosensitization do not diffuse through the lipid bilayer to label proteins other than rhodopsin. The high selectivity of the reaction is demonstrated by the observation that Ina is distributed throughout the ROS membrane and can label other membrane proteins, as is evident from the label-distribution pattern obtained following direct photoactivation at 314 nm (Fig. 4), and as was shown before by use of tritiated 5-azidonaphthalene (32).

The purpose of this work was to determine empirically whether sensitized photolysis of Ina could be used, under mild physiological conditions, to study the interaction of selected molecules in biological systems. The results indicate that it represents an extremely sensitive procedure to measure the partition of the photosensitizers into membrane loci where, upon photoactivation, they can collide with Ina to induce excitation transfer. This procedure can detect collisional encounters between sensitizer and azides with higher sensitivity than fluorescence quenching (Stern-Volmer) because energy absorbed by the azides leads to the formation of radioactively labeled products that can be detected at much lower concentrations. Furthermore, in fluorescence quenching, steady-state irradiation results in a constant signal that is dependent on the concentration of fluorophore and quencher. In contrast, in photosensitized labeling, the reactive species formed are trapped by their covalent attachment to the membrane proteins in a cumulative process that increases with time of irradiation.

The specific labeling of FluNCS-BSA and of rhodopsin demonstrates the usefulness of this method in labeling of sensitizer-bearing soluble and membrane proteins. More experiments are required to determine whether attachment of a sensitizer to a subunit of a multimeric protein will result in the labeling of only that subunit or, in addition, of its closest neighbors. It will be of interest to investigate the mechanism whereby sensitization generates Ina-derived species that behave in a manner indistinguishable from that formed by direct photolysis. Forster transfer is unlikely because of insufficient spectral overlap between the donors and acceptor. The sensitizers used are likely to behave as singlet sensitizers that may react by the formation of charge-transfer complexes (17). Irrespective of the mechanism, selective photosensitized labeling of proteins as described here, should be valuable in biochemical studies of protein structure and function.

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