Fluorescence Labeling of the Palmitoylation Sites of Rhodopsin†

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ABSTRACT: Two tandem cysteine residues in the carboxyl-terminal region of rhodopsin have been shown to be covalently linked to palmitate via thioester bonds (Ovchinnikov, Y. A., et al. (1988) *FEBS Lett. 230,* 1–5). We have synthesized a fluorescent analogue of palmitoyl coenzyme A (16-(9-anthroyloxy)hexadecanoyl coenzyme A ester) and incorporated the fluorescent derivative of palmitate into the protein in high yield (>40%) through pretreatment of bovine rod outer segments with 1 M hydroxylamine and subsequent incubation with the fluorescent label. Covalent incorporation of label into protein was demonstrated by SDS–polyacrylamide gel electrophoresis. Proteolytic digestion of labeled rhodopsin in the disc membrane with papain and thermolysin verified the C-terminal location of the label. Treatment of SDS-solubilized, labeled rod outer segments with 10% β-mercaptoethanol provided evidence that partial depalmitoylation may induce the formation of rhodopsin aggregates. Labeled, unbleached rhodopsin was purified by chromatography over hydroxyapatite and concanavalin A–agarose and reconstituted into dimyristoylphosphatidylcholine vesicles. SDS gels of the rhodopsin vesicle preparation verified that all unbound fluorescent label had been removed and that the thioester bond linking probe to protein was not labile.

Rhodopsin, the photoreceptor in the retinal rod cell, consists of a single polypeptide chain of 348 amino acids which putatively contains seven transmembrane α-helical segments (Hargrave et al., 1983; Nathans & Hogness, 1983). The amino terminus of rhodopsin is on the intradiscal side of the rod disc membrane, while the carboxyl terminus of the protein resides on the outer surface of the disc. Approximately 50% of the protein is within the hydrophobic core of the membrane, while the remainder of the protein is believed to form three water-exposed loops on both the cytoplasmic and the intradiscal side of the membrane (Figure 1A). Specific regions of the loops comprising the cytoplasmic domain of rhodopsin have been shown to provide binding sites for many of the proteins involved in the visual transduction process, i.e., transducin, rhodopsin kinase, and arrestin (Wilden et al., 1986; Palczewski et al. 1988, 1989; Konig et al., 1989; Franke et al., 1990). It has now been firmly established that two tandem cysteine residues near the carboxyl terminus of bovine rhodopsin are covalently attached to palmitic acid. Palmitoylation of rhodopsin was first demonstrated by O'Brien and co-workers (O'Brien & Zatz, 1984; O'Brien et al., 1987), who showed that palmitate is attached to rhodopsin via a thioester linkage and that the preferred fatty acid donor to the protein in retinae rod outer segments is palmitoyl coenzyme A. Ovchinnikov and co-workers showed that the sites of palmitoylation are at Cys 322 and Cys 323 and proposed that a fourth cytoplasmic loop would be formed if these two palmitates were anchored in the membrane (Ovchinniskov et al., 1988). The presence of palmitate on Cys 322 and Cys 323 of rhodopsin was later confirmed by the extensive mass spectroscopy study of Papac et al. (1992).

In this paper we describe the replacement of the endogenous palmitates of rhodopsin with a fluorescent derivative of the fatty acid and the subsequent purification of the labeled protein and its reconstitution into phospholipid vesicles. The introduction of a fluorescent reporter group into this region of rhodopsin should allow for direct physical measurements of the environment of the covalently bound fatty acids and may provide a sensitive probe of conformational changes occurring in rhodopsin during the visual transduction cycle.

MATERIALS AND METHODS

Synthesis and Purification of 16-(9-Anthroyloxy)hexadecanoyl Coenzyme A Ester. (A) Synthesis. 16-(9-Anthroyloxy)hexadecanoyl coenzyme A ester (AHCoA)[1] (Figure 1B) was synthesized from commercially available 16-(9-anthroyloxy)hexadecanoic acid (Molecular Probes). A modification of the procedure of Lapidot et al. (1967) was used to synthesize the *N*-hydroxysuccinimidyl ester (NHS ester) of the fatty acid analogue. Typically, 15 mg of 16-(9-anthroyloxy)-hexadecanoic acid (AHA) was dissolved in 1 mL of ethyl acetate (spectral grade) containing 6.0 mg of *N*-hydroxysuccinimide (NHS) (Aldrich Chemical Co.) recrystallized from ethyl acetate and 10.5 mg of dicyclohexylcarbodiimide (DCCD) (Aldrich). The NHS:DCCD:AHA molar ratio was 1.64:1.6:1. The sample was rotated gently for approximately 16 h at room temperature. Dicyclohexylurea was removed via centrifugation, and the ethyl acetate supernatant was streaked directly onto a silica gel TLC plate (EM Science, silica gel 60 without fluorescent indicator, 20 × 20 cm, 0.250-mm thickness). The plate was developed with anhydrous ethyl ether as solvent, and the fluorescent reaction products were briefly visualized with a long-wavelength UV light. The silica containing the NHS ester of (anthroyloxy)hexadecanoic acid, which represented the main fluorescent reaction product (R_f = 0.8–0.9), was scraped off the plate and suspended in

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[1] Abbreviations: 16-(9-anthroyloxy)hexadecanoic acid, AHA; coenzyme A, CoA; 16-(9-anthroyloxy)hexadecanoyl coenzyme A ester, AHCoA; *N*-hydroxysuccinimide, NHS; *N*-hydroxysuccinimidyl ester, NHS ester; dicyclohexylcarbodiimide, DCCD; critical micelle concentration, CMC; 50 mM Tris–acetate, pH 7.0 buffer, TAB; rod outer segments, ROS; sodium dodecyl sulfate–polyacrylamide gel electrophoresis, SDS–PAGE; column wash buffer (60 mM potassium phosphate, 40 mM sodium phosphate, 50 mM octyl glucoside, and 20 mM sodium chloride, pH 7.0), CWB; concanavalin A–agarose, Con-A; guanosine triphosphate, GTP.

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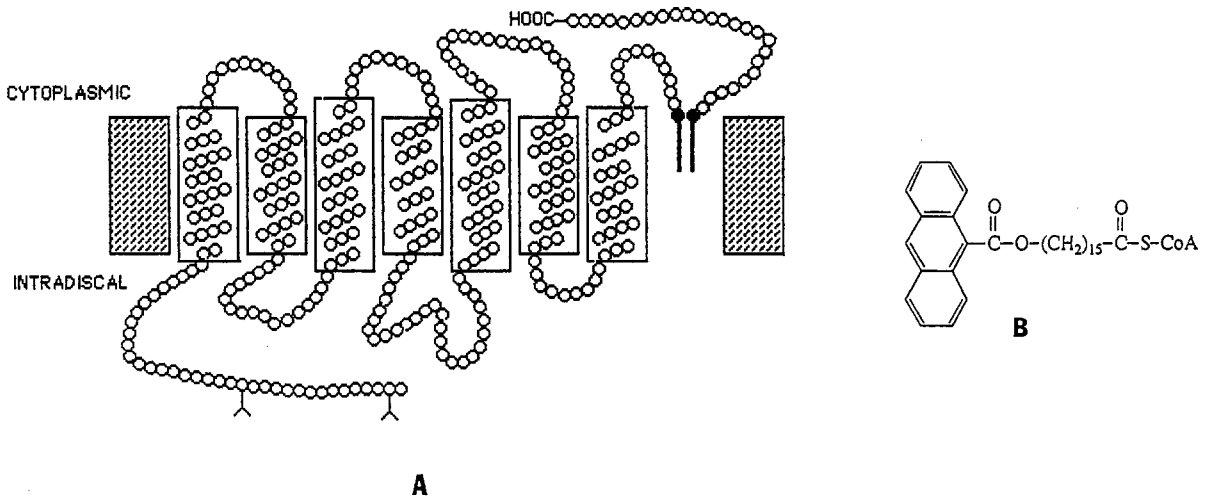

FIGURE 1: (A) "Snake diagram" of rhodopsin showing seven transmembrane helices (adapted from Hargrave et al., 1983). Blackened circles with tails represent the C-terminal palmitoylated cysteines. Portions of the lipid membrane are represented by shaded regions. (B) Structure of 16-(9-anthroyloxy)hexadecanoyl coenzyme A ester (AHCoA).

approximately 25 mL of anhydrous ethyl ether. The suspension was covered and incubated at room temperature with occasional stirring for several hours before being spun at 12000*g* for 15 min. The supernatant was transferred to a small beaker, and the ether was evaporated in a fume hood.

A modification of the procedure of Al-Arif and Blecher (1969) was followed to convert the NHS ester of 16-(9-anthroyloxy)hexadecanoic acid to the coenzyme A ester. Forty milligrams of coenzyme A (sodium salt) (Sigma) and the dried NHS ester were dissolved in 1–2 mL of water and 2–4 mL of 50:50 ethanol:ethyl acetate, respectively. The water:ethanol:ethyl acetate ratio was 1:1:1. The approximate CoA:NHS ester molar ratio was 3.5–4.0. The two solutions were mixed in a small glass vial to which 10–20 mg of solid sodium bicarbonate was added. The mixture was vigorously agitated for up to 8 h at room temperature. The reaction course was monitored periodically via analytical silica gel TLC (ethyl ether solvent) to detect the disappearance of the fluorescent NHS ester. The coenzyme A ester remains at the origin in this solvent system. When most of the NHS ester (>90%) was no longer detectable by TLC, the reaction mixture was dried under a stream of nitrogen, dissolved in 1–2 mL of distilled water, and spun at 12000*g* for 15 min to remove insoluble material.

(*B*) *Purification.* The sample of crude AHCoA was divided into two equal parts, and each sample was loaded onto a small (1 × 4 cm) hydroxyapatite (Bio-Rad Labs) column equilibrated in water. This column binds the coenzyme A ester. The columns were washed with approximately 10 mL of distilled water followed by 50–100 mL of 50 mM octyl glucoside and 10 mM sodium phosphate, pH 7.0, to elute any residual NHS ester and (anthroyloxy)hexadecanoic acid. The CoA ester was eluted with a minimal volume (10–15 mL) of 50 mM octyl glucoside and 300 mM sodium phosphate, pH 7.0, and diluted 1:3 with distilled water (below the CMC of octyl glucoside) before being extensively dialyzed against 50 mM Tris–acetate, pH 7.0 (TAB), to exchange the buffers and remove excess coenzyme A. Essentially no loss of product occurred at the dialysis step due to the low CMC for the formation of palmitoyl CoA micelles in aqueous solution (Zahler et al., 1968). If necessary, the AHCoA sample was concentrated by evaporating the water using a Speed-Vac concentrator (without heating) and redialyzing the decreased volume of sample against TAB. Final yields of 16-(9-anthroyloxy)hexadecanoyl coenzyme ester (determined from absorbance) were estimated to be 30% of the starting material, 16-(9-anthroyloxy)hexadecanoic acid, using an extinction coefficient of 4.7 $mM^{-1}cm^{-1}$ at 365 nm (Molecular Probes). The UV-vis spectrum of AHCoA synthesized by the above procedures was found to be indistinguishable from the spectrum of commercially available material (Molecular Probes). Small aliquots of the product were stored at −70 °C.

Labeling ROS with a Fluorescent Derivative of Palmitate. Unless otherwise noted, the following steps were performed in darkness or in the presence of red light. Frozen bovine retinae were purchased from Hormel (Austin, MN) or Excel (St. Louis, MO). Retinal rod outer segments (ROS) (1–10 mg), purified by the procedure of Smith et al. (1975), were washed with 50 mM Tris–acetate, (TAB), pH 7.0, and pelleted several times in an Eppendorf centrifuge to remove sucrose. When labeled rhodopsin was being prepared to be purified and reconstituted into lipid vesicles, ROS (8–10 mg) were first suspended in approximately 3 mL of 1 M hydroxylamine and 50 mM Tris–acetate, pH 6.5 (set with NaOH), and incubated at 37 °C for 30 min with gentle agitation using a wrist shaker. ROS were then pelleted and washed four times with TAB to remove hydroxylamine and resuspended in TAB containing 16-(9-anthroyloxy)hexadecanoyl CoA (AHCoA) (label:protein = 3). The sample was incubated at 37 °C for 1 h as described above and pelleted and washed with TAB. ROS (1–2 mg) were also incubated with AHCoA without prior treatment with hydroxylamine to determine the effects of hydroxylamine and bleaching on the level of label incorporation. In some cases, aliquots of samples were solubilized in 2% SDS and treated with 2% or 10% β-mercaptoethanol or with 1 M hydroxylamine, pH 6.5, to determine whether covalently bound label could be stripped from the protein or whether disulfide dimers were formed.

Covalent incorporation of label into protein was assessed by SDS–PAGE (Laemmli, 1970). AHCoA-treated ROS were pelleted, solubilized in 2% SDS, and run on 15% SDS gels at 4 °C. Fluorescence was visualized by backlighting gels with a short-wavelength (254 nm) UV light, and gels were photographed through a Corning 3–69 filter.

Estimation of the Percentage of Rhodopsin Bleached during Labeling and Reconstitution. ROS (approximately 4 mg) were solubilized in 4 mL of 50 mM octyl glucoside, 100 mM sodium chloride, and 10 mM sodium phosphate, pH 7.4, and insoluble material was removed by centrifugation. Solubilized ROS (2 mL) were bleached by exposure to room light for 15 min, and several samples of known ratios of unbleached to bleached rhodopsin were prepared. The absorbances at 500

and 420 nm were recorded for each sample using a Beckman DU-50 spectrophotometer, and the ratios of the absorbances were calculated for each sample and plotted against percentage of bleached protein (Figure 3). AHCoA does not absorb at these wavelengths. The standard curve thus generated was used to evaluate the extent of rhodopsin bleaching occurring during labeling and after purification of labeled rhodopsin. This absorbance ratio was also measured in samples of purified, labeled rhodopsin in detergent solutions as well as in the vesicle-reconstituted form of the labeled protein. To eliminate the contribution from light scattering, the absorbance ratio of the reconstituted sample was determined after detergent solubilization of the vesicles with a 1:2 dilution in the octyl glucoside buffer.

Estimation of the Average Stoichiometry of Label to Protein. A sample of ROS labeled with AHCoA as described above or of vesicle-reconstituted, purified, labeled rhodopsin was solubilized in 2% SDS and run on a 15% SDS gel along with a number of samples of AHCoA of known concentration and volume. Gel fluorescence was visualized with UV light and photographed as described previously. The photographic negative was scanned using a Beckman DU-64 spectrophotometer with a densitometer attachment, and the fluorescence intensity in each lane was recorded by cutting and weighing the paper on which the scans were plotted. A corresponding gel of solubilized, labeled ROS was run with bovine serum albumin protein standards and stained with Coomassie Blue R-250. The gel was scanned as described above except that a negative of the original photographic negative was used to generate a positive image. Alternatively, the Coomassie Blue stained gel was scanned directly. The resulting standard curves are shown in Figure 5.

Purification of Labeled Rhodopsin from ROS. All steps were performed in darkness or under red light and at 4 °C unless otherwise stated. After ROS were incubated with label and washed with TAB, they were solubilized in 2–3 mL of 50 mM octyl glucoside and 10 mM sodium phosphate, pH 7.0. The sample was left at room temperature for 10–20 min and then spun in an Eppendorf centrifuge to pellet any insoluble material. The sample was loaded onto a small (1 × 3 cm) hydroxyapatite column which had been equilibrated previously with 30 mM sodium cholate [cholic acid recrystallized from 70% ethanol in water by the procedure of Kagawa and Racker (1971)] and 40 mM sodium phosphate, pH 7.3. After the sample was loaded on the column, a time lag of approximately 1 h was allowed before washing with the same buffer at a rate of 70 mL/h until approximately 200 mL of buffer had passed through the column. The fluorescence intensity of the eluate was monitored using a hand-held 365-nm UV light. Greater than 90% of excess label was eluted at this point. The column was then washed with 10–20 mL of 50 mM octyl glucoside and 10 mM sodium phosphate, pH 7.0, and rhodopsin was eluted with 50 mM octyl glucoside in 60 mM potassium phosphate, 40 mM sodium phosphate, and 20 mM sodium chloride, pH 7.0 (CWB), into a Centriprep-10 concentrator container (Amicon Corp.) in a volume of approximately 15 mL. The sample was then concentrated to a volume of less than 2 mL by centrifugation of the Centriprep container and then loaded onto a small (1 × 3–4 cm) concanavalin A–agarose (Con-A) column (Sigma Type III ASCL) which had been equilibrated previously with CWB. After loading, a time delay of 1 h was allowed before the column was washed with approximately 50 mL of CWB to remove residual unbound label. Approximately 1 mL of CWB containing 0.75 M α-methyl mannoside (Sigma; recrystallized from methanol) was then added to the top of the column and allowed to run into the resin bed. After 3 h, the column was washed with approximately 14 mL of the same buffer to elute rhodopsin, which was collected into a 15-mL Centriprep container and concentrated to a volume of less than 2 mL.

Reconstitution of Labeled Rhodopsin into Phospholipid Vesicles. The following procedure is a slight modification of the method of Jackson and Litman (1985) and was carried out in the dark or under red light. Purified, labeled rhodopsin which had been eluted from the Con-A column and concentrated to a volume of less than 2 mL was dialyzed (for 8–12 h) against two changes of 100 mL of 30 mM octyl glucoside in CWB to remove α-methyl mannoside and excess octyl glucoside. The concentration of the protein solution was then recorded (with minimal bleaching) using a Beckman DU-50 spectrophotometer (the extinction coefficient of unbleached rhodopsin at 500 nm was taken to be 40 000 M^{-1} cm^{-1} (Shichi, 1970)], and dimyristoylphosphatidylcholine (Sigma; 99+%) was added at a molar ratio of lipid to protein of 150. Protein concentrations typically ranged from 2 to 15 μM, and under these conditions it was not necessary to add additional octyl glucoside to fully solubilize the lipid (Jackson & Litman, 1982, 1985). The lipid–protein–detergent mixture was vortexed in the dark for 5–10 min and allowed to sit on ice for at least 4 h. Vesicles were formed using the "octyl glucoside dilution" procedure described by Jackson and Litman (1985) as modified by Mitchell et al. (1991), and the solution was dialyzed for approximately 24 h against two changes of 4 L of TAB. Finally, the sample was concentrated using a Centriprep-10 concentrator.

Proteolysis of Labeled Rhodopsin as a Means of Locating the Labeling Sites. Labeled rhodopsin, in the disc membrane of the ROS, was digested with either papain or thermolysin using enzyme to substrate ratios of 1:20 to 1:5, and the reactions were terminated by the addition of specific inhibitors as described previously (Trayhurn et al., 1974; Pober, 1982). The major products of proteolytic digestion of rhodopsin by both of the enzymes are a large N-terminal fragment (approximately 24 kDa) called F1 and a smaller fragment containing most of the C-terminal amino acids (including Cys 322 and Cys 323) called F2 (Pober, 1982). Following digestion and enzyme inhibition, ROS were pelleted, solubilized in 2% SDS, and run on 15% SDS gels. The F1 fragment migrates on SDS gels only slightly ahead of holorhodopsin and, unlike the F2 fragment, is not partially or completely masked by the excess AHCoA which migrates near the ion front.

Measurements of Rhodopsin-Stimulated GTPase Activity. The rhodopsin-stimulated GTPase activity of labeled rhodopsin was measured in the laboratory of Dr. Richard A. Cerione at Cornell University by the method of Guy et al. (1990).

RESULTS

Labeling Rhodopsin with a Fluorescent Derivative of Palmitate. A fluorescent derivative of palmitoyl coenzyme A [16-(9-anthroyloxy)hexadecanoyl coenzyme A (AHCoA) Figure 1B] was used to label rhodopsin in rod outer segments (ROS). Several reports from other laboratories have indicated that the coenzyme A ester of palmitate is the preferred fatty acid donor to rhodopsin in the ROS and that a nonenzymatic exchange process may occur between palmitate bound to rhodopsin and free palmitoyl CoA (O'Brien et al., 1987; Morrison et al., 1991). Such a conclusion is consistent with the fact that the thioester bond linking the coenzyme A moiety to palmitate and palmitate to rhodopsin is a very high energy bond having a free energy of hydrolysis of –7 kcal/mol and, therefore, a high acyl group transfer potential.

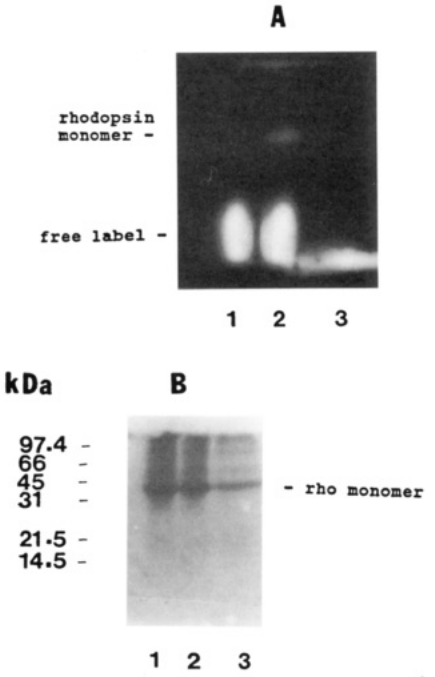

FIGURE 2: Detection of covalent modification of rhodopsin by fluorescent label using 15% SDS–PAGE. (A) Visualization of fluorescence on gel. Lane 1, ROS incubated with AHCoA; lane 2, ROS pretreated with hydroxylamine, washed, and incubated with AHCoA; lane 3, the same sample as in lane 2, except SDS-solubilized ROS were reincubated with 1 M hydroxylamine, pH 6.5, for 1 h at 37 °C. (B) The same gel as shown in panel A, stained with Coomassie Blue R-250.

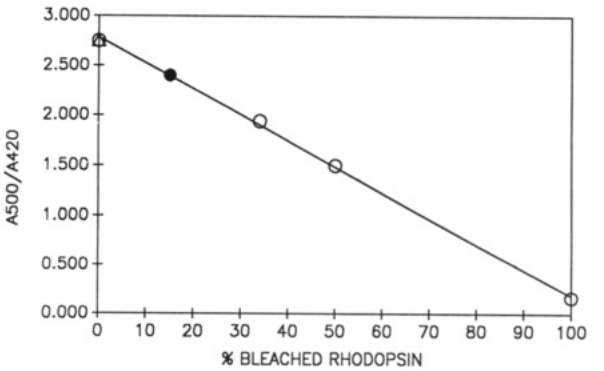

FIGURE 3: Standard curve of the A_{500}/A_{420} ratio versus the ratio of bleached to unbleached rhodopsin in samples of solubilized ROS. Correlation coefficient = 0.999. ROS were solubilized in 50 mM octyl glucoside, 10 mM sodium phosphate, and 100 mM sodium chloride, pH 7.4. ● indicates the A_{500}/A_{420} of ROS that have undergone hydroxylamine pretreatment and incubation with AHCoA. △ indicates the A_{500}/A_{420} of purified, labeled, detergent-solubilized rhodopsin and purified, labeled, vesicle-reconstituted rhodopsin which has been detergent-solubilized. ○ indicates the A_{500}/A_{420} of standards generated from mixtures of bleached and unbleached ROS.

In this study samples of unbleached ROS were incubated with AHCoA. The fluorescent anthroyloxy moiety was chosen, in part, because it is readily visualized when the gel is backlighted with a UV lamp. After incubation, the ROS were solubilized in 2% SDS and run on SDS gels. Under these conditions, free, unbound label will migrate near the ion front on the gel, while label that is covalently attached to protein will co-migrate with the protein. Figure 2, lane 1, shows the results of the above experiment. Barely detectable levels of fluorescence are associated with the rhodopsin band on the SDS gel, indicating that either a low level of exchange is occurring between the endogenously bound palmitates on rhodopsin and the fluorescent palmitoyl CoA or a small percentage of other sites on the protein are being labeled nonspecifically. Alternatively, a small population of depalmitoylated rhodopsin may be present in the native membrane which then becomes labeled by AHCoA. When fully bleached ROS were incubated with AHCoA, nearly identical data were obtained (data not shown). It has recently been shown that a substantial amount of the palmitate bound to rhodopsin can be gently stripped from the protein through treatment of ROS with solutions containing 1 M hydroxylamine (Morrison et al., 1991). When rhodopsin is treated with hydroxylamine under specific conditions, the thioester bond linking palmitate to the protein is cleaved, leaving a free sulfhydryl group on rhodopsin and forming the hydroxamic acid derivative of palmitate. Thioesters are more labile than esters and can be cleaved by neutral solutions of hydroxylamine, while O-esters are cleaved under more alkaline conditions (Kaufman et al., 1984). Under certain conditions, hydroxylamine is also reactive with the amide group in proteins, resulting in the formation of hydroxamates of asparagine and glutamine. It is unlikely that such a reaction has occurred under the mild conditions used here (Canova-Davis et al., 1992). Figure 2, lane 2, shows the results of an experiment in which ROS were first treated with 1 M hydroxylamine, pH 6.5, before being incubated with AHCoA. The majority of the hydroxylamine was removed by several centrifugation and washing steps before the addition of the fluorescent label. A comparison of lanes 1 and 2 in Figure 2, panels A and B, indicates that the incorporation of fluorescent label into rhodopsin is substantially greater in the hydroxylamine-treated sample. Such a result implies that labeling is predominantly at the native palmitoylation sites that become available for labeling after endogenous palmitate is stripped from rhodopsin. When the sample shown in Figure 2, lane 2, is solubilized with 2% SDS containing 1 M hydroxylamine, most of the fluorescent label is cleaved from rhodopsin (Figure 2, lane 3). This result indicates that the bond linking the fluorescent derivative of palmitate to rhodopsin is, most likely, a thioester.

Absorption Spectra of Solubilized ROS Labeled with AHCoA. Results from a previous study showed that long incubations of rhodopsin with hydroxylamine in the dark can result in the loss of the retinal prosthetic group from the protein (Dartnall, 1968). Recently, ROS which were treated with 1 M hydroxylamine at pH 7.9 were analyzed spectrophotometrically at 498 nm to quantitate the amount of unbleached rhodopsin, and the spectra were then normalized with respect to total protein. The results of this previous study indicated that, after treatment of rhodopsin with hydroxylamine, 85% of the remaining protein was in the unbleached form.

In the present work a sample of ROS was solubilized in the dark with a solution containing 50 mM octyl glucoside. One-half of the volume of this solution was bleached by exposure to room light for 15 min, while the other half was kept in the unbleached form. The two solutions were mixed in various ratios in the dark, and the absorption spectrum of each sample was recorded from 700 to 300 nm. ROS which had been pretreated with 1 M hydroxylamine and then incubated with AHCoA were solubilized in the same octyl glucoside containing buffer, and the absorption spectrum of this sample was also recorded. A standard curve was generated with the results obtained from the unlabeled samples of solubilized rhodopsin containing known quantities of bleached and unbleached protein by plotting the A_{500nm}/A_{420nm} ratio versus the percentage of bleached protein (Figure 3). The absorbance ratio obtained for the ROS sample which was pretreated with hydroxylamine and labeled with AHCoA is marked with a ● in Figure 3. This result indicates that only approximately 15% of the rhodopsin is bleached under these conditions.

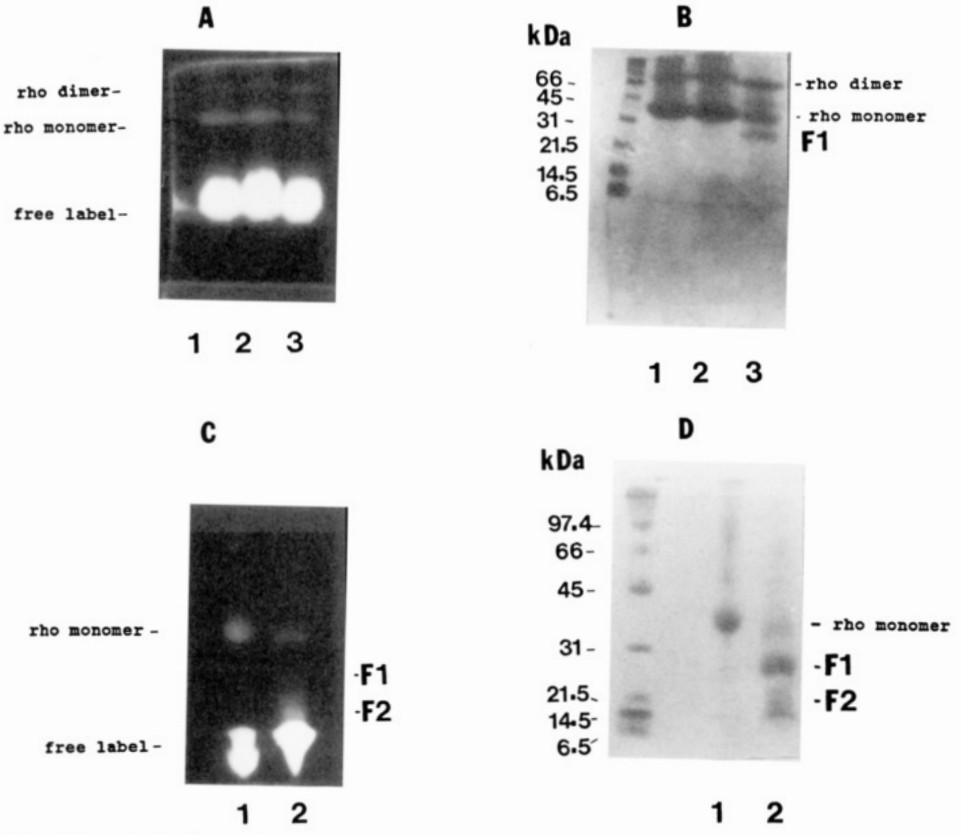

FIGURE 4: 15% SDS–PAGE analysis of papain and thermolysin digests of labeled rhodopsin in ROS. (A) Visualization of fluorescence on gel. Lanes 1 and 2, undigested, labeled ROS; lane 3, labeled ROS digested with papain (0.1 mg of papain to 2 mg of rhodopsin in 67 mM sodium phosphate, pH 7.0; 5 mM cysteine; 2 mM EDTA buffer incubated for 60 min at 37 °C.) (B) Gel shown in panel A after Coomassie Blue staining. (C) Visualization of fluorescence on gel. Lane 1, undigested, labeled ROS; lane 2, labeled ROS digested with thermolysin (0.1 mg of thermolysin to 2 mg of rhodopsin in 10 mM Tris–acetate, pH 7.0; 5 mM $CaCl_2$ buffer incubated for 60 min at 37 °C). (D) Gel shown in panel C after Coomassie Blue staining.

Proteolytic Cleavage of Labeled Rhodopsin in ROS into Two Main Fragments (F1 and F2). To more definitively identify the sites of the fluorescent palmitate attachment in rhodopsin, ROS labeled with AHCoA were digested proteolytically. Under fairly mild conditions, both thermolysin and papain cleave rhodopsin buried in the rod disc membrane into two main fragments, termed F1 (residues 1–240) and F2 (residues 241–327) (Papac et al., 1992). The remaining C-terminal amino acids (328–348) are readily cleaved from holorhodopsin and the F2 fragment. Located within the F2 fragment are the two palmitoylated cysteine residues. Figure 4 shows the results of an SDS–PAGE analysis of labeled ROS which have been digested with papain and thermolysin. Samples of undigested, labeled ROS are shown in lanes 1 and 2 of Figure 4, panels A and B. Fluorescence comigrates with two protein bands on the gel which correspond to the monomer and dimer of rhodopsin. The aggregation of rhodopsin will be discussed in more detail in a subsequent section. Labeled ROS which have been incubated with papain under conditions which result in partial proteolytic digestion of rhodopsin are shown in Figure 4, panels A and B. In lane 3 of Figure 4, panels A and B, the band corresponding to the dimer now migrates as if the molecular weight of the protein has decreased slightly. This result is most likely due to cleavage of C-terminal amino acid residues from the monomer proteins within the dimer. A new band migrates between the monomer and dimer bands which must also be a digestion product of the dimer (possibly dimer minus one F1 fragment). A fourth band migrating below rhodopsin monomer is seen on the protein gel (Figure 4B) but does not contain detectable amounts of fluorescent label and therefore is not visualized in Figure 4A. This band is the F1, or N-terminal, fragment. Although the F2 fragment is obscured by free AHCoA on the fluorescence gel (and does not stain well with Coomassie Blue), the absence of fluorescent label associated with the F1 fragment strongly suggests that label resides in the C-terminal (F2) fragment, which contains the endogenously bound palmitates. Figure 4, panels C and D, shows an SDS gel which corresponds closely to the gel in Figure 4, panels A and B, except that (1) thermolysin was used to digest the protein, (2) a more complete digestion of rhodopsin into F1 and F2 fragments was carried out, and (3) the F2 fragment is partially resolved from the band corresponding to free AHCoA. Figure 4C shows the fluorescence associated with the F1 and F2 digestion products of rhodopsin (lane 2) relative to the fluorescence associated with the undigested protein (lane 1). A small amount of holorhodopsin (or rhodopsin minus small C-terminal peptide fragments) is still observable in lane 2 (Figure 4C). The same gel stained to detect protein is shown in Figure 4D. It is clear that nearly all of the fluorescence in lane 2 of Figure 4C comigrates with either the holoprotein or the F2 fragment. This indicates that the labeling is primarily specific for the C-terminal region of rhodopsin. A very low level of fluorescence appears with the F1 fragment, suggesting that a small amount of nonspecific labeling is occurring. The level of nonspecific labeling is probably 5% on the basis of a comparison of the intensities of the fluorescent and Coomassie Blue stained bands of holorhodopsin and the F1 fragment in lane 2 of Figure 4, panels C and D. The F2 fragment also contains a solvent-exposed cysteine residue at position 316 which has been shown to be reactive to iodoacetamide (McDowell et al., 1979), and it could conceivably be a site for nonspecific attachment of fluorescent label. The results of labeling studies done without

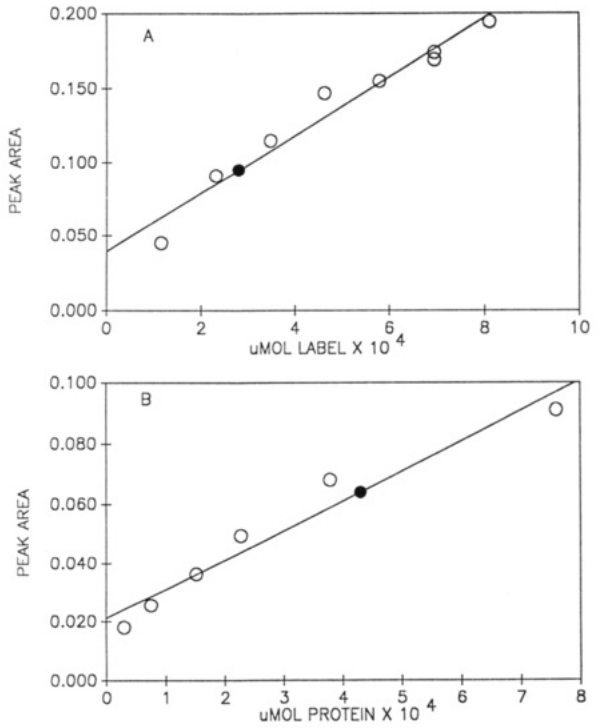

FIGURE 5: Estimation of the average stoichiometry of covalently incorporated fluorescent label to protein by 15% SDS–PAGE and densitometry. (A) Graph of the integrated intensity of the fluorescence samples of AHCoA run on an SDS gel versus the number of millimoles of each sample. ● indicates the integrated fluorescence intensity of a sample of labeled ROS. ○ indicates standard samples. (B) Graph of the integrated intensity of Coomassie Blue stained samples of bovine serum albumin run on SDS gels versus the number of millimoles of each sample. ● indicates the integrated intensity of a sample of labeled ROS (the same volume and concentration of ROS sample as described in (A)).

prior treatment of ROS with hydroxylamine (Figure 2A), however, indicate that such a possibility is not very likely.

Estimation of the Average Molar Ratio of Fluorescent Label Incorporated into Protein. To determine the average percentage of labeled rhodopsin molecules containing a bound fluorescent derivative of palmitate, both a fluorescence standard curve and a protein standard curve were generated as described above. Two representative standard curves are shown in Figure 5. Using this technique, it is estimated that 40–60 mol % of the rhodopsin molecules contain covalently attached fluorescent label. A value between 0 and 200% would be possible due to the two palmitoylation sites on each molecule of rhodopsin. The estimate of 40–60% labeling may represent a lower limit because residual *all-trans*-retinal comigrating with rhodopsin on SDS gels could quench the anthroyloxy fluorescence as a result of energy transfer. Unbound *all-trans*-retinal, however, can be detected visually migrating at the ion front and would not interfere with these measurements. It is important to mention that this method assumes that the quantum yield of the anthroyloxy group on the fatty acid in polyacrylamide gels is the same when bound to the CoA ester and when covalently attached to rhodopsin.

Aggregation of Rhodopsin. The SDS gel in Figure 4, panels A and B, shows the presence of variable amounts of both monomeric and dimeric forms of rhodopsin. Several studies of *S*-palmitoylated proteins which have been treated with reagents like hydroxylamine have revealed that disulfide dimers can sometimes form between protein monomers as a result of stripping endogenous fatty acids from cysteine residues. For example, deacylation of both human tissue factor and the HLA-D-associated invariant chain have been shown to result in the formation of disulfide dimers (Koch & Hammerling, 1986; Bach et al., 1988). Figure 6, panels A and B, shows an SDS gel of hydroxylamine-pretreated, labeled ROS which have been subsequently solubilized in 2% SDS. SDS-solubilized, labeled ROS are shown in lane 1. In addition to the intensely fluorescent band corresponding to rhodopsin monomer, fluorescent label also comigrates with a protein band with a molecular weight corresponding to rhodopsin dimer and two other bands: one migrating slightly behind the dimer and the other at approximately 50 kDa. When the SDS-solubilized, labeled ROS sample is incubated with 2% β-mercaptoethanol for 1 h at room temperature (lane 2), most of the fluorescence associated with the dimer band is eliminated, while the fluorescence intensities of the other bands do not change substantially. A protein stain indicates that this loss of fluorescence is due to loss of dimer (Figure 6 B). It is known that while 2% β-mercaptoethanol is usually capable of breaking disulfide bonds, higher concentrations of this thiol-reducing agent are generally necessary to cleave thioester bonds. For example, Schmidt et al. (1988) showed that 1.4 M (10%) β-mercaptoethanol was necessary to release >90% of the bound fatty acids from the membrane glycoprotein E1 of Semliki Forest virus. The results shown in lanes 1 and 2 of Figure 6 are consistent with the formation of some disulfide dimers of rhodopsin.

When the rhodopsin is incubated at 37 °C in the presence of 10% β-mercaptoethanol, however, a different situation is observed. While the fluorescence intensity of the bands corresponding to the rhodopsin monomer (as well as the bands

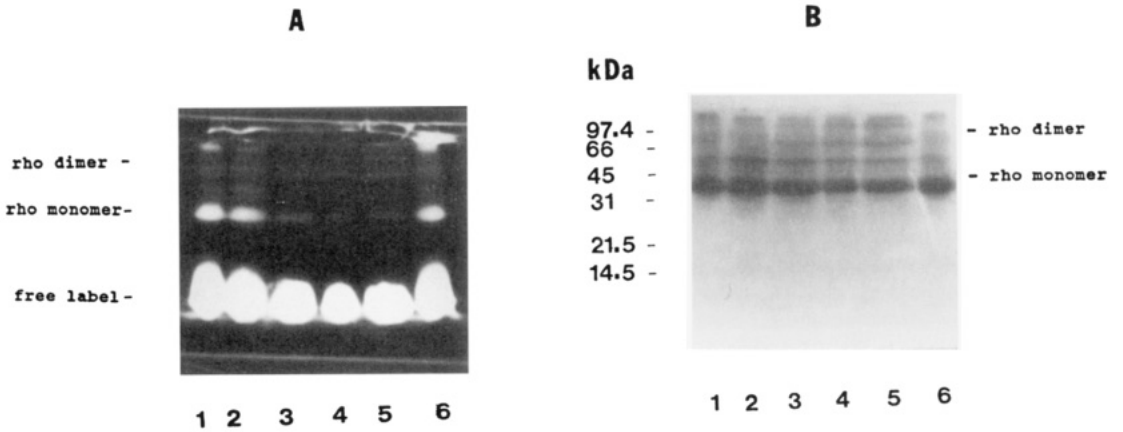

FIGURE 6: 15% SDS–PAGE analysis of the formation of rhodopsin aggregates. (A) Visualization of fluorescence on gel. Lane 1, labeled, SDS-solubilized ROS; lane 2, labeled, SDS-solubilized ROS treated with 2% β-mercaptoethanol for 1 h at room temperature; lanes 3, 4, and 5, labeled, SDS-solubilized ROS treated with 10% β-mercaptoethanol for 15, 45, and 60 min at 37 °C; lane 6, labeled, SDS-solubilized ROS incubated for 1 h at 37 °C. (B) Gel shown in panel A after Coomassie Blue staining.

at 50 kDa and above the dimer band) decreases, the fluorescence intensity of the dimer band becomes increasingly pronounced as the incubation proceeds. This is due to increased amounts of protein migrating at the dimer position. Lanes 3–5 of Figure 6 show SDS-solubilized, labeled ROS in the presence of 10% β-mercaptoethanol which have been incubated at 37 °C for 15, 45, and 60 min, respectively. Lane 6 contains SDS-solubilized, labeled ROS which have been incubated at 37 °C for 1 h. It is clear that neither heating the sample to 37 °C nor SDS solubilization is responsible for this aggregation and that these dimers are not due to the formation of disulfide bonds. As a control, the sample was also incubated with 10% ethanol at 37 °C, and no aggregation was observed (data not shown). Higher molecular weight aggregates corresponding most closely to rhodopsin trimer are also observed in lane 5 of Figure 6.

Purification and Vesicle Reconstitution of Labeled Rhodopsin. To perform fluorescence studies on rhodopsin labeled with a fluorescent derivative of palmitate, it is necessary to remove unbound fluorescent label. This was accomplished by solubilization of labeled ROS with octyl glucoside and purification of rhodopsin on hydroxyapatite and concanavalin A (Con-A) columns. The fluorescent derivative of palmitoyl CoA appeared to have some affinity for Con-A, possibly as a result of the hydrophobic binding pocket known to exist on Con-A (Goldstein & Hayes, 1978). Con-A was not capable of removing all traces of free fluorescent label from samples of solubilized, labeled ROS without prior chromatography of the sample over hydroxyapatite. The A_{500}/A_{420} ratio of purified, labeled rhodopsin eluted from Con-A in octyl glucoside containing buffer indicated that the sample was completely unbleached (Figure 3). This result is consistent with the work of Litman (1982), who found that only unbleached rhodopsin elutes from a Con-A column equilibrated with octyl glucoside. In addition, the rhodopsin-stimulated GTPase activity of the labeled rhodopsin, measured in the laboratory of Dr. R. A. Cerione, was not distinguishable from that of unlabeled rhodopsin. Yields of purified, labeled rhodopsin have generally been between 10 and 30% of the starting material in the ROS. The purified, labeled protein was reconstituted into lipid (dimyristoylphosphatidylcholine) vesicles using the octyl glucoside dilution method of Jackson and Litman (1985). Rhodopsin reconstituted into these vesicles has been shown to be capable of activating transducin (Mitchell et al., 1991).

The percentage of purified rhodopsin molecules containing fluorescent label was estimated to be 40–50% using the procedure described in Figure 5. When freshly prepared bleached vesicles were solubilized with 2% SDS and run on SDS gels, no free label was detected at the ion front (Figure 7, lane 1), although label comigrates with rhodopsin monomer, dimer, and higher aggregates. When the bleached vesicles were kept at room temperature for approximately 24 h and then solubilized and run on SDS gels, there was still no free label detectable, indicating that the thioester bond linking the fluorescent derivative of palmitate is not labile under these conditions (Figure 7, lane 2). However, rhodopsin aggregation is more pronounced in this sample relative to the sample in lane 1. The cause of this aggregation is still unknown, although the dimer is not disrupted significantly in the presence of 2% β-mercaptoethanol. In one experiment, ROS were incubated with a 10-fold excess of palmitoyl CoA following incubation with the fluorescent derivative of the fatty acid. This was done to relabel depalmitoylated protein with palmitate and to determine whether the presence of depalmitoylated protein was contributing to the formation of aggregates in the reconstituted sample. The reconstituted rhodopsin formed from this preparation, however, still showed the presence of protein aggregates.

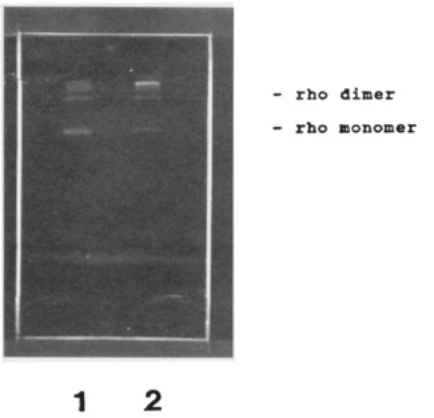

FIGURE 7: Visualization of fluorescence on 15% SDS gel of purified, bleached, vesicle-reconstituted, labeled rhodopsin. Lane 1, SDS-solubilized sample run immediately after bleaching of freshly prepared lipid vesicles containing labeled rhodopsin; lane 2, SDS-solubilized sample run after incubation of vesicles for 24 h at room temperature (sample bleached before incubation).

DISCUSSION

The attachment of palmitic acid to selected cysteine residues of a protein via thioester linkages is one of several ways that proteins are covalently modified by lipids. A diverse group of proteins have been found to be *S*-palmitoylated including ankyrin, major histocompatibility antigens, ras-like proteins, viral coat proteins, proteolipid protein of myelin, human tissue factor, neuronal growth cone protein GAP-43, transferrin receptor, β2-adrenergic receptor, and rhodopsin [for reviews, see Sefton and Buss (1987) and Towler et al. (1988)]. In most cases, the specific functional role played by the palmitate covalently attached to protein remains obscure. Palmitoylation of several soluble proteins, i.e., ras proteins and GAP-43, has been shown to increase their ability to bind to cell membranes (Hancock et al., 1989; Pearce et al., 1991; Skene & Virag, 1989; Sudo et al., 1992). A number of integral membrane proteins also have been found to be palmitoylated, e.g., β2-adrenergic receptor and rhodopsin. One way in which *S*-palmitoylation and *O*-palmitoylation (linkage of palmitate to a hydroxyl group on a protein) differ from other forms of lipid modification, e.g., *N*-myristoylation, isoprenylation, and the addition of glycosylphosphatidylinositol, is that the thioester (or ester) bond is potentially labile. In some cases, the palmitate has been shown to turn over with a much shorter half-life than that of the protein (Skene & Virag, 1989; Sefton & Buss, 1987; James & Olson, 1990; Mouillac et al., 1992). This high turnover rate indicates that the addition and removal of palmitate to and from protein may, in certain cases, represent a dynamic signaling process (Skene & Virag, 1989; Sudo et al., 1992).

In this work we have synthesized a fluorescent derivative of palmitoyl CoA (AHCoA) and labeled the palmitoylation sites (cysteine residues 322 and 323) in the C-terminal region of the membrane-bound receptor rhodopsin. This was accomplished by treating isolated retinal rod outer segments (ROS) with 1 M hydroxylamine to remove a portion of the endogenously bound palmitates. This was followed by incubation of the ROS with AHCoA. SDS–PAGE and proteolysis was used to demonstrate covalent attachment of label to protein, to show that the labeling site is located in the C-terminal region of the protein, and to estimate that an average of >40% of the protein molecules contain covalently

bound label. A purification procedure was developed to remove unbound fluorescent label from protein, and purified, labeled rhodopsin was reconstituted into lipid vesicles. No evidence was found to indicate that the thioester bond linking lipid to protein in samples of vesicle-reconstituted, purified, labeled rhodopsin is labile; i.e., no deacylation of protein was observed. It is possible, however, that the presence of a specific enzyme is required for deacylation of rhodopsin, although no enzymes catalyzing ester-linked acylation/deacylation of proteins have been purified to date (James & Olson, 1990). It is interesting that chemically deacylated rhodopsin readily incorporates palmitate from palmitoyl CoA (Morrison et al., 1991; this work). It had previously been demonstrated that, under certain conditions, low-level palmitoylation of rhodopsin can occur nonenzymatically (O'Brien et al., 1987).

Treatment of SDS-solubilized rod outer segments with reagents capable of cleaving thioester bonds resulted in the formation of aggregates of rhodopsin. The formation of rhodopsin aggregates as detected by SDS–PAGE has been observed for over a decade (DeGrip, 1982). We are unaware of any physical explanation of this phenomenon. A tempting, but very speculative, interpretation of these results is that aggregation is induced by partial depalmitoylation of the protein. Possibly, a hydrophobic patch may be uncovered when the protein is partially deacylated, and this might interact with a particular site on another rhodopsin molecule.

The preparation of vesicle-reconstituted rhodopsin containing a fluorescent label at the palmitoylation site of the protein is the first step toward detailed biophysical studies of this site. Since the palmitoylation sites are in close proximity to the binding regions for coupling and regulatory proteins, fluorescent probes at these sites may provide important reporter groups for protein–protein interactions mediating signal transduction.

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