Fluorescence Studies of the Location and Membrane Accessibility of the Palmitoylation Sites of Rhodopsin†

Susan J. Moench, Julie Moreland, David H. Stewart, and T. Gregory Dewey*

Department of Chemistry, University of Denver, Denver, Colorado 80208

Received October 4, 1993; Revised Manuscript Received March 4, 1994®

ABSTRACT: Fluorescent fatty acid labels have been incorporated into the palmitoylation sites of rhodopsin and used to probe the membrane accessibility and location of these sites. The fluorescence properties of anthroyloxy and pyrenyl fatty acids bound to rhodopsin were investigated in a reconstituted vesicle system. Collisional quenching of fluorescence by stearic acid (DSA) labeled with doxyls in the 16, 12, and 5 positions was used to determine the membrane accessibility and disposition of the modifying fatty acids. To properly determine the membrane concentration of these quenchers, the dependence of the Stern–Volmer parameters on both quencher and vesicle concentration was determined. An analysis of these dependences provided a correction for partitioning of the quencher between the aqueous phase and the membrane. After this correction, the relative effectiveness of doxyl quenchers was 16-DSA > 12-DSA > 5-DSA. Parallel studies on free anthroyloxy and pyrenyl fatty acids incorporated into the reconstituted system showed the same dependence on quencher position. These results indicate that the labels at the palmitoylation sites of rhodopsin are situated in the membrane much as a free fatty acid. This anchoring of the palmitates in the membrane results in the formation of a fourth cytoplasmic loop.

Rhodopsin is a 40-kDa integral membrane protein which resides in the discs of the rod cells of the retina. It is the G-protein-coupled receptor responsible for visual transduction. A variety of approaches have indicated that the protein contains seven transmembrane helices that create three water-exposed loops on both the cytoplasmic and intradiscal sides of the disc membrane (Hargrave et al., 1983; Nathans & Hogness, 1983). Rhodopsin is posttranslationally modified by two palmitates that are covalently bound through thioester linkages to cysteines 322 and 323 in the C-terminal cytoplasmic region of the protein (O'Brien et al., 1987; Ovchinnikov et al., 1988; Papac et al., 1992). Although the function of rhodopsin palmitoylation is still unclear (Morrison et al., 1991; Karnik et al., 1993; Traxler & Dewey, 1994; Moench et al., 1994), it has been suggested that the palmitates bound to rhodopsin might anchor the carboxyl tail of the protein to the membrane. Such a modification would substantially alter the protein structure, creating an additional, fourth loop on the cytoplasmic side of the membrane. The presence of such a loop could have significant functional implications since the C-terminal cytoplasmic face of rhodopsin is the binding site for a number of proteins involved in the regulation of visual transduction (Wilden et al., 1986; Palczewski et al., 1988, 1989; Konig et al., 1989; Franke et al., 1990).

The present work utilizes techniques developed in the accompanying paper (Moench et al., 1994) to probe the environment of the palmitoylation sites. In this work, the endogenous palmitates of rhodopsin were replaced with fluorescent fatty acid derivatives and the fluorescent properties of the labels were explored. Collisional quenching by membrane probes and fluorescence resonance energy transfer were measured for fluorescent labels in the palmitoylation sites of rhodopsin reconstituted into phospholipid membranes. A comparison of the quenching results with those of free, unbound fatty acid labels allows an assessment of the influence of the protein on the location of the label in the membrane. It is seen that these modifying fluorescent palmitates are in a membrane environment much like that of a free fatty acid.

MATERIALS AND METHODS

Synthesis and Purification of 1-Pyrenedodecanoyl Coenzyme A Ester. 1-Pyrenedodecanoyl coenzyme A ester (PDCoA)[1] was synthesized and purified by a slight modification of the procedure used in the accompanying paper (Moench et al., 1994). 1-Pyrenedodecanoic acid (PDA) was purchased from Molecular Probes. The NHS ester of this fatty acid was synthesized and purified by the procedure of Lapidot et al. (1967) as modified by Moench et al. (1994) except that the reaction of the fatty acid with DCCD and NHS was carried out using THF as solvent. The ratio of both DCCD and NHS to the fatty acid was 3.2:1, and the NHS ester was purified on silica gel TLC plates with chloroform as the chromatographing solvent. The portion of the silica gel plate containing the NHS ester of the fluorescent fatty acid (R_f approximately 0.5) was scraped off, suspended in anhydrous ethyl ether, and treated as described previously (Moench et al., 1994). The CoA ester was formed by dissolving the dried NHS ester in approximately 2 mL of DMSO and adding the solution to 2 mL of a solution of coenzyme A, sodium salt (Sigma), dissolved in water (the coenzyme A:NHS ester ratio was approximately 4:1). DMSO was then added dropwise to the resulting cloudy solution until the solution became clear. Approximately 10–20 mg of sodium bicarbonate was added, and the solution was

† This work is supported by a grant from the American Heart Association of Colorado (S.J.M.) and by NSF Grant No. DMB-9002084 (T.G.D.). D.H.S. is supported by the NSF REU program.

* Author to whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

[1] Abbreviations: 16-(9-anthroyloxy)hexadecanoic acid, AHA; 1-pyrenedodecanoic acid, PDA; coenzyme A, CoA; 1-pyrenedodecanoyl coenzyme A ester, PDCoA; *N*-hydroxysuccinimide, NHS; dicyclohexylcarbodiimide, DCCD; dimethyl sulfoxide, DMSO; tetrahydrofuran, THF; concanavalin A, Con-A; doxylstearic acid, DSA; sodium dodecyl sulfate, SDS; dimyristoylphosphatidylcholine, DMPC; dipalmitoylphosphatidylcholine, DPPC; polyacrylamide gel electrophoresis, PAGE.

0006-2960/94/0433-5791$04.50/0

agitated at room temperature for approximately 8 h. Formation of the CoA ester was monitored by TLC on silica gel using chloroform as solvent. Under these conditions, the CoA ester remained at the origin. The CoA ester was purified by chromatography over two hydroxyapatite columns as described previously (Moench et al., 1994) except that the CoA reaction mixture was added directly to the hydroxyapatite columns equilibrated with water. Detergent, residual coenzyme A, and other small impurities were removed from the PDCoA after elution by dialysis against 50 mM Tris–acetate, pH 7.0. Yields, estimated by absorbance and by assuming an extinction coefficient of 40 mM^{-1} cm^{-1} at 347 nm, were between 15 and 20% of the starting fluorescent fatty acid.

Preparation of Rhodopsin with Covalently Attached Derivatives of Palmitate and Reconstitution in DMPC Vesicles. Rhodopsin was labeled with either 1-pyrenedodecanoic acid or 16-(9-anthroyloxy)hexadecanoic acid using the coenzyme A esters of these fatty acids and was purified as described previously (Moench et al., 1994). Native rhodopsin (without fluorescent label) was purified over Con-A–agarose (Moench et al., 1994). Rhodopsin was reconstituted into DMPC vesicles using the procedure of Jackson and Litman (1985) as modified by Mitchell et al. (1991). The concentration of rhodopsin in the vesicle solution was determined by absorbance at 500 nm, assuming an extinction coefficient of 40 mM^{-1} cm^{-1} (Shichi, 1970). To reduce light scattering in these measurements, 1 mL of a solution of the vesicles was solubilized with 1 mL of 50 mM octyl glucoside and 10 mM sodium phosphate, pH 7.0. Concentrations of rhodopsin in vesicle preparations were generally between 2 and 3 μM. Prior to exposure to light, the rhodopsin in the preparations was in the unbleached form, as indicated by the A_{500}/A_{420} ratio (Moench et al., 1994).

Determination of Phospholipid Vesicle Concentration by Quantitation of Phosphorus. The concentration of phospholipids was determined by the method of Morrison (1964). Aliquots (0.2 and 0.4 mL) of solutions of rhodopsin reconstituted into DMPC vesicles were dried in Eppendorf tubes in a Speed-Vac concentrator and resuspended in 0.3 mL of concentrated H_2SO_4 before being transferred to Pyrex tubes for charring over an open flame. A single drop of 30% H_2O_2 was added to each tube after the sample had cooled to room temperature, and the samples were placed in boiling water for 1 min. After sample cooling, 3.2 mL of distilled water and 0.3 mL of 16.5%, w/v, anhydrous sodium sulfite were added to each tube and mixed well, followed by the addition of 1 mL of 2%, w/v, ammonium molybdate and mixing. Finally, distilled water was added to each tube to give a volume of 5 mL, and approximately 10 mg of sodium ascorbate was added to each tube and mixed well. The tubes were then placed in boiling water for 10 min and cooled. Absorbances were monitored at 822 nm and compared with a set of standard solutions containing various concentrations of sodium phosphate (Morrison, 1964). Standard solutions were prepared as described above with the exception of the charring step.

Phospholipid vesicle concentrations were calculated by assuming a value of 711 Å as the mean diameter of the vesicles, which were shown to vary in diameter between 560 and 850 Å (Jackson & Litman, 1985). The number of lipid molecules/vesicle was calculated to be 5.13×10^4 using the value of 62 $Å^2$/lipid. Measured surface areas of phospholipids cover a range of values. DPPC's surface area was determined to be 62 $Å^2$ (Nagle, 1993).

Fluorescence Measurements. All steady-state fluorescence experiments were performed at room temperature using a Spex Fluorolog 2 spectrofluorimeter. These studies included samples of rhodopsin-containing DMPC vesicles with covalently bound labels or free fatty acid labels. To prepare rhodopsin reconstituted vesicles containing free fluorescent fatty acids, small aliquots of label (~10 μL), dissolved in either DMSO (for AHA) or THF (for PDA) at a concentration of 1 mg/mL, were added to the vesicle preparation. The ratio of the concentration of free labeled fatty acid to rhodopsin in such vesicles was between 0.4 and 0.8. In these samples, rhodopsin contained endogenous palmitates. The fluorescent label to lipid molar ratio varied between 1:250 and 1:700. Solutions of vesicles were allowed to equilibrate with fluorescent label for at least 3 h at room temperature before spectral measurements were made.

Four different samples were studied. These were vesicles containing rhodopsin with bound or free PDA and vesicles containing rhodopsin with bound or free AHA. Sample conditions could be manipulated to explore rhodopsin in the unbleached, "meta II", and fully bleached (opsin) forms. Unbleached samples were exposed only to dim red light except for fluorimeter light exposure where narrow slit widths and brief measurement periods were employed. Absorbance spectra of these samples indicated that no detectable amount of rhodopsin bleaching had occurred during these manipulations. In all fluorescence experiments no detectable evidence of excimer formation was observed. Samples termed meta II were exposed to a 150-W xenon arc lamp for 20 s and then placed in the fluorimeter so that spectral measurements were generally completed within 1 min of initial exposure to light. Fully bleached samples were formed by exposure to a light box for 3 h at room temperature. Under these conditions, rhodopsin was converted completely to opsin.

Fluorescence Quenching Experiments. 5- and 12-doxyl stearic acid (DSA) were purchased from Molecular Probes, and 16-DSA was purchased from Sigma. The DSA samples were dissolved in DMSO in concentrations ranging from 0.3 to 10 mg/mL. Samples containing AHA were excited at 365 nm, and fluorescence emission was monitored at 460 nm. Samples containing PDA were excited at 347 nm, and fluorescence emission was monitored at 381 nm. These wavelengths represent the λ_{max} values for the two sets of excitation and emission spectra. Quenching experiments were performed by sequential addition and mixing of small aliquots (usually 1–2 μL) of a DSA solution to the fluorescent sample. Titrations of fully bleached samples with 5-, 12-, and 16-DSA solutions were carried out until at least 50% of the original fluorescence emission intensity was quenched. Final volumes of DMSO in the samples generally did not exceed 1% of the total sample volume, and fluorescence emission intensities were not corrected for dilution by DSA solutions. Background and light-scattering contributions were determined for samples with bound label by measuring the fluorescence intensity in the presence of saturating amounts of quencher (0.2 mg/mL 16-DSA). These intensities were subsequently subtracted from all fluorescence measurements. Typically, these corrections were 10% of the fluorescence signal. However, for the case of unbleached rhodopsin with bound PDA and bleached rhodopsin with bound AHA, corrections in the range from 25 to 50% were required. Light-scattering contributions to the fluorescence of vesicles with unbound label were measured directly before the addition of label. Titrations of each of the four types of fully bleached samples with each of the three DSA quenchers were performed in vesicle solutions at three concentrations which were prepared by making 1:2 and 1:4 dilutions of the most concentrated solutions of vesicles with 50 mM Tris–acetate, pH 7.0.

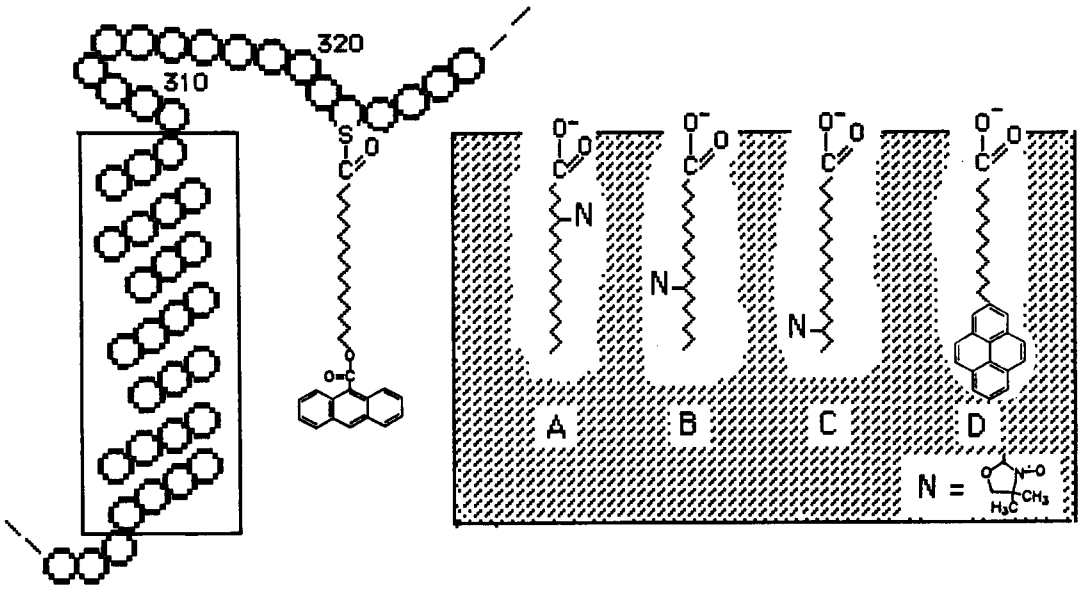

FIGURE 1: Carboxyl-terminal region of rhodopsin showing the replacement of the endogenous palmitates at Cys 322 and Cys 323 with covalently bound AHA. Also shown are the structures of (A) 5-DSA, (B) 12-DSA, (C) 16-DSA, and (D) PDA. The shaded area indicates lipid membrane.

Fluorescence quenching experiments were also done on unbleached samples of vesicles containing rhodopsin with bound PDA. A successive titration of the sample with aliquots of DSA was not possible because the rhodopsin was gradually bleached by the light beam of the fluorimeter. Consequently, small aliquots of the DSA quenchers were added under red light to individual (1.5 mL) samples of vesicles containing rhodopsin with bound PDA. For example, 1, 3, 5, or 10 μL of each of the three DSA solutions was added to twelve samples of vesicles containing rhodopsin with bound PDA. Each sample was kept in the dark until the fluorescence intensity was recorded at a single wavelength, thereby effectively preventing sample bleaching. The meta II form of each of the above samples was then prepared, and the fluorescence emission intensity was recorded as described in the previous section.

Fluorescence Resonance Energy Transfer. Fluorescence resonance energy transfer measurements were made on vesicle samples containing bound anthroyloxy and pyrenyl labels. The anthroyloxy label was excited at 365 nm, and the fluorescence emission was monitored at 460 nm. Corresponding measurements of samples containing the pyrene label were excited at 347 nm, and fluorescence emission was measured at 381 nm. Contributions from light scattering were subtracted from each of these values through independent measurements discussed in the previous section.

For both the anthroyloxy and pyrenyl labels bound to rhodopsin, considerable quenching occurred due to resonance energy transfer from the probe to the retinal in both the unbleached and the meta II state. The efficiency, E, of this energy transfer is defined by

$$E = 1 - \frac{F_{DA}}{F_D} = 1 - \frac{\tau_{DA}}{\tau_D} \quad (1)$$

where F_{DA} and F_D are the fluorescence intensity of the donor (anthroyloxy or pyrenyl) in the presence and absence of the acceptor (retinal), respectively. The efficiency can also be defined in terms of the fluorescence lifetimes, τ_{DA} and τ_D (Hammes, 1981). This latter relationship provides an estimate of the change in fluorescence lifetime of the donor between the unbleached and bleached state (opsin). These estimates were useful in the interpretation of the quenching data.

Table 1: Resonance Energy Transfer Parameters[a]

parameter	pyrenyl label	anthroyloxy label
E(unbleached)	0.75	0.93
E(meta II)	0.79	
R_o(unbleached)	36 Å	50 Å
R_o(bleached)	38 Å	36 Å
R(unbleached)	30 Å	32 Å
R(meta II)	30 Å	

[a] E is the efficiency of quenching relative to opsin state. R is the distance from the modifying fatty acid to the retinal acceptor in the indicated state. See text for details of meta II measurement. R_o is the characteristic Foerster distance and was determined as described in Hasselbacher and Dewey (1986). Quantum yields for anthroyloxy (0.55) and pyrenyl fatty acids (0.32) were determined previously in DMPC vesicles (Hasselbacher & Dewey, 1986). For the R_o calculations, the refractive index of the media was assumed to be 1.33, and κ^2, the orientation factor, was taken to be 2/3. J, the overlap integral, is determined from the fluorescence emission of free fluorescent fatty acids in organic solvent, and absorption spectra of the retinal acceptor were obtained using octyl glucoside solubilized rod outer segments. Absorption spectra of bleached rhodopsin were used to estimate the meta II spectra. Considerable error exists in the R_o measurement as a result of uncertainty in the orientation factor. This is a result of the restricted motion of both the retinal acceptor and the fatty acid donor. See text for more details.

Additionally, the distance, R, between the donor and acceptor can be calculated using E. The results of such a calculation are shown in Table 1. Values of F_{DA} were determined for bound fluorescent label in either the unbleached or the meta II state of rhodopsin. The fluorescence intensity, F_D, was determined for the bound label in bleached rhodopsin. No attempt was made to correct F_D for energy transfer to free retinal released from the opsin state.

RESULTS

Fluorescence Quenching Measurements. Fluorescence quenching by doxyl stearic acids in which the doxyl group is located at the 5, 12, or 16 position in the fatty acid chain was used to determine the membrane accessibility and relative depth of the fluorescent fatty acids modifying rhodopsin (see Figure 1). A typical Stern–Volmer plot (Figure 2) shows the quenching of the anthroyloxy moiety for free and rhodopsin-bound label. The quencher 16-DSA was used in this particular experiment. As is commonly observed, this data shows curvature at high quencher concentration. There are a number

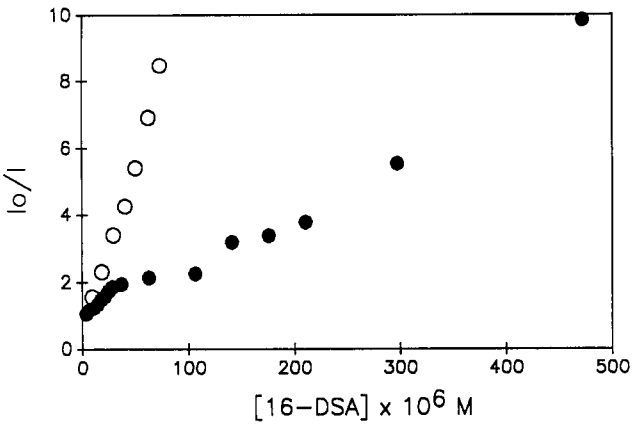

FIGURE 2: Stern–Volmer plots for the quenching of the fluorescence intensity of AHA in DMPC vesicles containing fully bleached rhodopsin. The quencher was 16-DSA, and the fluorescent species was free AHA (○) or rhodopsin-bound AHA (●). The buffer was 50 mM Tris–acetate, pH 7.0, and the vesicle concentration was approximately 4 nM for both samples.

of possible reasons for this curvature, but these were not explored in the present work. Typically, in membrane studies the linear region between 0 and 50% quenching is used to analyze the quenching behavior (Vermeir et al., 1992). This region was the focus of this work, and consequently, the results are pertinent only to this quenchable subpopulation. In Figure 2 the concentration given for the 16-DSA quencher was calculated as if the quencher was uniformly distributed in the bulk solution. This clearly is not the case because a large portion of the quencher will concentrate in the membrane. To accurately assess the quenching ability of a given molecule, the concentration in the membrane must be determined. To do this, the dependence of fluorescence intensity on both quencher and vesicle concentration must be measured.

To correct the Stern–Volmer plots for quencher partitioning between the aqueous and membrane compartments and/or binding to the vesicles, the analysis of Blatt and Sawyer (1985) and Encinas and Lissi (1982) [see also Vermeir et al. (1992)] was used. In this analysis, the quencher is assumed to exist in one of two compartments, aqueous (A) or lipid (L). Thus, the total bulk quencher concentration, $[Q]_T$, is given by

$$[Q]_T = [Q]_A + [Q]_L \quad (2)$$

The total concentration can be related to two constants, K and $\langle Q \rangle$, where $\langle Q \rangle$ is the average number of quenchers per vesicle. The constant K is called a "generalized" constant in that it accounts for both partitioning and specific site binding behavior of the quencher. From simple equilibrium considerations (Blatt & Sawyer, 1985) it is seen that

$$[Q]_T = \frac{\langle Q \rangle}{K} + \langle Q \rangle [\text{ves}] \quad (3)$$

where [ves] is the concentration of vesicles. Figure 3A shows a Stern–Volmer plot for the quenching of unbound anthroyloxy probe by 16-DSA using three different vesicle concentrations. The slopes of these plots become steeper as the vesicle concentration decreases. This is a result of increased quencher concentration in the lipid phase at decreased vesicle concentration. It is assumed that identical quenching (I_o/I) for all vesicle concentrations will occur when $[Q]_L$ is constant. Horizontal lines can be drawn across Figure 3A to give the condition of constant $[Q]_L$. From the intersection of these lines with the experimental curves, $[Q]_T$ and [ves] values can be determined at a constant $[Q]_L$. These values are used to construct the secondary plots (Figure 3B). These plots are

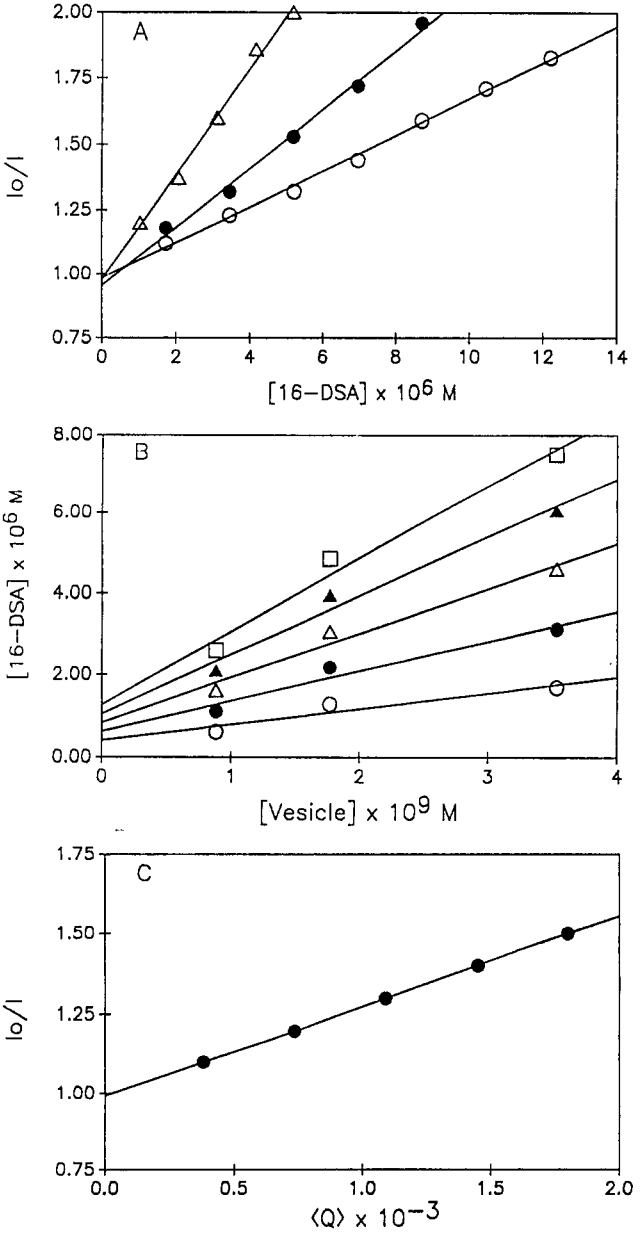

FIGURE 3: Quenching of the fluorescence intensity of free AHA in rhodopsin–DMPC vesicles by 16-DSA. (A) Stern–Volmer plots (I_o/I versus $[Q]_T$) for several vesicle concentrations (△, 0.88 nM; ●, 1.77 nM; ○, 3.53 nM). (B) Data plotted according to eq 5 with I_o/I = 1.5 (□), 1.4 (▲), 1.3 (△), 1.2 (●), and 1.1 (○). (C). Corrected Stern–Volmer plots (I_o/I versus $\langle Q \rangle$) showing the fluorescence intensity ratio as a function of the average number of quenchers per vesicle.

then fit to eq 3 and provide the constants K and $\langle Q \rangle$. The final plot of I/I_o versus $\langle Q \rangle$ (Figure 3C) now gives a Stern–Volmer plot that has been corrected for the distribution of quencher in the aqueous phase and is independent of vesicle concentration. This procedure is especially important when doxyl fatty acids are used, as the partitioning of these species is dependent on the position of the doxyl group (Blatt & Sawyer, 1985).

In the present work the constants K and $\langle Q \rangle$ were determined for all three doxyl quenchers for samples containing bound and unbound anthroyloxy or pyrenyl fatty acids in bleached rhodopsin vesicles. The constant $\langle Q \rangle$ determined above was used to directly generate the final, corrected Stern–Volmer plot. Figure 4 shows the corrected Stern–Volmer plots for free and bound anthroyloxy and pyrenyl labels in bleached rhodopsin vesicles. Figure 5 shows the original and corrected Stern–Volmer plots for the pyrenyl probe bound to unbleached rhodopsin. Values of K and $\langle Q \rangle$ obtained for

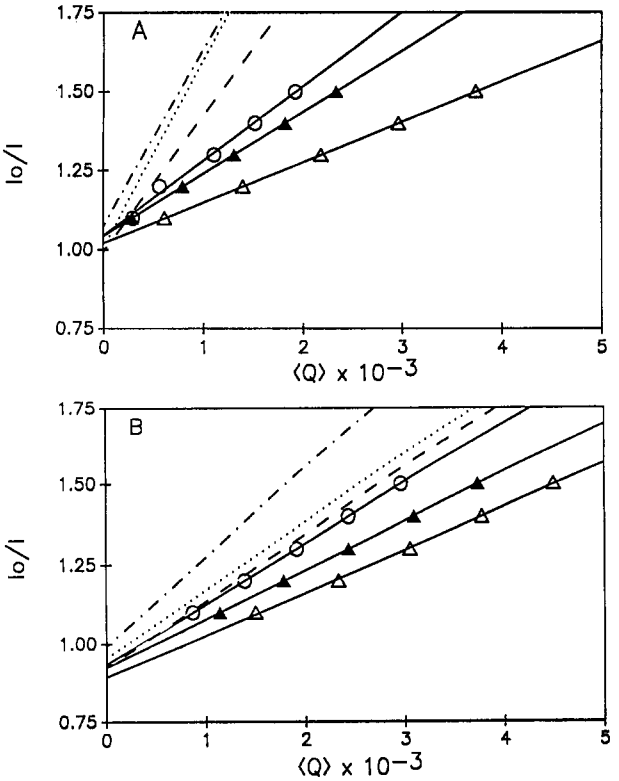

FIGURE 4: Corrected Stern–Volmer plots showing the fluorescence intensity ratio as a function of the average number of quenchers per vesicle, $\langle Q \rangle$. (A) Quenching of free and covalently linked PDA in bleached rhodopsin–DMPC vesicles. Data are not shown for free PDA. Lines indicate linear least-squares fits to the free PDA data for 16 DSA (– · –), 12-DSA (···), and 5-DSA (- - -). Data for bound PDA is given for 16-DSA (○), 12-DSA (▲), and 5-DSA (△). (B) Quenching of free and covalently linked AHA in bleached rhodopsin–DMPC vesicles. Data are not shown for free probe. Lines indicate least-squares fits to the free AHA data for 16-DSA (– · –); 12-DSA (···), and 5-DSA (- - -). Data for bound AHA is given for 16-DSA (○), 12-DSA (▲), and 5-DSA (△).

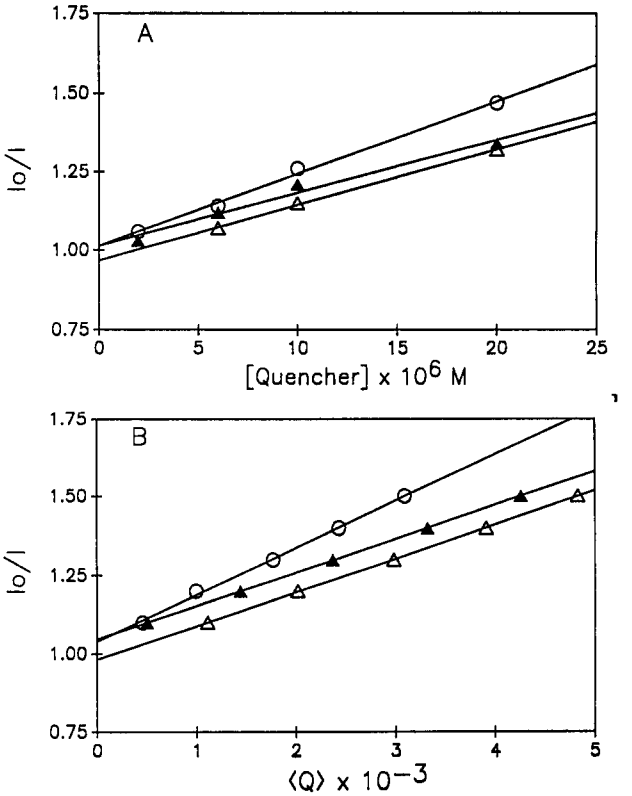

FIGURE 5: Quenching of the fluorescence intensity of covalently bound PDA in unbleached rhodopsin–DMPC vesicles. (A) Stern–Volmer plots (I_o/I versus $[Q]_T$) for the fluorescence intensity ratio as a function of total quencher concentration. Quenchers were 16-DSA (○), 12-DSA (▲), and 5-DSA (△). (B) Corrected Stern–Volmer plots (I_o/I versus $\langle Q \rangle$) showing the fluorescence intensity ratio as a function of the average number of quenchers per vesicle. Vesicle concentration was 6.45 nM.

fully bleached samples of rhodopsin with bound PDA were used to generate these corrected plots. Because of the large quenching of anthroyloxy resulting from resonance energy transfer to the retinal, the collisional quenching of this label could not be investigated in unbleached rhodopsin. For both of the labels, the fluorescent moiety is located at the end of the fatty acid chain (see Figure 1). It is seen that in all cases the effectiveness of quenching is 16-DSA > 12-DSA > 5-DSA over the concentration range of this investigation. This is anticipated for the free label and indicates that the fluorescent fatty acid is situated in the membrane much as a normal fatty acid would be. The same trend is seen for rhodopsin-bound label, indicating that its situation is also similar to that of a normal fatty acid. In all cases, the Stern–Volmer plots are less steep for the rhodopsin-bound label than for free label. This slope is equal to the product of the fluorescence lifetime, τ, and the bimolecular rate constant for quenching, k_Q. Changes in these slopes reflect changes in both of these parameters and are discussed more completely in the next section.

Resonance Energy Transfer to Retinal. Both the anthroyloxy and pyrenyl fatty acids will transfer energy to the retinal in rhodopsin via Foerster transfer. Table 1 shows that the Foerster distances for these donor-retinal pairs are relatively long and that significant quenching of the fluorescence will occur. In the case of the anthroyloxy label in unbleached rhodopsin, approximately 90% of the fluorescence is quenched by this mechanism. This is verified by the large fluorescence enhancement upon bleaching of the rhodopsin. While energy transfer will still occur to retinal in the bleached state of rhodopsin, opsin formation will result in diffusion of the retinal from its location within the rhodopsin to a distance far removed from the palmitate. This relatively slow process is monitored by a continual increase in fluorescence, which stabilized after a 4-h time period.

Resonance energy transfer in this system is not an optimal situation for distance determination. First, the Foerster distances are long relative to the distance between the retinal and the palmitates. This results in extremely high energy transfer efficiencies that are difficult to determine accurately. Additionally, there will be considerable errors in determining the Foerster distance because of uncertainty in the orientation factor, κ^2. This uncertainty is due to a lack of rotational averaging in the system. The retinal will be held rigidly within the protein, and the pyrenyl label is constrained so that its motion will be anisotropic. Consequently, there will be unacceptably large errors in the distances as a result of uncertainties in κ^2 (Dale et al., 1979). Nevertheless, it is important to characterize this energy-transfer effect, as it changes the fluorescence lifetime of the probe (see eq 5). Such changes are important in the interpretation of the quenching data. As will be seen in the next section, energy transfer effects alone are not sufficient to account for the observed changes in these other variables. For completeness, Table 1 shows the energy-transfer parameters for the system.

DISCUSSION

The results of this work show that, in a reconstituted system, the palmitates modifying rhodopsin behave much as a normal membrane fatty acid would. The fluorescence quenching data

Table 2: Comparison of Stern–Volmer Parameters

Comparison of Bound and Free, τ(bound)k_Q(bound) / τ(free)k_Q(free)				
		pyrenyl		
	anthroyloxy, bleached	bleached	unbleached	corrected unbleached[a]
16-DSA	0.68	0.42	0.20	1.08
12-DSA	0.74	0.33	0.18	0.72
5-DSA	0.66	0.30	0.25	1.00

Comparison of Anthroyloxy to Pyrene				
	free		bound	
	τ(AHA)k_Q(AHA) / τ(PDA)k_Q(PDA)	k_Q(AHA) / k_Q(PDA)	τ(AHA)k_Q(AHA) / τ(PDA)k_Q(PDA)	k_Q(AHA) / k_Q(PDA)
16-DSA	0.50	15.4	0.80	24.8
12-DSA	0.35	11.0	0.79	24.3
5-DSA	0.48	14.8	1.28	38.6

[a] Corrected for lifetime decreases due to resonance energy transfer to the retinal.

shows that the effectiveness of quenchers is 16-DSA > 12-DSA > 5-DSA for the concentration regime that was investigated. This dependence is identical to that for an unbound fatty acid. To further highlight this similarity, a number of quantitative features of the quenching data are considered. The slopes of the corrected Stern–Volmer plots will be proportional to τk_Q. In Table 2 the ratios of these slopes are considered for two situations. First, for bleached rhodopsin the ratios of the slopes of bound and free labels are obtained for identical quenchers. This ratio ranges from 0.18 to 0.74, depending on the specific label and the state of the rhodopsin. Since the fluorescent labels appear to be in a lipid environment, it is anticipated that the lifetime of the bound label in opsin will be comparable to that of the free label. The exception will be the case of label bound to unbleached rhodopsin. In this instance resonance energy transfer to the retinal will reduce the fluorescence lifetime. Using eq 1 and the data in Table 1, it is possible to correct for this effect, and this was done for the pyrenyl label bound to unbleached rhodopsin (see Table 2). With these considerations, it is seen that the bimolecular rate constant for quenching of bound fatty acid label is only slightly reduced from that of free label. This provides strong evidence that the label is in the membrane domain rather than buried in the protein. Regardless of the specific sample under consideration, the ratio τ_{bound}/τ_{free} is a constant, being independent of the particular quencher used. Interestingly, the ratio of the slopes also is essentially a constant. This means that the ratio of the bimolecular rate constants for quenching is independent of the quenching position. This result would be obtained if the modifying palmitates were held in the membrane as a free lipid with little interference from the protein.

Alternatively, the ratio of slopes for the anthroyloxy and pyrenyl curves can be considered. These are listed in Table 2. Fluorescence lifetimes of 14 ns (Blatt et al., 1986) and 208 ns (Hresko et al., 1986) have been observed for free anthroyloxy and pyrenyl fatty acids, respectively. However, the 208-ns value for pyrene is in a deoxygenated solution and, therefore, will be much longer than in our oxygenated samples (Vanderkooi & Callis, 1975). Assuming significant oxygen quenching, a value of 100 ns is assumed for the sake of argument. Using these estimates, the ratio of bimolecular rate constants is obtained and is listed in Table 2. With these lifetimes the ratio of bimolecular quenching rates for anthroyloxy and pyrenyl labels are determined for all quenchers and for both the free and the bound opsin cases. In both instances, the anthroyloxy label has a larger bimolecular rate constant than the pyrenyl label. The presence of the protein further enhances this effect. Interestingly, for a given sample, these ratios are essentially constant, independent of quencher position on the fatty acid. This is, again, suggestive of minimal interference from the protein structure.

The results of this work provide the first physical evidence of the membrane location of the palmitate groups modifying a membrane protein. One should bear in mind that these results were obtained on a reconstituted system and may not represent the behavior in the physiological setting, i.e., rod outer segments. It is known, however, that this rhodopsin reconstituted system is functionally active (Mitchell et al., 1991). Consequently, it is anticipated that the structure of rhodopsin is not dramatically altered by reconstitution. This work marks the first step in the biophysical characterization of the effects of palmitoylation on the structure of a membrane protein.

REFERENCES

Blatt, E., & Sawyer, W. H. (1985) *Biochim. Biophys. Acta 822*, 43–62.

Blatt, E., Chatelier, R. C., & Sawyer, W. H. (1986) *Biophys. J. 50*, 349–356.

Dale, R. E., Eisinger, J., & Blumberg, W. E. (1979) *Biophys. J. 26*, 161–194.

Encinas, M. V., & Lissi, E. A. (1982) *Chem. Phys. Lett. 91*, 55–57.

Franke, R. R., Konig, B., Sakmar, T. P., Khorana, H. G., & Hoffman, K. P. (1990) *Science 250*, 123–125.

Hammes, G. G. (1981) *Protein-Protein Interactions*, pp 257–287, Wiley, New York.

Hargrave, P. A., McDowell, J. H., Curtis, D. R., Juszczak, E., Fong, S. L., Mohana Rao, J. K., & Argos, P. (1983) *Biophys. Struct. Mech. 9*, 235–244.

Hasselbacher, C. A., & Dewey, T. G. (1986) *Biochemistry 25*, 6236–6243.

Hresko, R. C., Sugar, I. P., Barenholz, Y., & Thompson, T. E. (1986) *Biochemistry 25*, 3813–3823.

Jackson, M. L., & Litman, B. J. (1985) *Biochim. Biophys. Acta 812*, 369–376.

Karnik, S. S., Ridge, K. D., Bhattacharya, S., & Khorana, H. G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 40–44.

Konig, B., Welte, W., & Hofmann, P. (1989) *FEBS Lett. 257*, 163–167.

Lapidot, Y., Rappoport, S., & Wolman, Y. (1967) *J. Lipid Res. 8*, 142–145.

Mitchell, D. C., Kibelbek, J., & Litman, B. J. (1991) *Biochemistry 30*, 37–42.

Moench, S. J., Terry, C. E., & Dewey, T. G. (1994) *Biochemistry* (preceding paper in this issue).

Morrison, W. R. (1964) *Anal. Biochem. 7*, 218–224.

Nagle, J. F. (1993) *Biophys. J. 64*, 1476–1481.

Nathans, J., & Hogness, D. S. (1983) *Cell 34*, 807–814.

O'Brien, P. J., St. Jules, R. S., Reddy, T. S., Bazan, N. G., & Zatz, M. (1987) *J. Biol. Chem. 262*, 5210–5215.

Ovchinnikov, Y. A., Abdulaev, N. G., & Bogachuk, A. S. (1988) *FEBS Lett. 230*, 1–5.

Palczewski, K., Arendt, A., McDowell, J. H., & Hargrave, P. A. (1989) *Biochemistry 28*, 8764–8770.

Papac, D. L., Thornburg, K. R., Bullesbach, E. E., Crouch, R. K., & Knapp, D. R. (1992) *J. Biol. Chem. 267*, 16889–16894.

Shichi, H. (1970) *Biochemistry 9*, 1973–1977.

Stubbs, G. W., Smith, H. G., Jr., & Litman, B. (1976) *Biochim. Biophys. Acta 425*, 46–56.

Traxler, K. W., & Dewey, T. G. (1994) *Biochemistry 33*, 1718–1723.

Vanderkooi, J. M., & Callis, J. B. (1974) *Biochemistry 13*, 4000–4006.

Vermeir, M., Boens, N., & Heirwegh, K. P. M. (1992) *Biochim. Biophys. Acta 1107*, 93–104.

Wilden, U., Hall, S. W., & Kuhn, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1174–1178.