Depalmitylation with Hydroxylamine Alters the Functional Properties of Rhodopsin*

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Rhodopsin, the photosensitive protein found in rod photoreceptors, has two covalently attached palmitates that are thought to anchor a portion of the C terminus to the disc membrane, forming a fourth cytoplasmic loop. Using hydroxylamine (NH₂OH) to cleave the thioester linkage, we have characterized the effect of depalmitylation on certain functional properties of rhodopsin.

Treatment of rod outer segment membranes (prepared from rat retinas previously labeled in vivo with [³H]palmitate) with 1 M NH₂OH typically removed 75% of the [³H]palmitate initially bound to rhodopsin. Spectrophotometry of rod outer segment membranes that had been treated with 1 M NH₂OH indicated preservation of 85% of the native rhodopsin and no effect on the shape of the absorbance spectrum of rhodopsin. In vivo labeled rhodopsin that had been treated with 1 M NH₂OH did not reincorporate free endogenous [³H]palmitate over a 2-h incubation period. Both NH₂OH-treated and untreated rhodopsin incorporated [¹⁴C]palmitate from exogenously added [¹⁴C]palmitoyl-CoA. This incorporation was substantially greater in the NH₂OH-treated sample.

The palmitate by NH₂OH inhibited rhodopsin regeneration by 44% and increased the ability of rhodopsin to activate transducin’s light-dependent GTPase activity by 61%. However, the removal of palmitate from rhodopsin did not affect the light-dependent binding of transducin (Tα and Tβγ).

Rhodopsin, the membrane-spanning visual pigment of rod photoreceptors, has two palmitates covalently attached by thioester bonds to Cys³² and Cys³²⁻ near the C terminus of rhodopsin (1-5). The cysteines are palmitylated in both the rod inner segment rough endoplasmic reticulum, and in the rod outer segment (ROS)¹ plasma and disk membranes (1-3, 6). Palmitylation in the latter case occurs by a nonenzymatic exchange reaction, utilizing palmitoyl-CoA (2).

The cytoplasmic domain of rhodopsin is critical for the binding and activation of transducin (7-16). The membrane-spanning regions of rhodopsin form three cytoplasmic loops, two of which are critical for the activation of transducin (9-11, 17). In addition, the two covalently bound palmitates anchor a portion of the C terminus of rhodopsin, forming a fourth cytoplasmic loop (4, 5, 7). König et al. (7) have obtained direct evidence for a role of this fourth loop in the interaction of rhodopsin with transducin.

Investigations into the role of palmitylation in the function of the influenza virus spike glycoprotein (18) and the β-adrenergic receptor (19), suggest that covalently bound palmitates may be important for at least some of the functions of these proteins. Covalently bound palmitates may also play a critical role in the various functions of rhodopsin such as regeneration, and transducin binding and activation. To study the role of the palmitates in these functions, we used hydroxylamine (NH₂OH) to remove palmitate from the C terminus of rhodopsin. NH₂OH, a chemical known to cleave palmitate-protein linkages in other proteins (18, 20-22), has been used previously to study the role of palmitate in the function of the influenza virus spike glycoprotein (18). This approach complements experiments using site-directed mutagenesis as a way to modify the two cysteines required for palmitylation (4). By using NH₂OH, rhodopsin can be kept in the native environment of the ROS membrane, and conformational changes associated with amino acid exchanges in site-directed mutagenesis experiments can be eliminated.

EXPERIMENTAL PROCEDURES

Materials—Solvable⁴ tissue solubilizer, [9,10-³H]palmitate (60 Ci/mmol), and [γ-³²P]GTP (10 Ci/mmol) were obtained from Du Pont-New England Nuclear. [¹⁴C]Palmitoyl-CoA (54 mCi/mmol) was obtained from Amersham Corp. Enzyme immunosassay plates were obtained from Costar. Goat anti-rabbit IgG was obtained from Amersham Corp. Peroxide-anti-peroxidase was obtained from Accurate Chemical. α-Polyphenylamine (OPD) was obtained from Abbott. The biocinchoninic acid (BCA) protein assay reagents were obtained from Pierce Chemical Co. Concanaavan A (ConA)-Sepharose was obtained from Pharmacia LKB Biotechnology Inc. All other reagents were obtained from Sigma or Bio-Rad.

In Vivo Labeling—Procedures for the in vivo labeling of rat rhodopsin have been previously described (3). Sprague-Dawley rats (SASCO, 4-5 weeks old) were maintained in a 12-h light/12-h dark cycle. Twenty µCi of [³H]palmitate in 2 µl of ethanol was injected intravitreally into each eye during the light phase, 36 h prior to an experiment. Previous experiments have shown that maximal in vivo labeling of rat rhodopsin by [³H]palmitate occurs in 36-48 h (3).

Rod Outer Segment Membrane Preparation—ROS membranes were obtained from either [³H]palmitate-labeled or unlabeled retinas. All steps were performed under dim red light. Rats were sacrificed 1 h before the onset of the light cycle. The eyes were hemisected and
retinas placed in Buffer A (10 mM Tris, 2 mM MgCl₂, 200 mM NaCl, 10 μM pepstatin A, 10 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.9) containing 40% sucrose (4–6 retinas/3.5 ml). Retinas were vortexed six times for 10 s. ROS membranes were isolated by centrifugation (sucrose flotation) at 15,000 rpm for 15 min in a Beckman 50.5Ti rotor. The supernatants containing the ROS membranes were diluted with an equal volume of Buffer A, and centrifuged for 10 min at 30,000 rpm. Pellets were resuspended in Buffer A.

Urea-washed ROS membranes (25) were prepared by suspending dark-adapted ROS membranes in a buffer containing 10 mM Tris, 5 mM urea, and 1 mM EDTA (pH 7.9), and passed through a 20-gauge needle three times. The ROS membranes were incubated on ice for 60 min and then washed three times with a hypotonic buffer containing 10 mM Tris, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A (pH 7.9).

Incubation with NH₄OH—The standard incubations with NH₄OH followed the procedure previously described by O’Brien et al. (2). ROS membranes were suspended in Buffer A containing various concentrations of NH₄OH (pH 7.9) and incubated in the dark for 30 min at 37 °C. In some experiments zero-time incubations (control samples) were performed by suspending the ROS membranes in 1 M NH₄OH and immediately centrifuging the sample. Membranes were then washed three times and resuspended in Buffer A.

Spectrophotometric Determination of Rhodopsin—Aliquots of the isolated ROS membranes (25–50 μl) were added to spectrophotometry buffer (0.5 mM Tris, 0.5 mM NH₄OH, 1% (v/v) polyoxyethylene triethylene ether (PTE), pH 7.1) to a final volume of 400 μl. The solubilized rhodopsin was filtered on Whatman GF/A filters. Filters were washed with a 2.5 mM phosphate buffer. Percent regeneration was determined by spectrophotometry (24).

ConA Chromatography—Chromatographic procedures were performed under dim red light as previously described (2). All buffers were equilibrated to room temperature before use. A ConA-Sepharose column (0.5-ml bed volume) was washed with 20 ml of column buffer (50 mM Tris, 1 mM MgCl₂, 1 mM CaCl₂, pH 6.9) containing 0.1% (v/v) PTE. Samples were solubilized by adding 1.78 ml of column buffer and 20 μl of PTE to 200 μl of ROS membrane suspension (0.5–2 nmol of rhodopsin) on ice for 1 h. The samples were then centrifuged at 15,000 rpm in a Beckman JA-20 rotor for 15 min to remove particulate matter. The solubilized rhodopsin samples were loaded onto ConA-Sepharose columns in 200-μl aliquots. The eluates were collected and reapplied to the columns in 200-μl aliquots. The columns were then washed with 35 ml of column buffer containing 0.1% (v/v) PTE. Rhodopsin was eluted by adding column buffer containing 0.1% (v/v) PTE and 0.5 M α-methylmannoside. Fractions (0.5 ml) were collected and 0.25-ml aliquots analyzed for rhodopsin-bound [3H]palmitate by scintillation counting.

RESULTS

Concentration Dependence of Depalmitylation by NH₄OH—Fig. 1A shows the dependence of rhodopsin depalmitylation on NH₄OH concentration as determined by ConA chromatography. A single dark-adapted ROS membrane preparation was divided into equal aliquots and incubated for 30 min in the dark with Buffer A containing various concentrations of NH₄OH. NH₄OH concentrations less than or equal to 100 mM preserved a level of rhodopsin-bound [3H]palmitate similar to that of the control incubated in the absence of NH₄OH. NH₄OH concentrations above 100 mM significantly reduced the level of rhodopsin-bound [3H]palmitate. At 1 M NH₄OH, rhodopsin was about 75% depalmitylated. In the experiment shown in Fig. 1B, aliquots of the dark-adapted ROS membranes were incubated with the indicated NH₄OH concentrations. The ROS membranes were solubilized in SDS and analyzed by SDS-PAGE followed by autoradiography. The dependence of rhodopsin-bound [3H]palmitate on NH₄OH concentration was similar to that shown in Fig. 1A. SDS-PAGE also showed no degradation products or decrease in Mᵣ of rhodopsin after treatment with 1 M NH₄OH (data not shown).
possibility existed that under conditions required for optimal untreated) were used to quantitate rhodopsin spectrophoto-

depalmitylation (1 M NH₂OH, 37 °C, 30 min), rhodopsin could lose significant amounts of chromophore. The following experiments were performed to determine whether treatment with NH₂OH affects the incorporation of palmitate into rhodopsin in the native environment of a depleted ROS membrane preparation. Rat retinas were labeled in vivo with [³H]palmitate and the ROS membranes isolated from dark-adapted retinas. The ROS membranes were then washed with hypotonic buffer in the dark to remove peripheral proteins and soluble cofactors which may participate in the formation of palmitoyl-CoA (i.e. coenzyme A and ATP). A small aliquot was analyzed for bound [³H]palmitate by ConA chromatography to determine the extent of labeling of the rhodopsin. The sample was then treated with Buffer A containing 1 M NH₂OH (Fig. 2A) or Buffer A only (Fig. 2B) at 37 °C for 30 min, then washed several times to remove excess NH₂OH.

The treatment of rhodopsin with NH₂OH decreased the amount of bound palmitate from 2571 cpm to 167 cpm (94% depalmitylation) (Fig. 2A, pretreated and 0 min, open bars). [³H]Palmitoyl-CoA was then added to NH₂OH-treated and untreated ROS, and aliquots were analyzed for [³H]palmitate (Fig. 2, open bars) and [¹⁴C]palmitate (Fig. 2, filled bars) bound to rhodopsin at 0-min and 120-min incubation time. Over a 120-min incubation period, the amount of bound [³H]palmitate in the NH₂OH-treated ROS membranes decreased from 167 cpm to 95 cpm. Rhodopsin did, however, incorporate significant amounts of [¹⁴C]palmitate from added [¹⁴C]palmitoyl-CoA. Bound palmitate increased by 401 cpm over a 120-min incubation period (Fig. 2B, filled bars). Treatment of the ROS membranes with NH₂OH enhanced the incorporation of bound [¹⁴C]palmitate by 400 cpm (801 cpm incorporated during the 120-min incubation period; Fig. 2A, filled bars).

Regeneration of Rhodopsin—Rhodopsin consists of a chromophore, 11-cis-retinal, covalently attached to a protein, opsin. The chromophore is located in the hydrophobic pocket of opsin, which is buried in the disc membrane (30). Photoisomerization of 11-cis-retinal leads to a series of changes in the conformation of the chromophore and in the protein portion of rhodopsin. These changes eventually lead to the release of all-trans-retinal from opsin (bleaching). The process of rhodopsin regeneration involves the reattachment of 11-cis-retinal to opsin (30).

The following experiment was performed to determine whether NH₂OH-depalmitylation of rhodopsin affects the
regeneration of rhodopsin. Unlabeled rat ROS membranes were treated with Buffer A containing 1 mM NH₂OH for 30 min (treated samples) or 0 min (control samples) as described under "Experimental Procedures." Unlabeled rat ROS membranes were treated with Buffer A containing 1 mM NH₂OH for 30 min (treated samples) or 0 min (control samples) as described under "Experimental Procedures." Other peripheral proteins from the native membranes. After urea treatment, the ROS membranes were incubated with Buffer A containing 1 mM NH₂OH for 30 min (treated samples) or 0 min (control samples), as described under "Experimental Procedures."

In the transducin binding assay, samples (treated or control) containing 500 pmol of rhodopsin were reconstituted with 500 pmol of purified bovine transducin in a hypotonic buffer to decrease nonspecific binding of transducin to the ROS membranes. The samples were then incubated for 30 min in the dark, illuminated for 5 min, and incubated for 2 min more in the dark at 37 °C. Similar samples (treated and control) were incubated in darkness for the entire 7-min period. The membranes were then centrifuged and the supernatants removed. After diluting the supernatants, Ta and TPr remaining in the supernatants were quantitated by ELISA. The amount of Ta or TPr remaining in the supernatants was subtracted from the total amount of Ta or TPr added at the beginning of the assay to determine the amount of Ta or TPr bound to the ROS membrane. Light-dependent binding was determined by subtracting the amount of Ta or TPr bound to the dark-incubated samples from the amount of Ta or TPr bound to illuminated samples.

Treated membranes (87% depalmitylated) showed a light-dependent increase in the amount of Ta bound (from 244 pmol in the dark to 309 pmol upon illumination), and TPrγ bound (from 194 pmol in the dark to 299 pmol upon illumination) (Table II). Similarly, the control samples showed an increase in Ta bound (from 269 pmol in the dark to 315 pmol upon illumination) as well as TPrγ (from 192 pmol in the dark to 306 pmol upon illumination) (Table II). There was no significant difference in the light-dependent increase in the binding of Ta in treated and control samples (64 ± 24 pmol and 77 ± 27 pmol, respectively) or in the light-dependent binding of TPrγ (105 ± 29 pmol and 113 ± 30 pmol, respectively).

The effect of rhodopsin depalmitylation on transducin activation was also examined. Urea-washed ROS membranes were treated with NH₂OH as described above. Samples were analyzed for rhodopsin concentration by spectrophotometry before and after a 20-min bleach using 520 nm and infrared cut-off filters. Regeneration was initiated by the addition of 11-cis-retinal (moles 11-cis-retinal/moles opsin = 5/1). Aliquots were removed after defined periods of incubation, and regeneration was quenched by addition of the sample to spectrophotometry buffer. Percent regeneration was determined by difference spectrophotometry. Results were obtained from duplicate samples. Filled circles = treated ROS; open circles = control ROS.
TABLE II

Effect of NH$_2$OH treatment on transducin binding in rhodopsin

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control (pmol)</th>
<th>Treated (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bound $\alpha$ (pmol)</td>
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<td></td>
</tr>
<tr>
<td>Illuminated</td>
<td>315 ± 27</td>
<td>309 ± 19</td>
</tr>
<tr>
<td>Unilluminated</td>
<td>209 ± 26</td>
<td>244 ± 14</td>
</tr>
<tr>
<td>Light-dependent increase in binding</td>
<td>77 ± 27</td>
<td>64 ± 24</td>
</tr>
<tr>
<td>Bound $\beta y$ (pmol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Illuminated</td>
<td>306 ± 9</td>
<td>299 ± 18</td>
</tr>
<tr>
<td>Unilluminated</td>
<td>192 ± 29</td>
<td>194 ± 23</td>
</tr>
<tr>
<td>Light-dependent increase in binding</td>
<td>113 ± 30</td>
<td>105 ± 29</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of NH$_2$OH-depalmitylation of rhodopsin on transducin activation. Urea-washed ROS membranes were treated with 1 M NH$_2$OH for 30 min (treated samples) or 0 min (control samples) and washed. An aliquot was removed from each sample for the quantitation of palmitate bound to transducin. Transducin (63 pmol/assay tube) was preincubated with the treated or control ROS membranes (63 pmol of rhodopsin) for 5 min. ROS membranes were illuminated (circles) and GTP substrate added (20 $\mu$M GTP + 0.5 $\mu$Ci [y-32P]GTP/100-$\mu$l assay). Released phosphate was determined at the desired times as described under "Experimental Procedures." Similar samples (triangles) were incubated for equivalent time periods in the dark. Filled symbols = treated samples; open symbols = control samples.

DISCUSSION

Palmitylation is a widely occurring post-translational modification of membrane-bound proteins (20, 21). Recent studies of the $\beta$-adrenergic receptor have suggested a possible role of palmitate in the interaction of a receptor protein with its effector (19). Using site-directed mutagenesis, the putative site of palmitylation for the $\beta$-adrenergic receptor, Cys$^{231}$, was exchanged for a glycine. This mutation resulted in a receptor which had a reduced capability to couple with the adenylate cyclase-stimulating G-protein, Gs. Covalently bound palmates may play a similar role in the functioning of rhodopsin.

The present study has employed NH$_2$OH, a substance previously used to characterize other palmitylated proteins, to study the role of palmitate in rhodopsin function (5, 18, 20–22). By analyzing the amount of bound [H]palmitate using ConA chromatography before and after treatment with NH$_2$OH (Fig. 1A), we have shown that NH$_2$OH effectively depalmitylates rhodopsin. The depalmitylation of rhodopsin was dependent on the concentration of NH$_2$OH. The treatment of rhodopsin with 1 M NH$_2$OH (37°C, 30 min) typically removed ≥75% of the palmitate from rhodopsin. Similar results were obtained when bound palmitate was quantitated in rhodopsin bands excised from SDS-PAGE gels (Fig. 1B).

By three criteria, we have found that rhodopsin, depalmitylated by 1 M NH$_2$OH (37°C, 30 min), still resembles native rhodopsin. First, only 15% of the chromophore initially bound to rhodopsin was lost upon treatment with 1 M NH$_2$OH (Table I). Also, there was no effect on the $\lambda_{max}$ (498 nm), nor was there any effect on the intersection point (400 nm) of the spectra of bleached and unbleached rhodopsin (data not shown). Second, SDS-PAGE analysis of ConA-purified rhodopsin and silver staining of the resulting gel did not reveal any degradation products or decreases in the apparent molecular weight of rhodopsin as a result of NH$_2$OH treatment (data not shown). Third, over a 2-h incubation period, palmitate derived from palmitoyl-CoA was incorporated into both treated and untreated ROS membrane preparations (Fig. 2, A and B). Therefore, the treatment of rhodopsin with 1 M NH$_2$OH did not eliminate its ability to incorporate palmitate from palmitoyl-CoA.

A key aim of the present study was to determine whether the removal of palmitate influenced the physiological functions of rhodopsin. Specifically, the present experiments have focused on the effect of depalmitylation on the regeneration of rhodopsin and on the ability of rhodopsin to bind and activate transducin. We have shown that the depalmitylation of rhodopsin inhibited the reincorporation of 11-cis-retinal into opsins. The extent of rhodopsin regeneration was inhibited by 44% after a 20-min regeneration period (Fig. 3). Depalmitylation of rhodopsin also enhanced the light-dependent activation of transducin by 61% (Fig. 4) but had no effect on the light-dependent binding of transducin (Table II).

The results may be viewed in relation to data obtained when rhodopsin was treated with thermolysin, which removed either part or all of the C terminus (14, 31). In these experiments, the phosphodiesterase activity associated with thermolysin-treated rhodopsin was greater than that found in untreated rhodopsin. Furthermore, the removal of 12 amino acids from the C terminus of rhodopsin had no effect on the light-dependent binding of transducin in the absence of GTP (10). Therefore, it appears that proteolytically removing the C terminus (by the removal of the palmitate anchor), may affect a region of rhodopsin important for the regulation of the light-dependent GTPase activity of transducin, but not important for the regulation of the light-
dependent binding of transducin.

Recent data obtained by Boesze-Battaglia et al. (32) also raise the possibility that the mobility of the C terminus could be important in the regulation of the light-dependent GTPase activity of transducin. In the low cholesterol ROS disc membrane, the activity of phosphodiesterase was found to be greater than that found in the high cholesterol ROS plasma membrane. In low cholesterol membranes, where membrane fluidity is greater, the mobility of the palmitates in the lipid bilayer may have determined whether the C terminus itself was more mobile. The removal of the C-terminal palmitates of rhodopsin could mimic the effect of a more fluid membrane by increasing the mobility of the C terminus. This would increase the ability of rhodopsin to stimulate the GTPase activity of transducin, and, thus, to increase PDE activity.

The importance of rhodopsin structure on the regulation of transducin activation has been examined by Karnik et al. (4) using site-directed mutagenesis. Karnik et al. (4) found that a rhodopsin mutant, which had Cys323, Cys323, and Cys316 replaced with serines, showed no difference in its ability to activate transducin when compared with the activation by the wild-type receptor. However, several differences in the conditions of the experiments performed by Karnik et al. (4) and our experiments must be noted. First, the rhodopsin mutant and wild-type rhodopsin were solubilized in detergent. The solubilization of membranes would not maintain a planar lipid bilayer, which may be important for the physiological function of the palmitate anchor. For example, solubilization of rhodopsin may have increased the mobility of the C terminus of rhodopsin and thus, may have interfered with the regulatory function of the palmitate anchor. This could increase the GTPase activity of transducin, masking the stimulatory effect that the depalmitylation of rhodopsin had on GTPase activity. Second, the mutant rhodopsin, which had serines replacing the cysteines at residues 322 and 323, also had a serine replacing Cys316. The effect of a serine substitution at Cys316 on the ability of rhodopsin to regulate the GTPase activity of transducin is currently unknown. It is possible that this substitution could have masked the effect that substitutions at Cys322 and Cys323 had on the GTPase activity of transducin.

A previous study which has utilized NH4OH as a depalmitylating reagent, has examined the role of covalently attached palmitates in the proper functioning of the influenza virus spike glycoprotein (18). The results of this study indicated that while the removal of palmitate, using NH4OH, affected one function of the protein (hemolytic activity), another function was not affected (hemagglutinin activity). Similarly, it appears that depalmitylation of rhodopsin, using NH4OH, affects certain functions of rhodopsin (regeneration and transducin activation) and leaves other functions unaffected (transducin binding).

Finally it is interesting to note that rhodopsin differs from the visual pigments of cone receptors, with respect to the cysteine sites of palmitylation. That is, at the positions homologous to amino acid residues 322 and 323, the pigments of red- and green-sensitive cones lack cysteine, and that of the blue-sensitive cones contains only one cysteine (33). It is conceivable that the absence or reduced extent of palmitylation in the cone visual pigments may be correlated with distinctive properties of the cone pigments in the processes of bleaching and regeneration (34, 35), or transducin activation.