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Quantitation of the Effect of Hydroxylamine on Rhodopsin Palmitylation†

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Abstract

Rhodopsin (the photosensitive rod visual pigment) has been a model for photobiologic studies of the opsins as well as a structural model for G-protein-coupled receptors. The two palmitate groups attached to cysteines 322 and 323 are thought to serve as membrane anchors for the rhodopsin C-terminus, but the absence of the palmitates does not alter membrane localization. However, removal of the palmitates affects rhodopsin function. Therefore, it is important to quantitate the stability of rhodopsin palmitates to hydroxylamine, which is a widely utilized reagent in biochemical preparations of the apoprotein. We have developed a mass spectrometric method to quantitate the resulting opsin palmitylation. Our data show that both of the bovine rhodopsin palmitates are labile to hydroxylamine, with significant depalmitylation occurring at concentrations of ≥ 100 mM, with an EC_{50} of 220 mM L^{-1} . The palmitate at position 322 is the more stable to hydroxylamine. Samples prepared in the presence of >50 mM should therefore be considered to be at least partially depalmitylated and the results interpreted accordingly.

INTRODUCTION

G-protein-coupled receptors (GPCR) are membrane-associated proteins containing seven transmembrane spanning regions and are found in every species from bacteria, yeast, algae and plants to humans (for a recent review, see Kobilka [1]). The rod visual pigment rhodopsin is the first GPCR for which the crystallographic structure was obtained (2). Due to its abundance and resolved structure, rhodopsin has been widely studied and termed the prototypical GPCR.

Visual pigments are the light-sensing GPCRs of the photoreceptor cells of the retina. The phototransduction cascade, which eventually leads to vision (for a recent review, see Ridge and Palczewski [3]), utilizes conformational changes on the cytoplasmic surface of the pigment induced by the absorption of a photon. Rhodopsin, the rod cell pigment, was first shown to be modified by palmitylation by O'Brien and Zatz (4) and the sites later confirmed to be at positions 322 and 323 on two adjacent cysteine residues (5,6) (Fig. 1). These residues are proposed to anchor the C-terminus to the membrane and reduce the flexibility of the hydrophilic C-terminal tail of the protein.

Until recently, rhodopsin palmitylation has been thought to be static and of little physiologic significance, although fatty acid acylations/deacylations have been shown to be dynamic processes in several other GPCRs and to have a role in signaling processes (7). However, the report of Sachs *et al.* (8) suggested a role for the palmitates in creating a secondary retinoid

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biding site on rhodopsin. A mouse model lacking rhodopsin palmitate groups (the *Palm*^{-/-} mouse) was created by replacing the rhodopsin cysteines 322 with threonine and 323 with serine to determine the effects of the loss in palmitylation (9). The C-terminus of rhodopsin in these animals was found to be heavily phosphorylated on bleaching, which produced an enhanced shutoff of phototransduction (9). Only minor morphologic effects were observed, with a slight increase in the rate of decreased thickness of the rod outer nuclei layer with age being noted.

Five classes of opsins (10) have been identified. Interestingly, in addition to their functional differences, these opsin classes also differ in the state of C-terminal palmitylation (11). The best studied opsin class other than that of rhodopsin (Rh1) is the red cone opsin (M/LWS). The M/LWS opsins contain no cysteines in positions homologous to C322 or 323 of rhodopsin. As expected, no palmitylation was observed on this opsin (11). The blue opsins (SWS2), which are not present in higher mammals, do contain a cysteine at a position homologous to 323 of rhodopsin, but only partial palmitylation has been found on this site in the salamander SWS2 opsins (11). The SWS1 cone pigments have a cysteine at a position homologous to rhodopsin 322, and the mouse and salamander pigments were found to be unpalmitylated (11).

This present study utilized mass spectrometry (MS) for the analysis of opsin palmitylation. In this method, proteins were digested to obtain peptide fragments, the fragments were separated by high-pressure liquid chromatography (HPLC), and the effluent introduced into the mass spectrometer which recorded both mass spectra and sequences (12,13). With this technique it was possible to revisit the previous observation that hydroxylamine removes palmitates from opsin (4,14,15), and for the first time to provide quantitative information on opsin palmitylation and to determine the more labile of the two palmitates. Biochemical studies reported in the literature have often been performed on opsins exposed to high concentrations of hydroxylamine, and our data suggest that in many cases these opsins may not have their full complement of palmitates, which can affect the results of these studies.

MATERIALS AND METHODS

Sample preparation

Rod outer segments (ROS) were prepared as previously described (16). The rhodopsin concentration was determined spectroscopically after the addition of 11-*cis* retinal to insure complete regeneration. A stock of 5 M hydroxylamine (Sigma Aldrich, St. Louis, MO) in Tris-phosphate buffer (70 mM, pH 7.4) was freshly prepared for each experiment and diluted to achieve the correct concentration. ROS were incubated with varying concentrations of hydroxylamine for 30 min at 22°C in the light.

Reduction of disulfide bonds

Disulfide bonds in samples of 4 μ L of ROS preparations (20 μ g total protein) with or without hydroxylamine treatment were reduced using Tris[2-carboxyethyl]phosphine hydrochloride (TCEP; Pierce Chemical, Rockford, IL). Samples (200 μ L) were incubated with 1 μ L TCEP (0.5 M, pH 7.4) for 1 h at room temperature in a shaker. Cysteines were then labeled with 4-vinyl pyridine (Sigma Aldrich) as described previously (13).

Cyanogen bromide (CNBr) digest

CNBr cleavage was conducted as previously described (13). The CNBr reaction was quenched by an excess (3X) of deionized, HPLC grade water (Thermo Fisher Scientific, Waltham, MA). The samples were then vacuum dried (Speed Vac SC110; Sevant), and reconstituted in 5 μ L trifluoroacetic acid (TFA), 42 μ L acetonitrile (Thermo Fisher), 84 μ L isopropyl alcohol (Thermo Fisher) and diluted to 5 mL using HPLC grade water.

Asp-N digest

In order to obtain shorter (12 amino acid) palmitylated peptides, the Asp-N (Sigma) enzyme was used to remove the last 19 amino acids from rhodopsin and that peptide was then cleaved with CNBr. A ROS preparation (4 μ L in 100 μ L water; 20 μ g rhodopsin) was centrifuged and the supernatant discarded. Tris buffer (180 μ L, 10 mM, pH 7.5) was added to the pellet, followed by acetonitrile (20 μ L) and AspN (1 μ L, 50 ng). The sample was incubated at 37°C overnight, centrifuged at 50 000 *g* for 5 min at 4°C, and the pellet digested by CNBr as described above.

HPLC/MS/MS

Peptide separation was performed using a binary HPLC pump system (Agilent Technologies, Santa Clara, CA) in normal flow mode with a Brownlee Aquapore C18 reverse phase column (OD-300, 100 mm $\times$ 2.1 mm; Bodman Industries, Aston, PA). The HPLC solvents were: Solvent A, 0.05% TFA in HPLC grade water, and Solvent B, 0.05% TFA in 2:1 isopropanol/acetonitrile. The column gradient was from 2% B to 98% B over 75 min. The flow rate was set to 200 μ L min⁻¹, but a 10:1 split was utilized to reduce effluent flow into the Ion Max source of an LTQ linear ion trap mass spectrometer (Thermo Fisher). MS and MS/MS data were collected with Xcalibur version 1.4SR1 software. For MS/MS analysis, dynamic exclusion was used. Selected ion chromatograms (SICs) were collected by scanning the entire chromatogram for a single mass. Unpalmitylated singly palmitylated and doubly palmitylated peptides were quantitated by measuring and comparing areas under peaks in the SIC for the corresponding rhodopsin peptides. The dependence of palmitylation on hydroxylamine concentration was analyzed using Prism (GraphPad Software Inc., San Diego, CA).

RESULTS

Quantitation of rhodopsin palmitylation

Greater than 80% of the protein content in the ROS preparation is rhodopsin (17), and no further biochemical purification is needed for MS analysis. To facilitate peptide sequencing, the protein is treated with CNBr which cleaves the peptide chain at the C-terminus of methionines, leaving a homoserine lactone or homoserine moiety. The 348 amino acid chain of bovine rhodopsin provides 17 CNBr fragments (Fig. 1a) and has been completely mapped as described previously (12).

The present study focused on the C-terminal, 17th CNBr fragment. This fragment contains 31 amino acids including cysteines 322 and 323, which are available for palmitate modifications (Fig. 1b). Three distinct molecular weight species of the rhodopsin C-terminal peptide with highly different solubility properties, and hence HPLC retention times, are possible—unpalmitylated, singly palmitylated and doubly palmitylated. The singly palmitylated species can be a mixture of palmitylated C322/unpalmitylated C323 (CpC) or unpalmitylated C322/palmitylated C323 (CCp), which are not resolved through HPLC. As the samples were alkylated with 4-vinyl pyridine to block reactive sulfhydryl groups from forming disulfide bonds, the unpalmitylated cysteines appeared as pyridylethyl-cysteines in these analyses.

The SICs of the distinctive molecular weight species of the C-terminus (+3 charge state) are shown in Fig. 2. The SIC chromatograms trace the abundances of a selected individual *m/z* ion throughout the entire time course of the experiment and contain two pieces of basic information—the elution time of the ion of interest (the SIC peak), and the absolute amount of the detected ion (the area under the SIC peak). The SIC for *m/z* 1112 at 32.01 min corresponds to the CC form of the opsin C-terminal CNBr fragment (unpalmitylated), *m/z* 1156.9 at 59.49 min corresponds to the CCp or CpC form (singly palmitylated) and *m/z* 1200.9 at 80.55 min corresponds to CpCp (doubly palmitylated). The peak identifications in Fig. 2 were also

confirmed with collision-induced disassociation (CID) sequence information (MS/MS) of the individual ions. There is no evidence in the present study, or in other studies in the literature, for palmitylation at another site.

The SICs of the rhodopsin C-terminal peptides were used to derive quantitative information on palmitylation. The areas under the peaks of the unpalmitylated, singly palmitylated and doubly-palmitylated peptides in Fig. 2 (designated as A_0 for the unpalmitylated, A_1 for the singly palmitylated and A_2 for the doubly palmitylated species) are proportional to the concentrations of these individual peptides in the sample. Thus, the sum of these areas ($\Sigma A = A_1 + A_2 + A_3$) is proportional to the total concentration of the rhodopsin C-terminal CNBr peptide (which is necessarily similar to that of the total opsin concentration). Therefore, within the sample, the relative abundance of unpalmitylated, singly palmitylated and doubly palmitylated species is $p_1 = A_1/\Sigma A$, $p_2 = A_2/\Sigma A$ and $p_3 = A_3/\Sigma A$, respectively. The palmitate/opsin ratio is calculated from the relative abundance by multiplying it with the number of palmitates on the individual species:

$$(0 \times p_0) + (1 \times p_1) + (2 \times p_2) = \frac{\sum_{i=1}^2 i \times A_i}{\Sigma A} \quad (1)$$

The level of palmitylation in ROS preparations untreated with hydroxylamine was found to be 1.97 ± 0.02 mol palmitates/mol of opsin, with 2.98% of rhodopsin being singly palmitylated and the rest doubly palmitylated. Unpalmitylated opsin was also detected in these samples, but could not be quantitated. In order to demonstrate our ability to quantitate opsin palmitylation from the collected data, instead of an untreated sample, Fig. 2 shows the SICs for an ROS preparation treated with 100 mM hydroxylamine.

Hydroxylamine removal of rhodopsin palmitates

The effect of increasing concentrations of hydroxylamine on the number of palmitate groups on bovine opsin is shown in Fig. 3. The data in Fig. 3 were fitted with an equation for sigmoidal dose-response:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC_{50} - X)^{\text{Hill slope}}}} \quad (2)$$

The EC_{50} value obtained from the fitting was 220 mM, and the Hill slope was 1.77.

From Fig. 3 it can be seen that the opsin remained mostly doubly palmitylated up to concentrations of 10 mM hydroxylamine. However, at concentrations ≥ 100 mM, significant depalmitylation occurred. The significant difference in the Hill slope from 1 indicates that the two palmitylation sites have different sensitivities toward hydroxylamine. The loss of the first palmitate enhances the lability of the second.

Cysteine 323 palmitate is more labile than 322

Theoretically, the CID sequences for the intact opsin C-terminal CNBr peptides (such as the ones in Fig. 2) could provide quantitative information on the ratio of CCp/CpC peptides within the singly palmitylated peak. However, the 31 amino acid peptide (Fig. 1b) is too long and hydrophobic for routine sequencing. Therefore, a dual digest approach was applied. First Asp-N was used, which cleaves at the N-terminus of aspartates to remove the last 19 amino acids of rhodopsin (still in its native membrane); this is then followed by a secondary CNBr cleavage of the remaining protein. This cleaved a shorter (12 amino acid) fragment that was more reliably

sequenced (Fig. 1c). The predicted molecular weight for the singly palmitylated 12 amino acid peptide is m/z 709 at 15 min retention for CC, m/z 775 at 45 min for CCp/CpC and m/z 841 at 75 min for CpCp (all in +2 charge states).

A representative singly palmitylated MS/MS full-spectrum and the predicted fragmentation patterns for both singly palmitylated peptides (CCp and CpC) are shown in Fig. 4. In order to evaluate the relative labiality of the two palmitates, the ROS sample for Fig. 4 was treated with 100 mM hydroxylamine prior to the mass spectrometric analysis. Underlining within the figure identifies the detected fragments, and red numbering indicates the y_7 and b_5 ions, which constitute the differences between the CCp and CpC palmylation patterns. The ratio of the two singly palmitylated forms was quantitated using the y_7 ions of the two species (m/z 793 for CpC and m/z 927 for CCp). This methodology allowed for an accurate determination of the relative ratio of the two singly palmitylated species.

The relative stability of the palmitates on the two cysteines was determined by the above described sequence analysis of six opsin samples treated with 100 mM hydroxylamine (Fig. 5). The abundance of CpC was more than twice that of CCp (2.2:1; $P \leq 0.02$), demonstrating that C322 is the more stable palmylation site in this native membrane preparation.

DISCUSSION

These data support the well-established reports that rhodopsin contains two palmitate groups at cysteines 322 and 323, and that no other cysteines are palmitylated. Other acyl groups have been reported to be associated with rhodopsin (18). However, we did not find evidence for the presence of acyl groups other than palmitates on cysteines 322 or 323, or at other sites, in the bovine rhodopsin ROS preparations using MS analysis. However, our data do not eliminate the possibility of acylation by groups other than palmitates at less than 1% relative levels.

The purpose of this study was to quantitate the loss of opsin palmitates upon treatment with hydroxylamine under conditions commonly used for biochemical preparations of rod opsin and to establish whether there are significant levels of unpalmitylated or singly palmitylated rhodopsin in the native ROS preparations. We now have established a methodology that accurately quantitates the levels of unpalmitylated, singly palmitylated and doubly palmitylated opsins. The majority of the rod opsin is doubly palmitylated, but ~3% is singly palmitylated. This result is quite consistent between samples that have not been treated with hydroxylamine or other ester-hydrolyzing agents. In many GPCRs, the acyl modification is a dynamic process and sometimes is involved in signaling processes (7). To date there is no evidence supporting that rhodopsin palmylation is a dynamic process, but this consistent, low level of unpalmitylated material is intriguing.

Our second aim was to determine which rhodopsin palmylation site is the more labile of the two sites. Our data on the singly palmitylated rhodopsin show that the palmitate at 323 is the more labile palmitate. These experiments were conducted on rhodopsin within its native membrane. Both singly palmitylated forms were obtained, indicating that both are relatively stable. Karnik *et al.* studied recombinant rhodopsin mutants in detergent (19), and reported that in the C322S mutant in which position 322 cannot be palmitylated, C323 is also not palmitylated. However, in a C323S rhodopsin mutant, the cysteine at 322 is partially palmitylated. These findings are reasonable in light of our results, namely that the 322 site is the more stable site for palmylation on rhodopsin.

The finding that in rod opsin the C323 palmylation site is the more labile of the two rhodopsin cysteines is in contrast to the data on cone opsin palmylation. In the salamander blue cone opsin (SWS2 family) a single cysteine aligning to site 323 has been shown to be partially palmitylated (11). However, the UV opsins (SWS1 family) with a single cysteine aligning to

the 322 rhodopsin site (which is the more stable site in rhodopsin) contain no palmitoylation (11). These conflicting data on the rod and cone palmitoylation sites are another indication that there are subtle structural and/or conformational differences between these various opsins.

Evidence from several laboratories demonstrates that the loss of palmitates affects several functional aspects of the rod opsin. For example, in samples that were treated with 1 M hydroxylamine, rhodopsin regeneration was inhibited by 44%. In addition, the transducin activation was reported to be enhanced by 61% (14). Traxler and Dewey found only marginal effects on rhodopsin regeneration, but reported a significant change in the rate of thermal denaturation in the absence of palmitates (20). This result was interpreted as evidence for the stabilizing effect of the loop resulting from the addition of palmitates. Sachs *et al.* (8) observed differences in the binding of all-*trans* retinal and suggested that palmitates might have a role in a secondary chromophore-binding site. Results from the study of Heck *et al.* (21) on the difference in transducin activation by native or depalmitoylated opsins with the all-*trans* retinal agonist is quite striking. Finally, light-activated rhodopsin extracted from *Palm*^{-/-} mice showed greatly enhanced phosphorylation (9) compared with wild type animals, rather resembling the levels found in light-activated cone pigments (22). This again points to the importance of the palmitate groups in the stability and function of rhodopsin.

Biochemical experiments conducted on pigments often utilize opsin that is regenerated with 11-*cis* retinal or other retinal-like compounds. In many of these experiments, the opsin apoprotein itself was generated in the presence of high levels of hydroxylamine. This is the first study that quantifies the levels of opsin palmitoylation in samples treated with hydroxylamine. These samples were from ROS preparations in native membranes undisturbed by detergents. The conditions used (30 min at room temperature) are comparable to that used in many studies, and milder than is often used. Our conclusion is that if concentrations of hydroxylamine are being employed that exceed 50 mM, the samples are likely to contain unpalmitoylated material, which will affect G-protein activation, phosphorylation levels, pH rate profiles for opsin/all-*trans* retinal catalyzed transducin activations, as well as rates of regeneration on samples prepared in the native membranes. If possible, for studies on rhodopsin, conditions that hydrolyze acyl linkages should be avoided and the full palmitoylated state of the protein should be confirmed.

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Figure 1.

(a) Primary structure of bovine opsin and its peptide fragments. The rhodopsin sequence contains 348 amino acids. Numbers correspond to each of the 17 cyanogen bromide (CNBr) cleavage fragments. The sites of native palmitoylation (C322 and C323), chromophore linkage (K296), disulfide bond (between C110 and C187) and glycosylation (N2 and N15) are also indicated. The N-terminus of the protein is acetylated. (b) The 31 amino acid, 17th CNBr fragment (as shown) of rhodopsin, is palmitylated at sites 322 and 323. (c) The two different singly palmitylated 12 amino acid AspN fragments of CNBr fragment 17. These peptides have the same molecular weight, but as the sequences are different, the sequence was used for determination of the stability of the individual palmitoylation sites to hydroxylamine. C = cysteines with no palmitates; C_P = palmityl-cysteines.

Figure 2.

Ion chromatograms of bovine opsin digested with cyanogen bromide (CNBr). The rod outer segment sample was treated with 100 mM hydroxylamine before analysis by HPLC/MS. (a) Base peak chromatogram for the sample, showing the most abundant ion eluting from the HPLC column. This trace is derived from the database of individual mass spectrums taken periodically throughout the gradient (several times per second). The peaks are labeled with the retention time (top) and m/z value (bottom) of the eluting ion. This pattern is characteristic of opsin with peaks 1112, 688, 588, 1100, 1415 and 1201 representing the most abundant opsin peptides; peaks 599 and 428 are lipid contaminants. The remainder of the opsin peptides was also detected, but they do not form peaks above the base peak background. (b–d) Selected ion chromatograms derived from the same database. Here, the traces represent the three different mass peaks for the 31 amino acid long 17th CNBr fragment of opsin in the individual mass spectrums taken during the entire gradient. (b) The unpalmitylated CC fragment (m/z 1112.4, retention time 32 min); (c) the singly palmitylated fragment (either in the C_pC or CC_p form) (m/z 1156.9, retention time 60 min); and (d) the doubly palmitylated C_pC_p fragment (m/z 1200.9, retention time 81 min)

Figure 3.

Hydroxylamine-induced loss of palmitates from rhodopsin. Treatment of rod outer segment samples with hydroxylamine results in a dose-dependent reduction in palmitates per opsin. The x -axis is a logarithmic scale of hydroxylamine concentration; the y -axis represents the molar ratio of palmitates/opsin. The data (circles) were collected from calculating the palmitate/opsin ratio (Eq. 1) for selected ion chromatograms of samples treated with various concentrations of hydroxylamine. The collected data were fitted with a sigmoidal function for dose-dependent association (Eq. 2). From the fitting parameters, the EC_{50} is 220 mM L^{-1} , the Hill slope is 1.77. The incubation with hydroxylamine was for 30 min at 22°C .

Figure 4.

Mass spectra of the singly palmitylated opsin peptides. In order to obtain these peptides, a dual Asp-N and cyanogen bromide cleavage strategy was employed. Prior to the cleavages, the rod outer segment sample was treated with 100 mM hydroxylamine. The figure shows the spectrum after the mass spectral ion for single palmitylation (m/z 775 at 45 min for coeluting both C_pC and CC_p forms of the peptide) was fragmented through collision-induced dissociation. Therefore, the pattern is a mixture of the C_pC and CC_p sequences, which are indistinguishable through their intact molecular weight and elution time. Blue spectral lines correspond to b ions; green lines correspond to the y ions of the fragmentation pattern. The y_7 ion, which differentiates the two forms of the peptide (C_pC and CC_p), is highlighted in red. The top right inset shows the amino acid sequences and expected fragmentation patterns for both peptide forms (C_pC and CC_p). Underlining within the inset indicates the detected amino acids. Top left inset is a magnified area of the two different y_7 ions showing the 793 mass peak to be about twice as abundant as the 927 mass peak at this concentration of hydroxylamine

Figure 5.

Relative abundance of singly palmitylated rhodopsin. The data were derived from the sequence analysis of six independent experiments on rod outer segment samples treated with 100 mM hydroxylamine for 30 min at 22°C. The CpC (palmitylation at 322) to CCp (palmitylation at 323) ratio is 2.16 ($P = 0.02$), indicating that the palmitate at position 323 is more labile to these conditions