Palmitoylation of bovine opsin and its cysteine mutants in COS cells
(fatty acylation/visual pigment/transducin/site-directed mutagenesis/structure-function relationships)

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ABSTRACT Previously, bovine rhodopsin has been shown to be palmitoylated at cysteine residues 322 and 323. Here we report on palmitoylation of bovine opsin in COS-1 cells following expression of the synthetic wild-type opsin gene and several of its cysteine mutants in the presence of [14C]maltose acid. Two moles of palmitic acid are introduced per wild-type opsin molecule in thioester linkages. Palmitoylation is abolished when both Cys-322 and Cys-323 are replaced by serine residues. Replacement of Cys-322 by serine prevents palmitoylation at Cys-323, whereas replacement of the latter with serine allows palmitoylation at Cys-322. Opsin mutants that evidently do not contain a Cys-110/Cys-187 disulfide bond and presumably remain in the endoplasmic reticulum are not palmitoylated. Replacement of Cys-140 or Cys-185 reduces the extent of palmitoylation of the opsin. Lack of palmitoylation at Cys-322 and/or Cys-323 does not affect 11-cis-retinal binding, absorption maximum or extinction coefficient of the chromophore, the bleaching behavior of the chromophore, or the light-dependent binding and activation of transducin. Mutants containing serine substitutions at Cys-140 or Cys-323 showed reduced light-dependent phosphorylation by rhodokinase.

Posttranslational modification of several classes of proteins by fatty acylation or polyprenylation is well established (1, 2). Rhodopsin and the β2-adrenergic receptor are examples of such proteins (3–8). Bovine rhodopsin has been shown to be palmitoylated at the vicinal cysteine residues 322 and 323 (5, 8). In a previous report, Karnik et al. (9) prepared and characterized a variety of cysteine → serine substitution mutants of rhodopsin. We have now used these and other mutants (Fig. 1 and Table 1) to study the palmitoylation of bovine opsin following expression in COS-1 cells. Several features of the process have been observed and are herein reported. First, we show that COS-1 cell opsin, like rod outer segment (ROS) rhodopsin, contains two palmitoyl groups in thioester linkages. Replacement of Cys-322 and Cys-323 by serine residues completely abolishes palmitoylation of the opsin. Cys-322 is the primary acceptor for the palmitoyl group since its replacement by serine abolishes palmitoylation. The mutants C110S and C187S (mutants in which Cys-110 or Cys-187 has been replaced by serine), which presumably stay in endoplasmic reticulum because they are not correctly folded, show very low levels of palmitoylation. Further, mutants in which Cys-140 or Cys-185 is replaced show reduced palmitoylation, which raises the possibility that these residues are involved in the palmitoylation process. Finally, in the present in vitro experiments, we have not detected any effect of the lack of palmitoylation on chromophore formation or the signal-transducing properties of rhodopsin as measured by transducin binding and activation or phosphorylation by rhodokinase.

MATERIALS AND METHODS

Materials. Palmitic acid (PA), methyl palmitate, and methyl stearate were purchased from Aldrich. Hydroxylamine hydrochloride was from Sigma, and n-dodecyl β-maltoside (DM) was from Anatrace. Sequenase (version 2.0) was from United States Biochemical, and N-glycosidase F was from Boehringer Mannheim. Silica gel plates were from Baker, and nitrocellulose filters (HAWP 25) were from Millipore. Bovine retinene were from J. A. Lawson Co. (Lincoln, NE). 11-cis-Retinal was a gift of P. Sorter (Hoffman-La Roche) and R. Crouch (Medical University of South Carolina and the National Eye Institute). [9,10-3H]HMPA (60 Ci/mmol; 1 Ci = 37 GBq), [9,10-3H]myristic acid (30 Ci/mmol), [35S]ATP (3000 Ci/mmol), deoxyadenosine 5'-[α-35S]thio)triphosphate (500 Ci/mmol), [γ-32P]GTP (5000 Ci/ mmol), and guanosine 5'-[γ-32P]thio)triphosphate (1000 Ci/ mmol) were obtained from Du Pont–New England Nuclear.

The Opsi Gene and Its Mutants. The synthetic bovine opsin gene in the expression vector pMT3 and mutants carrying cysteine → serine replacements (Table 1) have been described (9, 10). The mutants Cys-140 → alanine, histidine, and tyrosine were constructed by replacement of the Spe I–Aha II restriction fragment in the synthetic opsin gene (11) with the appropriate synthetic DNA duplexes containing the desired codon alteration. All mutations were confirmed by dideoxynucleotide sequencing of CsCl-purified plasmid DNA (12).

Expression and Purification of Rhodopsin Mutants. Procedures for the culturing and transient transfection of COS-1 cells with the synthetic opsin gene have been reported (13). COS-1 cells were plated at a density of 1.25–1.5 × 105 cells per 150 × 25 mm culture dish and transfected 10–14 h later with 12.5 μg of CsCl-purified plasmid DNA. The cells were harvested 70–80 h after transfection, washed with 10 mM NaH2PO4 (pH 7.0) containing 150 mM NaCl (PBS), and incubated with 5 μM 11-cis-retinal for 3 h at 4°C in the dark. After solubilization in 1% DM, the pigments were purified by immunoaffinity adsorption on 1D4-Sepharose (13). The resin was thoroughly washed with (i) 10 mM Tris-HCl (pH 7.0) containing 150 mM NaCl and 0.1% DM or (ii) 2 mM NaH2PO4 (pH 6.0) containing 0.1% DM. The bound rhodopsin was eluted with 35 μM C1–18 peptide (a synthetic peptide corresponding to the carboxyl-terminal 18 amino acids of rhodopsin) in the appropriate buffer. Bovine rhodopsin from ROS was purified by the same procedure. Rhodopsin preparations typically had absorbance ratios (A340/A600) of 1.6–2.0. Purified protein samples were analyzed by SDS/PAGE (14) with a 5% stacking and a 10% or 12% resolving gel and visualized by silver staining (15).

Abbreviations: ROS, rod outer segment; PA, palmitic acid; DM, n-dodecyl β-maltoside; G protein, guanine nucleotide-binding protein.

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Labeling of Opsin in COS-1 Cells with [3H]PA. Forty-eight hours after transient transfection with the wild-type and mutant opsin genes, the cells were starved in serum-free medium. After 12 h, the cells were incubated for 30 min at 37°C in medium containing 1% fetal calf serum and then for an additional 2 h at 37°C in the same medium containing [3H]PA or [3H]myristic acid (100 μCi/ml). In some cases, pulse-labeled cells were washed with PBS and grown in high serum medium (10% fetal calf serum) without [3H]PA for the specified chase period. The cells were thoroughly washed with cold PBS and solubilized in 1% DM, and the opsin was purified on 1D4-Sepharose. Treatment of [3H]PA-labeled opsin with 100 mM hydroxyamine hydrochloride (pH 7.0) was done in 100 mM Tris-HCl for 2 h at 23°C (16).

Isolation and Analysis of [3H]PA-Labeled Opsin. Purified opsin (500 pmol in 0.5 ml), expressed in COS-1 cells in the presence of [3H]PA, was extracted three times with hexane/methanol, 2:1 (vol/vol), and the aqueous phase was incubated with Pronase (50 μg/ml) in the presence of (i) 20 mM 2-mercaptoethanol (pH 8.0) or (ii) 100 mM hydroxyamine hydrochloride (pH 7.0) for 16 h at 20°C. The released fatty acyl derivatives were extracted three times with hexane/methanol, 2:1, the organic layers were pooled, and the solvent was evaporated under reduced pressure. The dry residue was dissolved in 50 μl of methylene chloride and analyzed by TLC (20-cm silica gel plates). The chromatogram was developed in chloroform/methanol/water, 65:25:4 (vol/vol), cut into 0.5-cm strips, and analyzed for 3H radioactivity by scintillation spectrometry.

Preparation and Analysis of Palmitoyl Methyl Esters. Purified ROS and COS-1 cell rhodopsins (50 μg) were extracted with hexane/methanol, 2:3, to remove any residual phospholipids. The extracted opsin was then proteolyzed as above, and the digests were treated with 1 M KOH for 8 h at 37°C. After acidification with HCl (to pH 2), 5 vol of methanol was added, and the mixture was incubated at 80°C for 4 h in sealed tubes under argon to convert PA to the methyl ester. After neutralization of the solution, the samples were extracted three times with hexane/methanol, 2:1, and dried in vacuo. As a control, 1 and 2 nmol of PA were subjected to the above treatment.

Simultaneous detection and quantitation of methyl esters were performed with a gas chromatograph (Varian model 3700) equipped with a flame ionization detector and electronic recorder (Varian 4290 integrator). Samples dissolved in ethyl acetate were injected into a fused silica capillary column (0.25 × 300 mm, 0.25-μm film thickness; J & W Scientific). The retention time and the relationship of peak area to concentration were calibrated by using 1 μM solutions of methyl palmitate (7.4 min) and methyl stearate (8.2 min).

Spectral Characterization of Rhodopsin Mutants. Spectroscopic measurements were performed with a Perkin–Elmer L7 UV/visible spectrophotometer. Stability of the chromophore toward hydroxyamine in the dark was examined by adding a solution of neutral hydroxyamine hydrochloride to a final concentration of 100 mM.

Binding and Activation of Transducin. The ability of wild-type and mutant rhodopsins to bind transducin was measured at equilibrium and by initial rate of GTP–GDP exchange (17) as described (18). For initial rate assays, the reaction mixtures (250 μl) contained 1 nM pH 6.0-purified wild-type or mutant rhodopsin and 10 μM guanosine 5'-[γ-32P]triphosphate in 10 mM Tris-HCl, pH 7.5/0.012% DM/100 mM NaCl/5 mM MgCl2/2 mM dithiothreitol. After illumination (>495 nm) for 1 min at 20°C, the reactions were initiated by addition of transducin (0.5–4 μM). Aliquots (50 μl) were removed at 7.5, 15, 22.5, and 30 s and filtered through nitrocellulose filters. The filters were washed five times with 5 ml of the above-mentioned buffer, dried, and assayed. For the equilibrium assays, the same reaction mixture containing 4 μM transducin was illuminated (>495 nm) for 1 min at 20°C and allowed to remain in the dark for an additional 2 h. The extent of the exchange reaction was measured as described above.

Activation of transducin by wild-type and mutant rhodopsins as measured by GTPase activity was done essen-
tially as described (13, 19). The reaction mixtures (100 μl) contained 1.25–5 nM pH 6.0-purified wild-type or mutant rhodopsin and 2 μM transducin in 20 mM Tris-HCl, pH 7.2/0.012% DM/100 mM NaCl/5 mM MgCl₂/1 mM dithiothreitol/20 μM [γ-32P]ATP.

**Phosphorylation by Rhodopsin Kinase.** The light-dependent phosphorylation of wild-type and mutant rhodopsins by rhodopsin kinase was done essentially as described (19). The phosphorylation reactions (2.0 ml) contained 100 nM pH 6.0-purified wild-type or mutant rhodopsin and ~200 units of rhodopsin kinase extract in 70 mM NaH₂PO₄, pH 7.0/0.012% DM/3 mM MgCl₂/1 mM dithiothreitol/100 μM [γ-32P]ATP/ 10 μM GTP.

**RESULTS**

**Palmitoylation of Bovine Opin Expressed in COS-1 Cells.** Bovine opsin was expressed in transiently transfected COS-1 cells in the presence of [3H]PA or [3H]myristic acid. After purification, opsin contained 3H radioactivity only when expressed in COS-1 cells in the presence of [3H]PA. The 3H label in the purified opsin was not removed upon extraction with n-hexane or with 0.2% aqueous SDS. However, about 50% of the 3H label in the opsin was removed when the cells were subjected to a 30-min chase (Fig. 2, lane 3). Longer chase periods (60 and 120 min) showed no further decrease in the extent of palmitoylation (Fig. 2, lanes 4 and 5). The PA ester was susceptible to hydroxylamine. Thus, treatment of the radioactively labeled protein with neutral hydroxylamine yielded a form of palmitohydroxamate (R₂ ~ 0.59) as shown by TLC (Fig. 3). Similarly, treatment of the 3H-labeled opsin with 2-mercaptoethanol (pH 8.6) released the radioactivity as PA (R₂ ~ 0.74). The release of the palmitoyl by the above reagents shows that the palmitoyl group(s) was bound to the opsin in a thioester linkage.

Gas chromatographic analysis revealed that COS-1 cell rhodopsin released 1.97 mol of PA per mol of protein, while under similar treatment, ROS rhodopsin yielded 2.0 mol of PA per mol of protein. A PA standard processed through the same reaction steps to obtain methyl palmitate yielded ~97% recovery.

**Palmitoylation of Cysteine Mutants of Bovine Opin.** The opsin mutant in which both Cys-322 and Cys-323 were replaced by serine residues (C322,323S) was not labeled by [3H]PA in COS-1 cells (Fig. 4 and Table 1). This result shows that only Cys-322 and Cys-323 are the sites of palmitoylation in bovine opsin (5, 8). Surprisingly, individual replacement of Cys-322 abolished palmitoylation at Cys-323; however, replacement of Cys-323 did not affect the acylation of Cys-322 (Fig. 4 and Table 1). These results suggest that Cys-322 is the primary palmitoylation site in bovine opsin.

We also considered the possibility that replacement of cysteine residues that do not directly participate in acylation might influence the extent of palmitoylation. Mutants with single replacements at Cys-222, Cys-264, and Cys-316 (Fig. 4 and Table 1) showed no significant effect on palmitoylation. In contrast, individual replacement of Cys-110 or Cys-187 by serine virtually abolished palmitoylation of opsin (Fig. 4 and Table 1). These mutations result in incorrectly folded opsin (9, 20) and may therefore affect the transport of the protein from the endoplasmic reticulum to the Golgi where palmitoylation is believed to occur (21).

A third intradiscal cysteine mutant, C185S, showed reduced palmitoylation to ~60% the level of wild-type opsin.
Further, similarities in extinction coefficient (Table 2) and bleaching behavior suggest that these amino acid substitutions do not affect the folding or stability of the protein. Like wild-type rhodopsin, the C322,323S mutant was also stable toward hydroxylamine in the dark. A similar finding has also been observed with ROS rhodopsin depalmitoylated with hydroxylamine (7).

Functional Properties of Mutants with Cysteine → Serine Substitutions. The ability of the cysteine mutants to bind and activate the guanine nucleotide-binding protein (G protein) transducin and undergo phosphorylation by rhodopsin kinase was investigated. For these assays, wild-type and mutant rhodopsins purified on 1D4-Sepharose in low salt buffer at pH 6.0 were employed. We have observed that this purification protocol results in the effective separation of regenerated pigment from opsins and other mutants (K.D.R. and H.G.K., unpublished work). All of the mutants showed nearly wild-type levels of transducin binding as measured by guanine nucleotide exchange at equilibrium (Table 2). Further, none of these mutations affected the initial rates of transducin binding because they showed apparent $K_m$ and $V_{max}$ values similar to that of wild-type rhodopsin (Table 2).

Activation of transducin by these mutants was examined by measuring the light-dependent hydrolysis of GTP (Table 2). With the exception of C316S, which showed a 40% reduction in GTPase activity, these cytoplasmic cysteine mutants showed specific activities of light-dependent $P_i$ release similar to that of wild-type rhodopsin ($\sim 25$ mol of $P_i$ per mol of rhodopsin per min). Previously, Zozulya et al. (23) reported a nominal effect on the ability of the C316S mutant to stimulate transducin GTPase and cGMP phosphodiesterase activities.

The light-dependent phosphorylation of the cysteine → serine mutants by rhodopsin kinase showed that replacement of Cys-140 and Cys-323 reduced the level of $^{32}P$ incorporation by 43% and 19%, respectively (Table 2). Under the same conditions, the C316S, C322S, and C322,323S mutants were maximally phosphorylated to a level similar to that of wild-type rhodopsin (Table 2).

**DISCUSSION**

We have shown that COS-1 cells have the ability to palmitoylate bovine opsins. Two palmitoyl groups per opsin were incorporated, and palmitoylation was specific at Cys-322 and Cys-323 since replacement of these cysteine residues by serine residues abolished the acylation (Fig. 4 and Table 1).

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**Table 2. Spectral and functional properties of cysteine mutants of bovine rhodopsin**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$\varepsilon$ a</th>
<th>Chromophore formation, %</th>
<th>Equilibrium, mol</th>
<th>$V_{max}$ b (mol per mol of rhodopsin per s)</th>
<th>$K_m$ c (mM)</th>
<th>$P_i$ incorporation d (mol per mol of rhodopsin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>40,600</td>
<td>84</td>
<td>296 ± 11</td>
<td>2.8 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>24.4 ± 2.2</td>
</tr>
<tr>
<td>C140S</td>
<td>40,600</td>
<td>79</td>
<td>261 ± 18</td>
<td>2.4 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>23.7 ± 1.8</td>
</tr>
<tr>
<td>C316S</td>
<td>39,800</td>
<td>80</td>
<td>292 ± 11</td>
<td>3.0 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>14.7 ± 2.1</td>
</tr>
<tr>
<td>C322S</td>
<td>40,000</td>
<td>81</td>
<td>297 ± 13</td>
<td>2.7 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>25.1 ± 2.2</td>
</tr>
<tr>
<td>C323S</td>
<td>40,900</td>
<td>77</td>
<td>312 ± 16</td>
<td>2.9 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>23.0 ± 1.9</td>
</tr>
<tr>
<td>C322,323S</td>
<td>40,800</td>
<td>81</td>
<td>319 ± 11</td>
<td>2.8 ± 0.4</td>
<td>0.9 ± 0.2</td>
<td>25.0 ± 1.9</td>
</tr>
</tbody>
</table>

The pigments used in the GTP-GDP exchange, GTP hydrolysis, and phosphorylation assays were purified in 2 mM NaH$_2$PO$_4$ (pH 6.0) containing 0.1% DM. The amount of pigment assayed was based on the molar extinction coefficient. Values are presented as the means ± SEM from two or three independent experiments.

*a Molar extinction coefficients were determined as described (11, 19). The molar extinction coefficient of rhodopsin was assumed to be 40,600 M⁻¹·cm⁻¹ (22). All values were rounded to the nearest 100 M⁻¹·cm⁻¹.

*b The values were obtained by estimation of the intercepts from linear regression analyses of an initial velocity⁻¹ versus [transducin]⁻² plot (17, 18).

*c The values were obtained from the slope of the line by linear regression analysis of a velocity versus [rhodopsin] plot (13, 19).

*d Stoichiometry of light-dependent $^{32}P$ incorporation after a 60-min phosphorylation reaction.
Previously, Ovchinnikov et al. (5) characterized the same two cysteine residues as the sites of palmitoylation in ROS rhodopsin. This finding was recently confirmed by Papac et al. (8). Further, we have also shown the palmitoyl groups to be linked via thioester bonds (Fig. 3). Thus, cleavage with 2-mercaptoethanol or acetic acid released the expected palmitohydroxamate (16).

We have further studied the characteristics of palmitoylation by using several additional opsin mutants that carry cysteins → serine substitutions. The specific replacement of Cys-322 alone by serine abolished palmitoylation of Cys-323. In contrast, replacement of Cys-322 by serine had no effect on the palmitoylation of Cys-322 (Fig. 4 and Table 1). Therefore, Cys-322 appears to serve as the primary acceptor of the palmitoyl group, and the palmitoylation of Cys-323 occurs subsequent to palmitoylation at Cys-322. A sequential or ordered addition of palmitate might be taking place in which Cys-322 is the primary acceptor because of prefered reactivity or accessibility of its sulfhydryl group. The mechanism of this reaction deserves further study, but there are analogies in other acylation systems. For example, in the aminoacylation of 3'-terminal adenosine in tRNA, a class of tRNA synthetases exists that first acylates the 2'-hydroxyl of the nucleoside and the amino acyl group subsequently migrates to the 3'-hydroxyl (25). Similarly, the transfer of acetyl groups to chloramphenicol by chloramphenicol acetyltransferase takes place by a sequential mechanism (26, 27).

The mutants containing either of the two substitutions, C110S or C187S, showed very low palmitoylation (Fig. 4 and Table 1). Such mutants have previously been shown not to fold correctly (9) and, as a consequence, may remain in the endoplasmic reticulum. Absence of palmitoylation in these mutants is consistent with the conclusion that palmitoylation requires transport of the proteins to the cis Golgi cisternae, where acylation is believed to take place (21). Minor acylation of C110S and C187S (≈10% and ≈20%, respectively) might reflect the extent of "leakiness" in the transport of these misfolded mutant proteins.

The mutants C140S and C185S both showed reduced levels of palmitoylation, ≈70% and 60% relative to that of wild-type opsin, respectively (Fig. 4 and Table 1). Replacement of Cys-140 by serine restored a histidine to tyrosine phenotype similar to that of the C140S mutant. The influence of the Cys-140 and Cys-185 mutants on acylation is rather surprising. We have previously shown that these residues do not play a critical role in the folding of rhodopsin. In the C185S mutant, a significant reduction in transducin activation was previously observed (9). The multiple substitutions made at Cys-140 suggest a structural role for this amino acid in rhodopsin, possibly through interactions with the C-terminal tail. The negative effect of the serine substitution at this position on phosphorylation by rhodopsin kinase (Table 2) supports this hypothesis. Indeed, it has been suggested that in the folded structure of rhodopsin the second cytoplasmic loop (Fig. 1) may interact with the C-terminal tail (28).

What role does palmitoylation play in rhodopsin structure and function? The suggestion has been made that the palmitoyl groups anchor the C-terminal tail in the membrane, thus generating a fourth cytoplasmic loop (5, 29). A similar role has been proposed for the palmitoyl group at Cys-341 of the β2-adrenergic receptor (6). In a recent study (7), direct removal of palmitate from rhodopsin in ROS membranes with hydroxylamine was found to markedly reduce the extent of chromophore regeneration following photobleaching and to increase the light-dependent activation of transducin. No effect on the light-dependent binding of transducin was observed. In the present study, the nonpalmitoylated opsin mutants C322S and C322,323S bound 11-cis-retinal to a level similar to that of wild-type opsin (Fig. 5 and Table 2). Further, although we also did not detect any change in the level of transducin binding with these mutants, no increase in the light-dependent GTPase activity of transducin was apparent (Table 2). The reasons for the difference between our results and those of the above workers (7) are not clear, but these could be due to the conditions used to prepare the nonpalmitoylated rhodopsins or those used in the in vitro assays.

Finally, although a role in phosphorylation was observed with the C323S mutant, the nonpalmitoylated C322S and C322,323S mutants had virtually no effect (Table 2). Therefore, the functional efficiency of the proximal part of C terminus may not be dependent on the presence of the palmitoyl groups in rhodopsin. It is plausible that interaction(s) with other peptide loops affords sufficient stability to this region, and hence removal of the palmitoyl groups has no major effect on the interaction with transducin or rhodopsin kinase.

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