

# Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin

(heterologous expression/glycosylation/transmembrane receptor/site-specific mutagenesis/synthetic gene)

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**ABSTRACT** To investigate the role of different cysteine residues in bovine rhodopsin, a series of mutants were prepared in which the cysteine residues were systematically replaced by serines. The mutant genes were expressed in monkey kidney cells (COS-1) and the mutant opsins were evaluated for their levels of expression, glycosylation patterns, and ability to form the chromophore characteristic of rhodopsin and to activate transducin. Substitution of the three cytoplasmic cysteines (Cys-316, Cys-322, and Cys-323) and the four membrane-embedded cysteines (Cys-140, Cys-167, Cys-222, and Cys-264) produced proteins with wild-type phenotype. Also, single substitutions of Cys-185 gave rise to a wild-type phenotype. In contrast, substitution of the three intradiscal cysteines (Cys-110, Cys-185, and Cys-187) or single substitution of Cys-110 or Cys-187 gave proteins that were expressed at reduced levels, glycosylated abnormally, and unable to bind 11-*cis*-retinal. Thus, of the 10 cysteines in bovine rhodopsin, only intradiscal Cys-110 and Cys-187 are essential for the correct tertiary structure of the protein.

Rhodopsin is the photoreceptor pigment of vertebrate rod cells (1). On absorption of light, it undergoes a series of structural changes that are initiated by the *cis*–*trans* isomerization of the chromophore, 11-*cis*-retinal. One of the photointermediates, *meta*-rhodopsin II, activates the guanine nucleotide-binding regulatory protein (G protein) transducin. Transducin exchanges bound GDP for GTP and the  $\alpha$  subunit then dissociates from rhodopsin and the  $\beta\gamma$  subunits of transducin. The complex of the  $\alpha$  subunit of transducin and GTP activates the rod outer segment (ROS) cGMP phosphodiesterase. The resulting decrease in cGMP levels leads to the closing of cGMP-gated channels in the ROS plasma membrane. Thus, photoactivation of rhodopsin culminates in hyperpolarization of the rod cell followed by a neuronal signal (2).

Rhodopsin is an integral membrane protein with a single polypeptide chain of 348 amino acids. The primary structure of bovine rhodopsin is now known both from protein and cDNA sequencing (3–5). The protein is believed to consist of seven transmembrane segments (6). A similar structural motif has been proposed for many other membrane receptors that are coupled to G proteins (7).

Structure–function studies of rhodopsin are clearly important to an understanding of its multiple activities that include interactions with a number of soluble proteins. In initiating such studies, we have described the synthesis of a gene for bovine rhodopsin (8), the expression of the gene in COS-1 cells to produce functional rhodopsin (9), and the construction of rhodopsin mutants to study transducin activation (10). We have now carried out a systematic study of the role of different cysteine residues in rhodopsin structure and function. Rhodopsin contains 10 cysteine residues, 6 of which

appear to be in the free sulfhydryl form (11). The presence of disulfide bonds has been proposed on the basis of chemical modification studies and homologies with other G-protein-coupled receptors (12, 13). We have now prepared 10 rhodopsin mutants in which sets of cysteine residues or individual cysteines were replaced by serines (Table 1 and Fig. 1). The mutants were expressed in COS-1 cells and evaluated for their levels of expression and extent of glycosylation and for their abilities to bind 11-*cis*-retinal and to activate transducin. We show that only 2 of the 10 cysteine residues, Cys-110 and Cys-187, are essential for the formation of the correct structure in rhodopsin.

## MATERIALS AND METHODS

**Materials.** Fully protected deoxynucleoside (*N,N*-diisopropylamino)phosphoramidites were obtained from Applied Biosystems and American Bionetics (Hayward, CA). Radionuclide compounds were from Amersham. Bovine retinas were from J. A. Lawson (Lincoln, NE). Endo-*N*-acetylglucosaminidase H (endo H, EC 3.2.1.96) and glycopeptidase F (EC 3.2.2.18) were from Boehringer Mannheim.

**Immunoaffinity Support.** Anti-rhodopsin monoclonal antibody, rho 1D4 (14), was purified from myeloma cell culture supernatant by ammonium sulfate precipitation followed by DEAE-cellulose chromatography (15) and was coupled to Sepharose 2B (9).

**Purification of Transducin and Rhodopsin from Bovine Retinas.** ROSs were prepared from frozen bovine retinas by the procedure of Hong and Hubbell (16). Transducin was purified from a ROSs preparation according to Fung *et al.* (17). ROS was solubilized in 1% dodecyl maltoside and rhodopsin was purified by immunoaffinity adsorption on rho 1D4-Sepharose 2B. The typical  $A_{280}/A_{500}$  ratio for the purified rhodopsin preparations was 1.7–1.8.

**Preparation of Oligonucleotides.** Reagents and solvents were purified according to standard procedures (8, 18). Oligonucleotides were synthesized by using an Applied Biosystems model 380A DNA synthesizer and were purified by denaturing polyacrylamide gel electrophoresis. After 5'-end phosphorylation, oligonucleotides were characterized by gel electrophoresis and 5'-end analyses (8).

**Construction of Rhodopsin Mutants.** Mutations were introduced into the synthetic rhodopsin gene (8) by restriction fragment replacement (18). In all cases, a cysteine codon, TGT or TGC, was replaced by the serine codon TCT. The rhodopsin mutants CysI–CysVII (Table 1) were constructed by successive restriction fragment replacement in a pUC-derived cloning vector. After sequencing the DNA regions containing the codon changes, the mutant genes were trans-

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Abbreviations: ROS, rod outer segment; G protein, guanine nucleotide-binding regulatory protein; endo H, endo-*N*-acetylglucosaminidase H.

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Table 1. Rhodopsin mutants: Cys → Ser replacements

Mutant (Cys → Ser)	Residues changed
<b>Multiple</b>	
CysI	316, 322, and 323
CysII	140, 167, 222, and 264
CysIII	316, 322, 323, 140, 167, 222, and 264
CysIV	110, 185, and 187
CysV	110, 185, 187, 140, 164, 222, and 264
CysVI	110, 185, 187, 316, 322, and 323
CysVII	All 10 cysteines
<b>Single</b>	
Cys-110 → Ser	110
Cys-185 → Ser	185
Cys-187 → Ser	187

For topological locations of cysteines see Fig. 1

ferred into the expression vector as described below. The single cysteine mutants (Cys-110 → Ser, Cys-185 → Ser, and Cys-187 → Ser) were constructed by restriction fragment replacement directly in the expression vector.

**Cloning of the Rhodopsin Mutants into the Expression Vector pMT-2.** The cloning of the synthetic rhodopsin gene into the expression vector has been reported (10). All of the cysteine codons in the rhodopsin gene were within a 1022-base-pair *Kpn I*-*BstXI* restriction fragment. After the introduction of multiple cysteine mutations (CysI-CysVII), as described above, this restriction fragment was transferred into the expression vector.

**DNA Sequence Analysis.** The nucleotide sequence of DNA fragments used to introduce mutations was confirmed by the chain-terminator method for DNA sequencing on CsCl-purified plasmid DNA (19). In areas of band compression, DNA sequencing by the chemical degradation method was carried out (20). The entire mutant CysVII gene was resequenced to confirm that the only nucleotide changes present were those introduced by mutagenesis.

**Expression of Mutant Genes.** The wild-type rhodopsin gene and cysteine mutant genes were expressed in transiently transfected COS-1 cells as reported (9, 10).

### Purification and Characterization of Rhodopsin Mutants.

After the addition of 11-*cis*-retinal (40  $\mu$ M), the transfected COS-1 cells were solubilized in 1% dodecyl maltoside. The wild-type rhodopsin and mutants were purified essentially as before by using immunoaffinity adsorption (9) and characterized by UV/visual spectroscopy and by NaDodSO<sub>4</sub>/gel electrophoresis (21) followed by immunoblotting (22) with a rabbit polyclonal anti-rhodopsin IgG. Rhodopsin quantitation was based on  $A_{495}$  by using an extinction coefficient of 42,700 M<sup>-1</sup>cm<sup>-1</sup> (16).

**Transducin Activation Assay.** The purified rhodopsin mutants were assayed for their ability to enhance the GTPase activity of ROS transducin. The assay was performed as described (9, 10), except that dodecyl maltoside replaced digitonin. Typically, the assay mixture contained 1 pmol of purified rhodopsin, 120 pmol of transducin, and 500 pmol of [ $\gamma$ -<sup>32</sup>P]GTP.

**Endo H and Glycopeptidase F Digestions.** Endo H cleaves between the two proximal GlcNAc residues only on high-mannose oligosaccharides (23). Thus, it can be used to distinguish high-mannose intermediates from complex glycans. Glycopeptidase F cleaves all N-linked glycans from a glycoprotein (24). The purified rhodopsin and the mutant proteins were digested with the enzymes in the light and the products were analyzed by immunoblotting. The binding of the polyclonal antibody used for the immunoblots was not affected by the glycosylation pattern.

## RESULTS

### Rhodopsin Gene Mutants Containing Multiple Cys → Ser Codon Changes.

The 10 cysteine residues in rhodopsin are located in the three domains of the secondary structure model (Fig. 1). Seven rhodopsin gene mutants were constructed in which cysteine residues were replaced by serines, systematically in each domain (Table 1). Cytoplasmic cysteines (Cys-316, Cys-322, and Cys-323) located on the carboxyl-terminal tail were all replaced by serines in mutant CysI. All four membrane-embedded cysteines (Cys-140, Cys-167, Cys-222, and Cys-264) were substituted in CysII. Both the

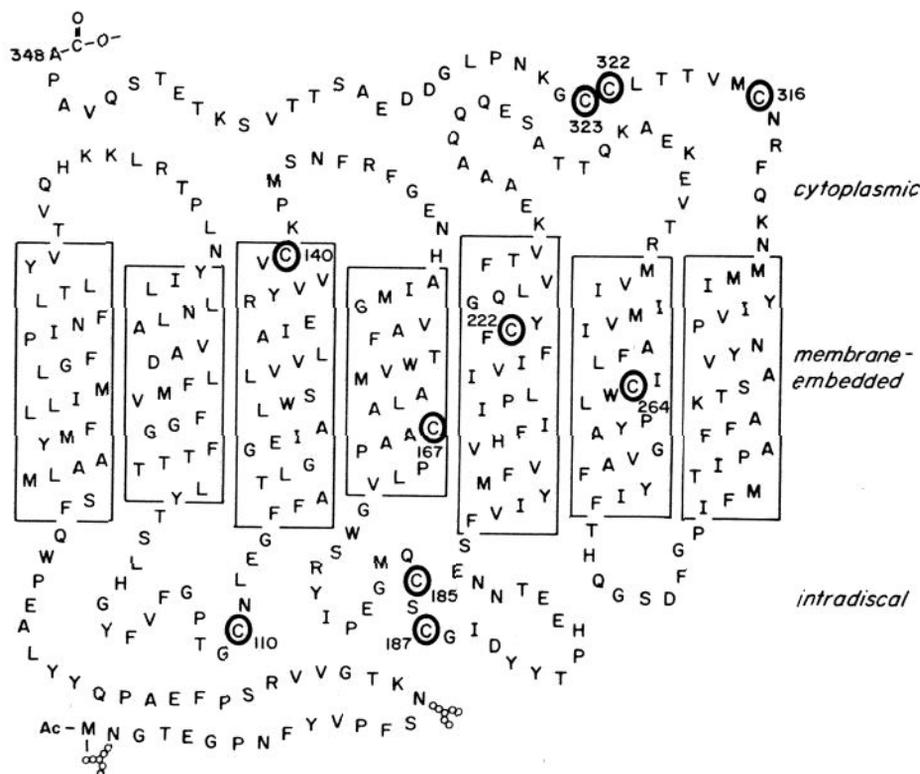


FIG. 1. Secondary structure model for the polypeptide chain of bovine rhodopsin (6). The amino acids in the seven transmembrane helices are within the vertical boxes. Also shown are the amino acid sequences connecting the successive helices and forming loops in the cytoplasmic and intradiscal space. The 10 cysteine residues are circled. According to this model, three cysteines are on the intradiscal side, four are membrane-embedded, and three are on the cytoplasmic side. The single-letter amino acid code is used.

cytoplasmic and membrane-embedded cysteines were replaced in CysIII. The 3 intradiscal cysteines (Cys-110, Cys-185, and Cys-187) were replaced in CysIV. The intradiscal and membrane-embedded cysteines were replaced in CysV, and intradiscal and cytoplasmic cysteines were replaced in CysVI. All 10 cysteines were replaced in mutant CysVII.

**Expression and Properties of the Mutant Proteins Containing Multiple Cys → Ser Replacements (CysI–CysVII) Expression in COS-1 Cells.** The level of expression varied for the different mutants. In general, two patterns were observed. For example, CysI and CysII were expressed at a level of 4–5  $\mu\text{g}$  of rhodopsin per  $10^7$  cells, which was comparable to the wild-type level. The second group of mutants (CysIII–CysVII) produced the proteins at reduced levels. Expression levels observed with different mutants are shown in Table 2.

**Glycosylation Patterns of Wild-Type and Rhodopsin Cys → Ser Mutants Synthesized in COS-1 Cells.** Bovine opsin synthesized in COS-1 cells was heterogeneously glycosylated. On NaDodSO<sub>4</sub>/gel electrophoresis, the wild-type protein was shown to contain a major 41-kDa band and several minor bands with slower electrophoretic mobilities (Fig. 2). After treatment with glycopeptidase F, the protein migrated as a single 31-kDa band, suggesting that the heterogeneity seen in gel electrophoresis patterns was due to glycosylation. To estimate the ratio of high mannose to complex glycan in the protein, the opsin was treated with endo H. Only 50% of the purified COS-1 cell rhodopsin was sensitive.

The cysteine mutants (CysI–CysVII) fell into two classes based on gel electrophoretic mobility of the proteins formed (Fig. 2). Expression of CysI, CysII, and CysIII gave products similar to those obtained with the wild type. Thus, each protein gave a predominant band at 41 kDa on gel electrophoresis, about 50% of which was endo H-sensitive. On the other hand, the mutant genes CysIV, CysV, CysVI, or CysVII produced much less opsin, and the products appeared as three distinct bands on gel electrophoresis at 41, 36, and 31 kDa. The 31-kDa band corresponded to unglycosylated opsin and the 36-kDa band corresponded to some intermediate of glycosylation at the two N-linked glycosylation sites in the protein. When treated with glycopeptidase F, each of these mutant proteins ran as a single 31-kDa band. Treating the same mutants with endo H also hydrolyzed all of the glycan, showing that only high-mannose moieties were present. Thus, these mutant opsins were not further glycosylated to form the endo H-resistant complex glycan.

**Spectral properties of Mutant Opsins.** The ability of opsin to bind 11-*cis*-retinal indicates proper folding of the polypep-

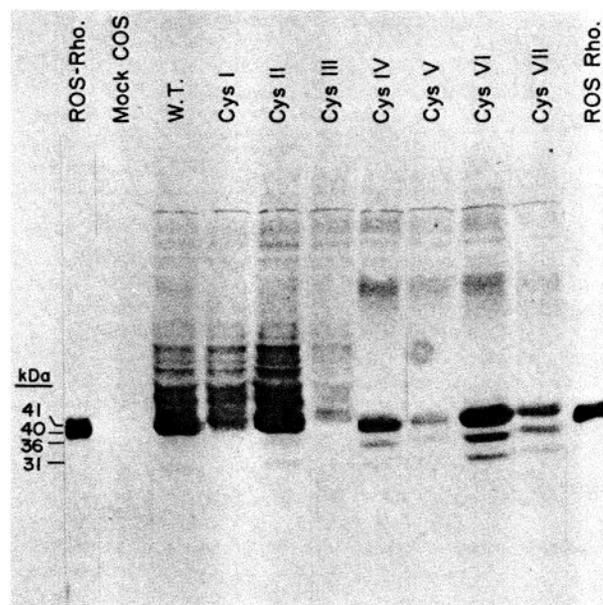


FIG. 2. Expression of the cysteine mutants of rhodopsin (CysI–CysVII) in COS-1 cells. Total cellular proteins present in the solubilized fraction were resolved on NaDodSO<sub>4</sub>/polyacrylamide gels and visualized by immunoblotting. The 31-kDa band corresponded to unglycosylated opsin. Rhodopsin from bovine ROS (ROS-Rho.) moves as a 40-kDa band on this gel. Mock COS, mock-infected COS cells.

ptide chain with consequent formation of the correct chromophore-binding pocket. A UV/visible absorption spectrum of wild-type rhodopsin purified from COS-1 cells in dodecyl maltoside is shown in Fig. 3A. The  $\lambda_{\text{max}}$ , 495 nm, was identical to that observed for bovine rhodopsin in the same detergent. The ratio of  $A_{280}/A_{500}$  was 2.0. The opsins obtained from the mutants CysI, CysII, and CysIII bound 11-*cis*-retinal and after purification under identical conditions yielded chromophores with a  $\lambda_{\text{max}}$  of 495 nm. The  $A_{280}/A_{500}$  ratios obtained were 2.2, 2.0, and 2.5 for CysI, CysII, and CysIII, respectively. The spectrum of CysIII is compared to that of wild type in Fig. 3. The opsins from the mutants CysIV–CysVII did not bind 11-*cis*-retinal. The sensitivity of rhodopsin detection in our spectrophotometric assay is about 1.25  $\mu\text{g}$ . Therefore, protein (5–20  $\mu\text{g}$ ) from each of these mutants was purified, after the standard addition of 11-*cis*-retinal. No absorbance in the visible range was detected. To exclude the possibility of 11-*cis*-retinal not reaching the inner membranes of the COS-1 cells, total cell membrane was prepared from cells transiently transfected with the wild-type rhodopsin gene or the CysIV gene by homogenization followed by centrifugation. The membrane fractions were incubated with 11-*cis*-retinal and then solubilized in 1% dodecyl maltoside. After purification, a normal chromophore ( $A_{280}/A_{500} = 2.1$ ) was obtained from rhodopsin expressed from the wild-type gene. However, the membrane fraction from the cells expressing CysIV showed no rhodopsin-like chromophore, although it yielded 9  $\mu\text{g}$  of purified protein. Thus, the absence of chromophore in the opsin from CysIV was not due to inaccessibility to 11-*cis*-retinal in whole cells. This conclusion also applies to the proteins from the other mutants, CysV–CysVII, that also were expressed at lower levels and failed to bind 11-*cis*-retinal.

**Activation of Transducin.** The proteins expressed from CysI–CysIII were regenerated with 11-*cis*-retinal, purified, and assayed for their ability to stimulate the GTPase activity of purified bovine transducin. Under the conditions of the assay, the wild-type rhodopsin released 0.5 pmol of  $P_i$  per min

Table 2. Phenotypes of rhodopsin mutant proteins

Gene	RHO expression	Glycan type	$\lambda_{\text{max}}$ , nm	Activity	
				Dark	Light
Wild type	4.8	I	495	0.40	3.80
CysI	4.5	I	495	0.51	3.13
CysII	4.9	I	495	0.47	2.87
CysIII	2.1	I	495	0.41	1.84
CysIV	1.2	II	ND	ND	ND
CysV	1.1	II	ND	ND	ND
CysVI	1.2	II	ND	ND	ND
CysVII	1.1	II	ND	ND	ND
Cys-110 → Ser	1.1	II	ND	ND	ND
Cys-185 → Ser	4.1	I	495	0.29	2.24
Cys-187 → Ser	1.2	II	ND	ND	ND

Rhodopsin (RHO) expression is presented as  $\mu\text{g}$  per  $10^7$  solubilized cells, based on immunoblot analysis. Type I glycan was 50% endo H-resistant. Type II glycan was endo H-sensitive (see Figs. 2 and 4 for gel electrophoresis pattern). Activity is a measure of GTP hydrolysis and is presented as pmol of  $P_i$  per min per pmol of rhodopsin. ND, not detected.

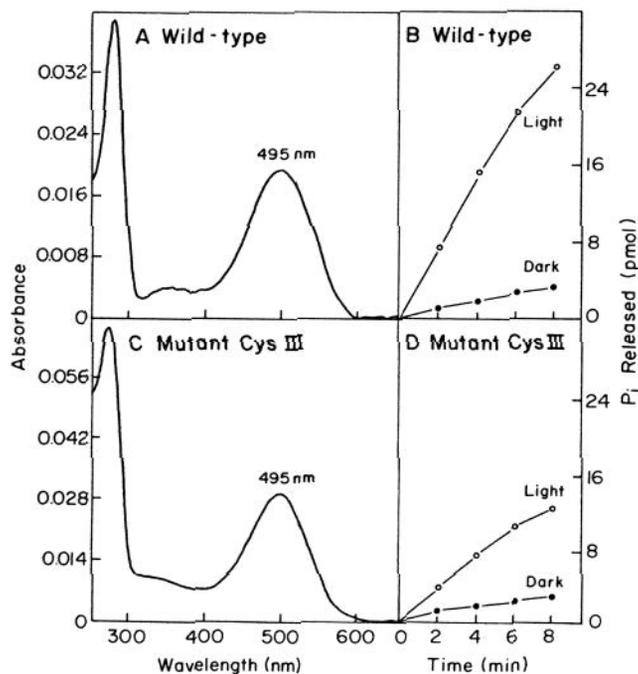


FIG. 3. (A and C) UV/visible absorption spectra of rhodopsin from the wild-type gene (A) and from mutant CysIII (C) are shown. The proteins were purified from COS-1 cells as in text. The absorption spectra of mutant proteins from CysI and CysII were essentially wild type with respect to  $\lambda_{max}$  (495 nm) and  $A_{280}/A_{500}$  ratio (Table 2). (B and D) Kinetics of transducin activation as stimulated by the wild-type protein (B) and the mutant CysIII protein (D) in 0.02% dodecyl maltoside. The activity shown is for 1 pmol of rhodopsin per assay in dark or light. The values given are not corrected for the decay of *meta*-rhodopsin 11 during the course of the assay. The specific activities (Table 2) were calculated from the slopes of the curves.

per pmol of the chromophore in the dark and 3.8 pmol of  $P_i$  per min per pmol of the chromophore in the light. COS-1 cell rhodopsin and bovine ROS rhodopsin have been shown (9) to have identical specific activities. The purified mutant proteins from CysI and CysII showed activities comparable to the wild-type level. The protein from CysIII was less active than wild type (0.4 pmol of  $P_i$  released per min per pmol of chromophore in the dark with a 4-fold stimulation in the light) (Fig. 3). The results of the transducin activation assays are summarized in Table 2.

**Rhodopsin Mutants Containing Single Cys  $\rightarrow$  Ser Substitutions.** Mutants in which the three intradiscal cysteines (Cys-110, Cys-185, and Cys-187) were replaced simultaneously by serines were defective in level of expression, glycosylation, and chromophore binding. Each of the intradiscal cysteines was next singly replaced by serine. Thus, three mutant opsin genes containing single substitutions (Cys-110  $\rightarrow$  Ser, Cys-185  $\rightarrow$  Ser, or Cys-187  $\rightarrow$  Ser) were prepared (Fig. 1 and Table 1). On expression, Cys-110  $\rightarrow$  Ser and Cys-187  $\rightarrow$  Ser produced a band pattern on gel electrophoresis that was identical to that produced by CysIV (Fig. 4). This band pattern was characteristic of protein with incomplete glycan processing. Further, these mutant proteins failed to generate a chromophore when incubated with 11-*cis*-retinal and, therefore, did not activate transducin in the presence of light. The Cys-185  $\rightarrow$  Ser mutant, on the other hand, produced opsin with a wild-type glycosylation pattern. It displayed a wild-type rhodopsin-like absorption spectrum ( $\lambda_{max}$  = 495 nm) and activated transducin (Table 2). These results show that Cys-185 is not obligatory for rhodopsin structure and function. However, both Cys-110 and Cys-187 are required.

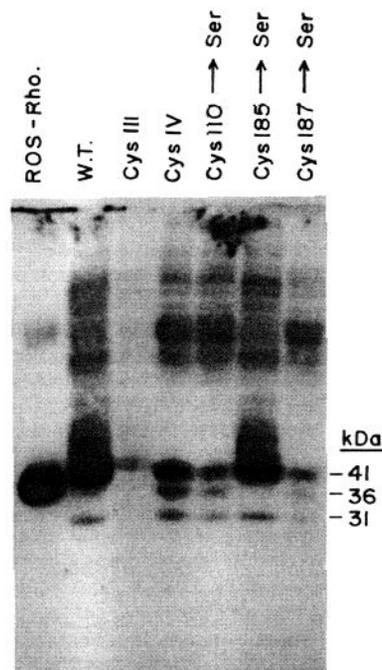


FIG. 4. Immunoblot analysis of proteins expressed from rhodopsin mutants containing individual substitutions of the intradiscal cysteines. The proteins on the gel were probed with rabbit anti-rhodopsin IgG and  $^{125}I$ -labeled protein A. Lanes: ROS-Rho., rhodopsin from bovine ROS; W.T., wild type; CysIII and CysIV, mutants CysIII and CysIV, respectively; Cys-110  $\rightarrow$  Ser, Cys-185  $\rightarrow$  Ser, and Cys-187  $\rightarrow$  Ser, rhodopsins with a Cys  $\rightarrow$  Ser substitution at the position indicated.

## DISCUSSION

The structural motif of seven transmembrane helices has been proposed for a large number of membrane receptors. These include rhodopsin and other visual pigments (25), adrenergic receptors (26), the family of muscarinic receptors (27), yeast pheromone receptors (28), and a number of others. In each case, a G protein couples the receptor to the appropriate effector enzyme—e.g., adenylate cyclase, phospholipase C, or cGMP phosphodiesterase—or an ion channel. The apparent similarities in structure and function among the seven helix receptors suggest the possibility of a common underlying mechanism of action for this class of receptors. The proposal that the ligand-binding site of the  $\beta$ -adrenergic receptor is in the membrane-embedded domain of the protein (29, 30) analogous to the 11-*cis*-retinal binding pocket in rhodopsin, lends support to the idea of a unitary mechanism. The binding of ligand to receptor, like the *cis*-*trans* isomerization in rhodopsin, initiates a structural change in the helical cluster that propagates to the nonmembranous domains and allows interactions with the G protein and other proteins. To understand the mechanism of signal transduction in this class of receptors, we need to obtain insights into the structural interrelationships of the receptor domains. The results with the cysteine residues herein reported are important in this regard.

**Cys-110 and Cys-187 Are Essential to Rhodopsin Structure.** Systematic mutations of the different cysteine residues showed two types of proteins. The first was similar to wild type. The second was characterized by a lower level of protein expression, incomplete glycan processing, and an inability to bind 11-*cis*-retinal. In all cases, the abnormal proteins correlated with replacement of Cys-110 or Cys-187. The lower levels of protein expression with these mutants may be due to an increase in degradation of an incorrectly folded protein. Glycosylation was also defective in the

mutants with replacement of Cys-110 or Cys-187. The processing of the high-mannose glycan did not take place, presumably due to the lack of transport of the proteins from endoplasmic reticulum to the Golgi. The importance of disulfide bonds in membrane proteins for glycosylation and cellular transport has been proposed. Thus, a deletion of four amino acid residues in the cysteine-rich extracellular domain of the low density lipoprotein receptor resulted in a mutant receptor that failed to be transported to Golgi from endoplasmic reticulum and was incompletely glycosylated (31).

Replacement of Cys-110 or Cys-187 also prevented binding of 11-*cis*-retinal to the rhodopsin mutants. A disulfide bond in the intradiscal domain may be critical in directing correct helix-helix interactions that form the 11-*cis*-retinal binding pocket. A qualitatively similar effect between cysteine mutagenesis and ligand binding was reported by Dixon *et al.* (32).

Cysteine residues corresponding to Cys-110, Cys-187, and Cys-316 in bovine rhodopsin are conserved in all vertebrate visual pigments and seven helix hormone receptors (12, 13). Thus, an essential disulfide bond may exist in these receptors as well. In  $\beta$ -adrenergic receptors, certain concentrations of dithiothreitol decreased agonist binding (33) and treatment with thiol compounds caused thermolability (34), implying a direct functional role for disulfide bond(s).

Our results indicate the presence of a specific disulfide linkage between Cys-110 and Cys-187 in bovine rhodopsin so that Cys-185 cannot substitute for Cys-187.

**The Role of "Nonessential" Cysteines in Rhodopsin.** The replacement of the four membrane-embedded cysteines (Cys-140, Cys-167, Cys-222, and Cys-264) had no significant effect on the function of the protein. Apparently, these cysteines do not directly interact with the chromophore nor do they seem to be involved in disulfide bond formation. This conclusion is consistent with the results of the previous chemical modification studies of ovine rhodopsin (35, 36). The replacement of three cytoplasmic cysteines also did not affect the properties of the protein. The presence of a vicinal disulfide bond between Cys-322 and Cys-323 has been proposed (37), whereas another study concluded that these two residues were palmitylated and served to anchor the carboxyl-terminal tail in the disc membrane (38). Site-specific mutagenesis cannot disprove the presence of a nonessential disulfide bond between Cys-322 and Cys-323, but the presence of palmityl groups essential for activity on these cysteines is improbable. The observation that the mutant protein CysIII, in which seven cysteines (Table 1) were replaced, activated transducin at only about 50% of wild-type level is not understood. Perhaps the removal of each one of the seven cysteines has a cumulative effect on the ability to activate transducin.

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