Structure and Function in Rhodopsin. Separation and Characterization of the Correctly Folded and Misfolded Opsins Produced on Expression of an Opsin Mutant Gene Containing Only the Native Intradiscal Cysteine Codons†

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ABSTRACT: Previous mutagenesis studies have indicated the requirement of a tertiary structure in the intradiscal region with a disulfide bond between Cys-110 and Cys-187 for the correct assembly and/or function of rhodopsin. We have now studied a rhodopsin mutant in which only the natural intradiscal cysteines at positions 110, 185, and 187 are present while all the remaining seven cysteines in the wild-type bovine rhodopsin have been replaced by serines. The proteins formed on expression of this mutant in COS-1 cells bind 11-*cis*-retinal only partially to form the rhodopsin chromophore. We show that this is due to the presence of both correctly folded chromophore-forming opsin and misfolded opsins. Methods have been devised for the separation of the correctly folded and misfolded forms by selective elution from immunoaffinity adsorbants. Using several criteria, which include SDS−PAGE as well as UV/visible and CD spectroscopy, we find that the correctly folded mutant protein is indistinguishable in its spectral properties from the wild-type rhodopsin. Further, reaction of sulfhydryl groups in the correctly folded mutant pigment with *N*-ethylmaleimide indicates that alkylation of a single sulfhydryl requires denaturation or illumination, while reaction with an additional two sulfhydryl groups occurs only after reduction. The misfolded mutant opsins are characterized by reduced α-helical content, sulfhydryl reactivity under native conditions in the dark, and also the presence of a disulfide bond. We conclude that the failure to bury Cys-185, or the formation of a disulfide between an alternative pair of cysteine residues, precludes establishment of the correct intradiscal structure required for the binding of retinal.

Rhodopsin, photoreceptor of the rod cell, is a seven-helical integral membrane protein containing three distinct regions, the intradiscal, the membrane-embedded, and the cytoplasmic (Figure 1). Clearly, each of these three regions has a unique role and is capable of undergoing a conformational change that is coupled to the signal transduction process (Khorana, 1992; Kaushal et al. 1994; Davidson et al., 1994). Although previous studies have allowed the conclusion that the formation of an intradiscal structure comprised of the NH_2-terminal region, all three polypeptide segments connecting helices B and C, D and E, and F and G, respectively, and a Cys-110/Cys-187 disulfide bond is required for correct assembly and/or function (Karnik et al., 1988; Doi et al., 1990; Karnik et al., 1990), many questions remain concerning its formation and the nature of the conformational changes which occur upon photoactivation.

We now report a further study on the role of the intradiscal region in which we have used a bovine rhodopsin mutant that has only the three intradiscal cysteines at positions 110, 185, and 187. We designate this mutant as the Cys-mutant. We find that the protein expressed by this mutant in COS-1 cells forms the normal rhodopsin chromophore with 11-*cis*-retinal, but in yields less than theoretically expected. This finding, together with similar previous experiences with a large number of site-directed and natural mutants (Doi et al., 1990; Sung et al., 1991, 1993; Kaushal & Khorana, 1994; Kaushal et al., 1994; Anukanth & Khorana, 1994), suggested that the expressed mutant proteins contain mixtures of correctly folded opsins that regenerate the rhodopsin chromophore with 11-*cis*-retinal and misfolded forms that do not bind retinal. We have devised methods for the separation of the folded and misfolded forms obtained on expression of the Cys-mutant and studied their properties using a number of criteria. The correctly folded Cys-mutant protein exhibits spectral and structural features virtually identical to those of wild-type rhodopsin. Chemical modification studies under a variety of conditions show that the correctly folded Cys-mutant is inert toward *N*-ethylmaleimide (NEM)[1] alkylation unless denatured or illuminated and, like the wild-type protein, contains a disulfide bond. The misfolded Cys-mutant protein, on the other hand, shows significantly less α-helical structure and reacts with NEM under native conditions in the dark. However, we also find that the misfolded Cys-mutant contains a disulfide bond. Therefore, these results suggest that exposure of Cys-185, or formation of a disulfide bond between cysteine residues other than Cys-

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[1] Abbreviations: PBS, phosphate-buffered saline; DM, *n*-dodecyl β-D-maltoside; vis, visible; NEM, *N*-ethylmaleimide; ROS, rod outer segment; ER, endoplasmic reticulum.

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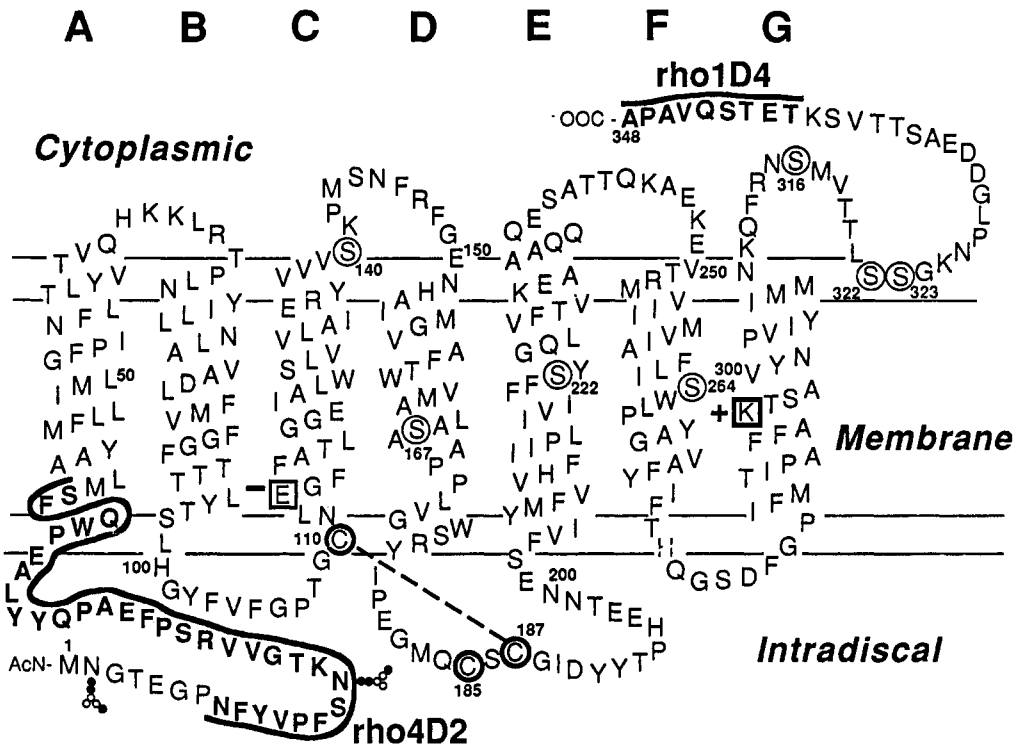


FIGURE 1: Secondary structure model of bovine rhodopsin showing the positions of the cysteines retained in the Cys-mutant and those replaced by serines. The seven helices are lettered A−G from left to right, and the membrane/solvent boundaries are shown approximately by the interrupted horizontal lines. The three intradiscal cysteines at positions 110, 185, and 187, retained in the Cys-mutant, are highlighted by boldface circles. The seven remaining cysteines in native rhodopsin were replaced by serine residues, their positions being circled. The dashed line between cysteines-110 and -187 shows the disulfide bond in the native rhodopsin. The presumed epitopes for the anti-rhodopsin monoclonal antibodies rho-1D4 and rho-4D2 are highlighted. Also shown in boxes are the attachment site of retinal (lysine-296) and the counterion for the protonated Schiff base (glutamate-113).

110 and Cys-187, interferes with correct assembly of the intradiscal structure.

MATERIALS AND METHODS

Materials. NEM was from Sigma, and tributylphosphine was from Fluka. *N*-[*ethyl*-2-^{3}H]ethylmaleimide (41.3−53.3 Ci/mmol) was from Dupont−New England Nuclear, and 11-*cis*-retinal was a gift of P. Sorter (Hoffmann-La Roche) and R. Crouch (Medical University of South Carolina and the National Eye Institute). The enhanced chemiluminescence detection system was from Amersham. Construction of the Cys-mutant opsin gene in the pMT3 expression vector has been described (Karnik et al., 1988). Sources of additional materials have been reported (Karnik et al., 1993).

Buffers. Phosphate-buffered saline (PBS) was 10 mM NaH_2PO_4, pH 7.0, containing 150 mM NaCl. Buffer A was 10 mM Tris-HCl, pH 7.0, containing 150 mM NaCl and 0.1% dodecyl maltoside (DM). Buffer B was 2 mM NaH_2PO_4, pH 6.0, containing 0.1% DM. Buffer C was 2 mM NaH_2PO_4, pH 6.0, containing 150 mM NaCl and 0.1% DM. During elution of the proteins, buffers A, B, and C contained 35 μM c′-octadecapeptide. Buffer D was 50 mM NaOAc, pH 3.0, containing 150 mM NaCl and 0.1% DM. Buffer E was 20 mM Tris-HCl, pH 8.0, containing 1 M NaCl, 2 mM ATP, 2 mM $MgCl_2$, and 0.1% DM. Buffer F was 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 2 mM EDTA, and 0.02% DM.

Antibodies. The rho 4D2 and rho 1D4 monoclonal antibodies, which are specific for the NH_2- and COOH-terminal sequences of rhodopsin (Figure 1), respectively, have been described (Molday & MacKenzie, 1983; Hicks & Molday, 1986). Antibodies against Hsp-72/73, GRP-78, Hsp-90, and GRP-94 were from Stress-Gen Biotechnologies Corp. (Victoria, B.C.).

Expression and Purification of Wild-Type and Cys-Mutant Rhodopsins. Procedures for the transient transfection of wild-type and mutant opsin genes in COS-1 cells have been reported (Oprian et al., 1987; Karnik et al., 1993). The transfected cells were harvested 52−56 h later, washed with 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 (Oprian et al., 1987). In some cases, the DM-solubilized extract was preincubated with 4D2-Sepharose prior to adsorption on 1D4-Sepharose. The 1D4-Sepharose was washed 5 times with 20 column volumes of buffer A (or buffer E when indicated) and then an additional 5 times with 20 column volumes of buffer A, B, or C. The bound rhodopsin was eluted in the appropriate buffer containing 35 μM c′-octadecapeptide. Rhodopsin from bovine rod outer segments (ROS) was purified by the same procedures.

Spectral Characterization of Wild-Type and Cys-Mutant Rhodopsins. UV/vis spectra were recorded at 20 °C with a Perkin-Elmer λ7 spectrophotometer. ϵ_{max} was determined as described (Sakmar et al., 1989; Bhattacharya et al., 1992). ϵ_{500} for the rhodopsin chromophore was 40 600 M^{-1} cm^{-1} (Wald & Brown, 1953) while that for the opsin apoprotein (ϵ_{280}) was 65 000 M^{-1} cm^{-1}. Chromophore stability toward hydroxylamine was measured in a 100 mM (pH 7.0) solution. Pigments were illuminated for 10 s at 20 °C with a 150-W light source through a >495-nm long-pass filter. CD spectra were recorded at 15 °C with an Aviv 60DS spectropolarim-

eter. A cell path length of 0.1 cm was used. Each spectrum was the average of five replicate scans in steps of 1 nm with a 1.0 s averaging time and was corrected by subtraction of the solvent spectrum obtained under identical conditions. All measurements are reported in degrees of centimeter squared per decimole. The ellipticity at 222 nm was used to estimate the α-helical content as follows: $[\theta]_{222} = (30\,300)f\alpha - 2340$ where $[\theta]_{222}$ is the mean residue ellipticity at 222 nm and $f\alpha$ is the fractional α-helical content (Chen et al., 1972).

SDS−PAGE Analysis of ROS, Wild-Type, and Cys-Mutant Rhodopsins. Protein samples were analyzed by nonreducing SDS−PAGE (Laemmli, 1970) with a 5% stacking and a 10 or 12% resolving gel and visualized by silver staining (Wray et al., 1981) or electroblotted onto nitrocellulose (Burnette, 1981). Immunoreactive protein was detected using the above-mentioned primary antibodies and goat anti-mouse horseradish peroxidase as the second antibody. The protein bands were visualized by chemiluminescence.

N-Ethylmaleimide Alkylation of ROS, Wild-Type, and Cys-Mutant Rhodopsins. [^{3}H]-*N*-Ethylmaleimide ([^{3}H]NEM) was extracted into 50 mM Hepes, pH 7.6, and the concentration adjusted to 6 mM by addition of unlabeled NEM (specific activity, 1.25−1.50 Ci/mmol). Alkylation of samples with [^{3}H]NEM was done using two different procedures. In the first procedure, the pigments were alkylated with a 100-fold excess of [^{3}H]NEM while bound to 1D4-Sepharose (Resek et al., 1993). Prior to alkylation, the 1D4-Sepharose-adsorbed proteins were washed with 20 column volumes of buffer F. After a 16 h reaction in the dark at 20 °C, the resin was extensively washed to remove unreacted [^{3}H]NEM, and the bound rhodopsin was eluted in buffer A, B, or C containing the octadecapeptide. The extent of alkylation was determined by taking a UV/vis spectrum of the sample and counting the ^{3}H radioactivity in an aliquot. In the second procedure, the 1D4-purified pigments were incubated in the dark or after illumination with an 80-fold excess of [^{3}H]-NEM in the absence or presence of SDS (1%, w/v). For reduction with tributylphosphine, the procedure used was similar to that of Finn et al. (1991). The purified samples were preincubated for 30 min in the dark with a 10-fold excess of tributylphosphine (above the expected half-cystines) in the presence of SDS prior to addition of [^{3}H]-NEM. After 15 min or 16 h in the dark at 20 °C, the alkylation reactions were quenched by addition of an 8000-fold excess of unlabeled NEM. To quantitate [^{3}H]NEM incorporation, samples were electrophoresed on 15% *N,N'*-diallyltartardiamide-cross-linked SDS gels. After visualization of the opsin bands with Coomassie blue and extensive washing to remove unincorporated [^{3}H]NEM, the protein was excised from the gel, dissolved in 25 mM periodate, and analyzed by scintillation counting. A sample of [^{3}H]NEM-alkylated wild-type rhodopsin (alkylated on 1D4-Sepharose) was used as a standard to estimate recovery of radioactivity from the gel. Samples were also analyzed by nonreducing SDS−PAGE and fluorography.

RESULTS

Expression of Cys-Mutant in COS-1 Cells. The Cys-mutant was expressed in COS cells, and after treatment with 11-*cis*-retinal, the protein was isolated by the standard method (procedure I, buffer A) (Karnik et al., 1988; Oprian et al., 1987). Reduced levels (40−70%) of 11-*cis*-retinal

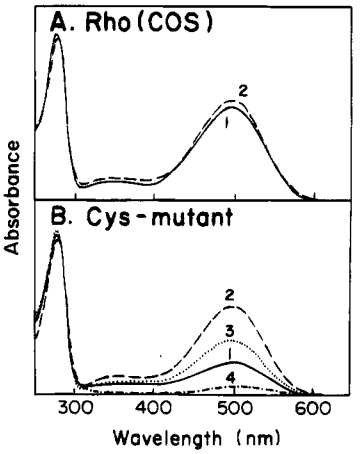
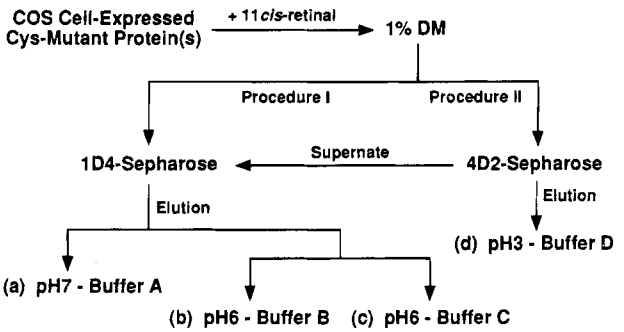


FIGURE 2: UV/vis absorption spectra of wild-type and Cys-mutant rhodopsins purified under different conditions. (A) Wild-type rhodopsin from COS cells [Rho (COS)] prepared according to procedure I and eluted with buffer A (—) or buffer B (— —). (B) Cys-mutant rhodopsin prepared according to procedure I and eluted with buffer A (—), buffer B (— —), or buffer C (-·-). Alternatively, Cys-mutant rhodopsin was prepared according to procedure II and eluted with buffer A (···). All spectra were normalized at A_{280}. The absolute A_{280} values were as follows: Rho (COS)-1, 0.07; Rho (COS)-2, 0.06; Cys-mutant-1, 0.075; Cys-mutant-2, 0.025; Cys-mutant-3, 0.06; Cys-mutant-4, 0.03. See Scheme 1 and Materials and Methods for details.

Scheme 1: Alternative Procedures Used for Separation of Protein(s) Expressed by the Cys-Mutant



binding were observed by UV/vis spectra after elution from the immunoaffinity matrix (Figure 2B, spectrum 1). This suggested the presence of Cys-mutant protein(s) which failed to bind 11-*cis*-retinal.

Separation of the Cys-Mutant Proteins into Rhodopsin and Non-Retinal Binding Opsin. In Scheme 1 are shown the alternative procedures investigated for the separation of the expressed proteins. Wild-type COS cell rhodopsin, purified by procedure I with buffer A or B, showed no significant difference in the absorption spectrum or the amount of folded pigment (Figure 2A, spectra 1 and 2). As indicated above, purification of the Cys-mutant by procedure I with buffer A gave a mixture of proteins. SDS−PAGE analysis of this preparation showed the presence of a heterogeneously glycosylated 41 kDa protein (Figure 3, lane 3) and three opsins of higher mobility (molecular masses 36, 33, and 30 kDa). The relative abundance of the latter opsins appears to be lower than the 41 kDa protein which is similar to that of COS cell-expressed wild-type opsin (Figure 3, lane 2). The 36 kDa opsin contains high-mannose oligosaccharide chains as it collapses to a 33 kDa nonglycosylated opsin upon endoglycosidase H treatment (data not shown). The 30 kDa opsin may represent a conformer of the 33 kDa opsin. These

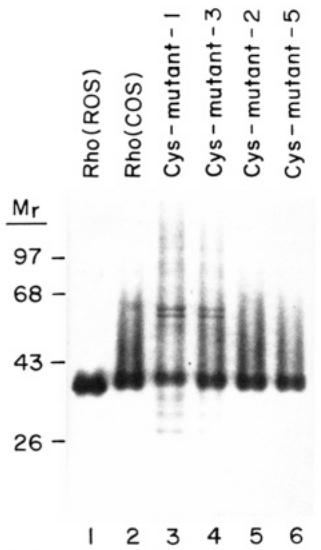


FIGURE 3: SDS−PAGE analysis of ROS, wild-type, and Cys-mutant rhodopsins purified under different conditions. Lanes 1−3, ROS [Rho(ROS)], wild-type [Rho(COS)], and Cys-mutant rhodopsins, prepared according to procedure I and eluted with buffer A, respectively; lane 4, Cys-mutant rhodopsin prepared according to procedure II and eluted with buffer A; lane 5, Cys-mutant rhodopsin prepared according to procedure I and eluted with buffer B; lane 6, Cys-mutant rhodopsin prepared according to procedure II and eluted from 4D2-Sepharose with buffer D. Equivalent amounts of protein (1 μg) were subjected to SDS−PAGE and visualized by silver staining. Positions of molecular size standards are shown at the left in kilodaltons. See Scheme 1 and Materials and Methods for details.

faster migrating opsins are similar to those previously observed in a number of site-directed and natural mutant preparations (Karnik et al., 1988; Doi et al., 1990; Karnik & Khorana, 1990; Kaushal & Khorana, 1994; Kaushal et al., 1994; Anukanth & Khorana, 1994). In procedure II, the DM-solubilized proteins were first passed through 4D2-Sepharose, and the unadsorbed protein was then purified by procedure I with buffer A. While a significant improvement in the absorbance ratio was observed (Figure 2B, spectrum 3), SDS−PAGE analysis revealed the band pattern to be similar to that obtained using procedure I directly (Figure 3, lane 4). Elution of the protein adsorbed to the 4D2-Sepharose resin with buffer D followed by UV/vis and SDS−PAGE analysis (Figure 3, lane 6) showed only the 41 kDa opsin which lacked a chromophore.

The pH and ionic strength of the buffer used for elution from 1D4-Sepharose proved to be critical for the nature and the recovery of the retinal binding pigment. Elution at pH 6 in low salt (buffer B) gave a dramatic improvement in the UV/vis absorbance ratio (Figure 2B, spectrum 2), and SDS−PAGE analysis of this preparation showed only the 41 kDa protein (Figure 3, lane 5). On the basis of its ϵ_{max} (37 600 M^{-1} cm^{-1}), the eluted pigment consisted only of the regenerated rhodopsin chromophore. Subsequent elution of the 1D4-Sepharose at pH 6 with high salt (buffer C) gave predominantly Cys-mutant opsins which did not form a chromophore with 11-*cis*-retinal (Figure 2B, spectrum 4). In the following, the Cys-mutant protein which forms the chromophore is referred to as Cys-mutant-2, while that which does not bind retinal is referred to as Cys-mutant-4.

Non-Opsin Proteins in the Cys-Mutant Preparations. Purification of the Cys-mutant by procedures I and II with buffer A showed the presence of additional proteins following SDS−PAGE. Two distinct proteins of 58−60 kDa, as well as other faint bands migrating at 70−80 and 90−100 kDa, were evident (Figure 3, lanes 3 and 4). Notably, these proteins were absent from preparations of wild-type and Cys-mutant-2 rhodopsin (Figure 3, lanes 2 and 5). Immunoblot analysis identified some of these proteins as cytosolic and endoplasmic reticulum (ER) resident members of the stress-70 and stress-90 families of chaperones (data not shown). Although some of these proteins could effectively be removed by addition of Mg/ATP to buffer A, presumably because of their intrinsic ATPase activity, complete removal of these non-opsin proteins also required the presence of high concentrations of salt in the column wash (buffer E).

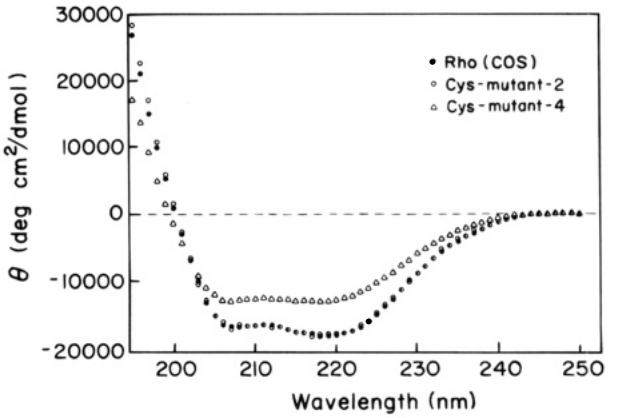


FIGURE 4: CD spectra of wild-type and Cys-mutant proteins purified under different conditions. Wild-type [Rho (COS)] (●) and Cys-mutant-2 (○) rhodopsins were prepared according to procedure I (washed with buffer E) and eluted with buffer B. Cys-mutant-4 (△) was prepared according to procedure I (washed with buffer E) and eluted with buffer C. The CD spectra were obtained as described under Materials and Methods.

Characterization of the Folded and Misfolded Cys-Mutant Proteins. (a) Stability to Hydroxylamine and Metarhodopsin II Formation in Wild-Type and Cys-Mutant-2 Rhodopsins. The sensitivity of the retinyl-Schiff base linkage in the Cys-mutant-2 and wild-type rhodopsins to hydroxylamine was compared. Both rhodopsins were stable in the dark but formed the 360-nm-absorbing retinaloxime upon illumination (>495 nm) for 10 s (data not shown). The bleaching behavior of the above pigments was also compared. Upon illumination, both pigments yielded a 380-nm species characteristic of metarhodopsin II (MII). Acidification of the illuminated samples caused the formation of the 440-nm-absorbing protonated retinyl-Schiff base (data not shown).

(b) CD Measurements of Wild-Type Rhodopsin and the Separated Cys-Mutant Proteins. Far-UV CD spectra of the wild-type, Cys-mutant-2, and Cys-mutant-4 proteins are shown in Figure 4. The CD spectra of the wild-type and Cys-mutant-2 rhodopsins were virtually superimposable, and both exhibited strong negative ellipticities at 222 and 208 nm, indicative of substantial α-helical structure. In contrast, the Cys-mutant-4 protein showed a very different spectral shape and a 30% decrease in molar ellipticity at 222 nm ($[\theta]_{222}$) relative to the wild-type and Cys-mutant-2 rhodopsins.

(c) Sulfhydryl Groups in Wild-Type Rhodopsin and the Separated Cys-Mutant Proteins. (i) Nondenaturing Conditions. The wild-type and Cys-mutant proteins were first tested in the dark at room temperature for reaction with [^{3}H]-NEM when bound to 1D4-Sepharose. Subsequent elutions were according to the procedures in Scheme 1. Previous

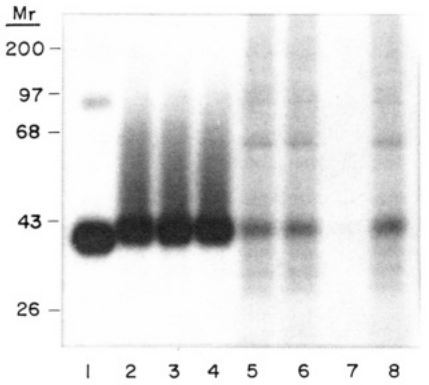


FIGURE 5: Fluorograph of ROS, wild-type, and Cys-mutant rhodopsins alkylated with [^{3}H]NEM in the dark while bound to 1D4-Sepharose. Lane 1, ROS rhodopsin prepared according to procedure I and eluted with buffer A; lanes 2−4, wild-type COS cell rhodopsin prepared according to procedure I and eluted with buffer A, buffer B, or buffer C, respectively; lane 5, Cys-mutant rhodopsin prepared according to procedure I and eluted with buffer A; lane 6, Cys-mutant rhodopsin prepared according to procedure II and eluted in buffer A; lanes 7 and 8, Cys-mutant rhodopsin prepared according to procedure I and eluted in buffer B or buffer C, respectively. Equivalent amounts of protein (0.5 μg) were subjected to SDS−PAGE and visualized by fluorography. Positions of molecular size standards are shown at the left in kilodaltons. See Scheme 1 and Materials and Methods for details.

Table 1: Incorporation of [^{3}H]-*N*-Ethylmaleimide into Wild-Type and Cys-Mutant Rhodopsins Purified under Different Conditions (Scheme 1)

	NEM, mol/mol of protein[b,c]	
procedure[a]	wild-type	Cys-mutant
I, buffer A	2.20 ± 0.20 (3)	0.20 ± 0.02 (2)
II, buffer A	1.85 (1)	0.19 ± 0.03 (2)
I, buffer B	2.14 ± 0.10 (3)	0.09 ± 0.05 (3)
I, buffer C	2.42 ± 0.30 (3)	0.30 ± 0.07 (3)

[a] The wild-type and Cys-mutant proteins were bound to 1D4-Sepharose, washed with buffer A, alkylated with [^{3}H]NEM, and eluted from the resin as indicated. [b] Based on A_{280} using a molar extinction of 65 000 M^{-1} cm^{-1}. [c] Experimental errors are shown after ± in each result. Numbers in parentheses correspond to the number of independent determinations.

studies have shown that under nondenaturing reaction conditions, only two of rhodopsins cysteines, Cys-140 and Cys-316, react with NEM (DeGrip et al., 1975; DeGrip & Daemen, 1982; Findlay et al., 1984). Alkylation of both ROS and wild-type COS cell rhodopsin with [^{3}H]NEM and purification by either process confirmed this finding (Figure 5, lanes 1−4, and Table 1). In agreement with the previously demonstrated inertness of Cys-185 in ROS rhodopsin, Cys-mutant-2 did not react with [^{3}H]NEM under these conditions (Figure 5, lane 7, and Table 1). However, since the different purification procedures (Scheme 1) gave varying mixtures of retinal binding and non-retinal binding Cys-mutant proteins, [^{3}H]NEM alkylation was observed in some cases and was apparent in all four opsin species (Figure 5, lanes 5, 6, and 8). Despite the different amounts of Cys-mutant-4 in the above preparations, the stoichiometry of alkylation was similar (Table 1). Further, as described above for Figure 3, these Cys-mutant preparations also contained several non-opsin proteins, some of which contain sulfhydryl groups that are alkylated by [^{3}H]NEM. Performing these alkylation reactions after removing the non-opsin proteins with buffer E and eluting the Cys-mutant proteins from 1D4-Sepharose with buffer B or C gave similar results (Table 2). These findings show that in contrast to Cys-mutant-2, a sulfhydryl group in the Cys-mutant-4 protein is accessible to NEM alkylation.

Table 2: Incorporation of [^{3}H]-*N*-Ethylmaleimide into Wild-Type and Cys-Mutant Rhodopsins Purified and Alkylated under Different Conditions

		NEM, mol/mol of protein[b,c]	
sample[a]	dark/ nondenaturing	denaturing	reducing/ denaturing
wild-type	1.70 ± 0.10 (3)	5.80 ± 0.30 (3)	7.53 ± 0.20 (3)
Cys-mutant-2	0.13 ± 0.10 (2)	0.80 ± 0.10 (2)	2.89 ± 0.10 (2)
Cys-mutant-4	0.45 ± 0.10 (2)	0.61 ± 0.10 (2)	2.59 ± 0.10 (2)

[a] The wild-type and Cys-mutant proteins were bound to 1D4-Sepharose, washed with buffer E, eluted from the resin with buffer B or buffer C (Scheme 1), and alkylated as described under Materials and Methods. [b] Based on A_{280} using a molar extinction of 65 000 M^{-1} cm^{-1}. [c] Experimental errors are shown after ± in each result. Numbers in parentheses correspond to the number of independent determinations.

(ii) Denaturing Conditions. Reactivity toward [^{3}H]NEM was next examined in the presence of SDS. The wild-type protein showed incorporation of 6 mol of [^{3}H]NEM/mol of rho (Table 2). Thus, as previously reported (DeGrip et al., 1975; DeGrip & Daemen, 1982), denaturation by SDS rendered four additional sulfhydryls in rhodopsin accessible to NEM alkylation. In the presence of SDS, Cys-mutant-2 also showed alkylation (0.8 mol of [^{3}H]NEM/mol of rho), presumably from modification of Cys-185. However, the addition of SDS to the Cys-mutant-4 showed only a slight increase in the incorporation of [^{3}H]NEM (Table 2).

(iii) Upon Illumination. Since previous work from this laboratory (Kaushal et al., 1994; Davidson et al., 1994) has shown that the intradiscal region of rhodopsin plays an active role in the signal transduction process, we examined whether Cys-mutant-2 could be alkylated after photoactivation. As is shown in Figure 6, brief exposure of Cys-mutant-2 to light (>495 nm) resulted in [^{3}H]NEM alkylation. The stoichiometry of [^{3}H]NEM incorporation was 0.6 ± 0.1 mol of [^{3}H]-NEM/mol of rho after a 15 min reaction, a time range which corresponds to the spectrophotometric half-life of MII in the Cys-mutant (S. Karnik and H. G. Khorana, unpublished observations).

(d) Disulfide Bonds in Wild-Type Rhodopsin and the Separated Cys-Mutant Proteins. The alkylation reactions were carried out after reduction of the putative disulfide bonds in the pigments and opsins in the presence of SDS. The wild-type rhodopsin showed an additional incorporation of 2 mol of [^{3}H]NEM/mol of rho (Table 2), as expected for a single disulfide bond in the folded rhodopsin (Karnik & Khorana, 1990). Reduction of the SDS-denatured Cys-mutant-2 and Cys-mutant-4 also resulted in an additional 2 mol of NEM incorporation (Table 2), consistent with the presence of a disulfide bond in these mutant proteins.

DISCUSSION

A significant accomplishment of the present work has been the separation of different forms of opsin produced on expression of a mutant gene which retained only the three intradiscal cysteines present in the native receptor molecule. We concluded previously that the proteins expressed by the present mutant and by a large number of other mutants contained mixtures of retinal binding and non-retinal binding opsins (Doi et al., 1990; Karnik et al., 1988). This was

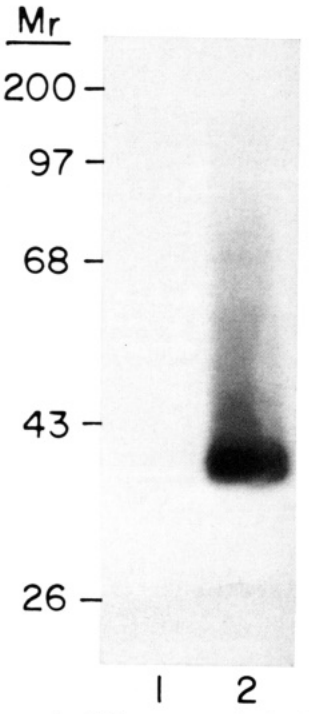


FIGURE 6: Fluorograph of Cys-mutant rhodopsin alkylated with [^{3}H]NEM in the dark and after illumination. Cys-mutant-2 rhodopsin was prepared according to procedure I (washed with buffer E) and eluted with buffer B. The pigment was alkylated with [^{3}H]-NEM in the dark (lane 1) or after 10 s of illumination (>495 nm) (lane 2) for 15 min. Equivalent amounts of protein (1 μg) were subjected to SDS−PAGE and visualized by fluorography. Positions of molecular size standards are shown at the left in kilodaltons. See Scheme 1 and Materials and Methods for details.

indicated by the absorbance ratios (A_{280}/A_{500}) for the mutant proteins that were much higher than 1.6, a value characteristic of pure rhodopsin (Hargrave, 1982). By this criterion, the retinal binding opsins constituted 20−70% of the total expressed proteins. The separation method now described has been applied with uniform success in the preparation of pure 500-nm-absorbing rhodopsin pigments from mixtures of expression products obtained from a variety of mutant opsin genes (Resek et al., 1993, 1994; Kaushal & Khorana, 1994; Kaushal et al., 1994; Davidson et al., 1994; Anukanth & Khorana, 1994).

The Cys-mutant-2 pigment (Figure 2B) has properties characteristic of the wild-type rhodopsin. First, it contains only one prominent band on SDS−PAGE with an apparent molecular mass of 41 kDa and has a glycosylation pattern that is similar to the wild-type (Figure 3). Second, the 500-nm chromophore of Cys-mutant-2 has a molar extinction similar to that of the wild-type rhodopsin (37 600 vs 40 600 M^{-1} cm^{-1}, respectively). The slight difference could be due to the substitution of one or more of the membrane-embedded cysteine residues. Third, Cys-mutant-2 is stable to hydroxylamine in the dark, indicating that the retinyl-Schiff base, like that in the wild-type rhodopsin, is not accessible to hydrolytic attack. Fourth, formation and decay of the 380-nm MII intermediate obtained upon illumination are similar in both pigments. Fifth, the α-helical content of Cys-mutant-2 is virtually identical to that of wild-type rhodopsin (Figure 4). Sixth, and perhaps the most cogent argument for correct folding in Cys-mutant-2, is that, as in wild-type rhodopsin, Cys-185 is silent toward [^{3}H]NEM alkylation in the dark (Figure 5; Tables 1 and 2). In both cases, SDS denaturation is required for this residue to be accessible for alkylation (Table 2). Similarly, reduction of a disulfide bond with tributylphosphine occurs under denaturing conditions (Table 2). Finally, upon illumination, Cys-mutant-2 undergoes a conformational change which results in [^{3}H]NEM alkylation (Figure 6). This is clearly the result of a conformational change in the intradiscal region and is consistent with earlier observations that one or more cysteine residues become exposed upon illumination of rhodopsin (Wald & Brown, 1952; DeGrip et al., 1975; McDowell & Williams, 1976; Regan et al., 1978; Chen & Hubbell, 1978; McDowell et al., 1979; Khatami et al., 1981; DeGrip & Daemen, 1982; Rath et al., 1994). Thus, the total evidence leaves little doubt that the Cys-mutant-2 rhodopsin is indistinguishable from the wild-type in its folded structure.

Preparations of Cys-mutant-4 contain a 41 kDa opsin species and three opsins of higher mobility (36, 33, and 30 kDa) (Figure 5). The 41 kDa opsin most likely represents Cys-mutant-5 (Figure 3), which was adsorbed to 4D2-Sepharose (procedure II) and eluted with buffer D. This opsin has a glycosylation pattern characteristic of the mature opsin, suggesting that it exited the ER and was duly processed to the complex carbohydrate form in the Golgi. Since the rho-4D2 antibody preferentially binds photobleached or denatured opsin (Hicks & Molday, 1986), we infer that some subtle defect in the packing of the helices has rendered it incapable of binding retinal. The faster migrating opsins in the Cys-mutant-4 preparation have glycosylation patterns characteristic of ER-retained proteins. As previous work has demonstrated that glycosylation is not required for the correct folding of opsin (Kaushal et al., 1994), the above results suggest the presentation of a structurally altered apoprotein to the processing machinery. These findings are consistent with the CD data in Figure 4, which show that Cys-mutant-4 has considerably less α-helical content than the correctly folded Cys-mutant-2 and wild-type rhodopsins. This difference is not due to the absence of the retinal chromophore *per se*, as wild-type opsin shows essentially the same amount of α-helical content as the regenerated pigment (X.L. and H.G.K., unpublished observations).

The Cys-mutant-4 opsins, like the correctly folded Cys-mutant-2 and wild-type rhodopsins, also contain a disulfide bond (Table 2). However, in contrast to the Cys-mutant-2, the Cys-mutant-4 opsins incorporate [^{3}H]NEM under non-denaturing conditions and the reactivity shows only a modest increase upon denaturation (Table 2). Thus, these results raise the possibility that (i) Cys-185 is accessible for alkylation in the Cys-mutant-4 opsins or (ii) a disulfide is formed between an alternative pair of cysteine residues, such as Cys-110 and Cys-185 and/or Cys-185 and Cys-187. Finally, we draw attention to the fact that the preparations containing Cys-mutant 4 contained several non-opsin proteins (Figures 3 and 5), some of which have been identified as stress-70 and stress-90 chaperones. These non-opsin proteins have been observed in other mutant rhodopsin preparations (Anukanth & Khorana, 1994). For soluble and secreted proteins, direct evidence for the association between stress proteins and misfolded polypeptides has been widely documented (Gething & Sambrook, 1992). Thus, as anticipated, preparations of wild-type and Cys-mutant-2 rhodopsin did not contain these stress proteins (Figures 3 and 5).

Taken together, the present findings suggest that misfolding in the Cys-mutant protein is characterized by gross structural defects which preclude proper glycosylation, correct assembly and/or packing of the helices, and, possibly, formation of the Cys-110/Cys-187 disulfide bond. In addition, these structural alterations may also expose regions of the opsin that are normally buried, possibly resulting in the observed higher affinity for the immobilized rho 1D4 antibody under conditions of low pH and ionic strength.

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