# Mapping of the Amino Acids in the Cytoplasmic Loop Connecting Helices C and D in Rhodopsin. Chemical Reactivity in the Dark State Following Single Cysteine Replacements†

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ABSTRACT: The cytoplasmic loop connecting helices C and D in rhodopsin is a part of the region involved in protein−protein interactions during signal transduction. To probe the structure of the CD loop, we have replaced, one at a time, the amino acids 136−150 by cysteine residues. The cysteine substitution mutants contained only the introduced single reactive cysteines and were prepared from a base opsin mutant that retained only the three intradiscal cysteines. All of the cysteine substitution mutants formed the characteristic rhodopsin chromophore ($\lambda_{max}$, 500 nm) with 11-*cis*-retinal. They showed normal photobleaching characteristics and activated transducin in a light-dependent manner, albeit at lower levels than the wild-type pigment. The newly introduced cysteines in the substitution mutants all underwent alkylation in the dark with the membrane-permeant sulfhydryl reagent *N*-ethylmaleimide, but with varying rates. The cysteine substitution mutants also showed prominent differences in alkylation with membrane-impermeant *N*-polymethylenecarboxylmaleimides of various alkyl chain lengths. Notably, derivatization of the cysteines in the mutants was not observed with the polar sulfhydryl reagents iodoacetic acid or iodoacetamide. These findings highlight intrinsic differences in both the reactivity and accessibility of the different cysteine residues in the CD loop and support the important role for a structure in the second cytoplasmic region of rhodopsin.

Rhodopsin, the photoreceptor of the vertebrate rod cell, is assumed to undergo a conformational change upon light activation. This allows the binding of transducin ($G_T$)[1] on the cytoplasmic face, the first step in the ensuing biochemical cascade (Kühn & Hargrave, 1981; Bennet *et al.*, 1982). The latter culminates in the closing of the cation conductance channels in the plasma membrane, causing hyperpolarization of the rod cell (Fesenko *et al.*, 1985). The nature of the structural changes in rhodopsin occurring upon light activation are essentially unknown. Therefore, we are interested in developing approaches to the study of these presumed structural changes. One potentially useful approach is to introduce single cysteine residues by replacement of the naturally occurring amino acids at predetermined positions in the different domains of rhodopsin. The sulfhydryl groups in the cysteine residues then provide a handle for chemical and biophysical investigations. Previous studies along this line with bacteriorhodopsin proved to be very rewarding (Flitsch & Khorana, 1989; Altenbach *et al.*, 1989, 1990; Greenhalgh *et al.*, 1991; Steinhoff *et al.*, 1994).

The importance of the cytoplasmic loops (Figure 1) for the rhodopsin−$G_T$ interaction has been highlighted by a number of studies (Weiss *et al.*, 1988; Konig *et al.*, 1989; Sakmar *et al.*, 1989; Franke *et al.*, 1990, 1992; Resek *et al.*, 1994). In starting our investigation of the cytoplasmic surface of rhodopsin, we chose to work first with the loop connecting helices C and D (CD loop, amino acids 136−150). We have introduced cysteine residues, one at a time, by substituting everyone of the amino acids in this loop (Figure 1). The cysteine substitution mutants were constructed from a base mutant, designated Cys-mutant, in which Cys-140, -167, -222, -264, -316, -322, and -323 were replaced by serine (Karnik *et al.*, 1988). After expression in COS-1 cells, all of the mutants carrying cysteine substitutions folded to bind 11-*cis*-retinal, forming the characteristic 500-nm absorbing chromophore. We then systematically studied the reactivity/accessibility of the sulfhydryl groups in the introduced cysteines in the dark state by comparing their behavior toward membrane-permeant as well as polar and membrane-impermeant sulfhydryl reagents. All of the mutants reacted with *N*-ethylmaleimide (NEM) but showed differences in the rates of reaction. Differences in the accessibility of some sulfhydryl groups to carboxyalkyl maleimide derivatives (Griffiths *et al.*, 1981; Figure 2) were also evident. Further, derivatization of the sulfhydryl groups in these cysteines was not observed with iodoacetic acid or iodoacetamide. The results suggest an ordered, sterically restricted environment for the amino acids of the CD loop. In the accompanying paper (Farahbakhsh *et al.*, 1995), an electron paramagnetic resonance (EPR) study of the light-dependent structural changes in these amino acids following nitroxide spin-labeling of these sulfhydryl groups is reported.

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[1] Abbreviations: $G_T$, transducin; NEM, *N*-ethylmaleimide; DM, *n*-dodecyl-β-D-maltoside; MII, metarhodopsin II; ROS, rod outer segment; EPR, electron paramagnetic resonance.

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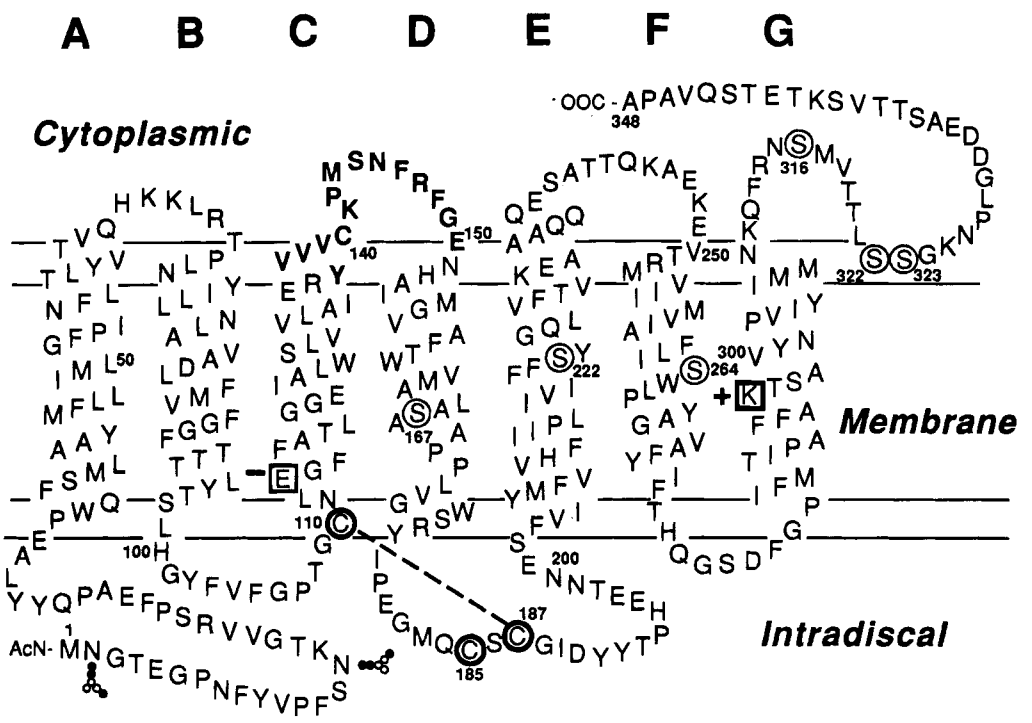


FIGURE 1: Secondary structure model of bovine rhodopsin. Endogenous cysteine residues at positions 167, 222, 264, 316, 322, and 323 have been replaced with serine while those at positions 110, 185, and 187 have been retained (circled residues). Single consecutive cysteine residues have been introduced at positions 136−139 and 141−150 in the second interhelical (C−D) polypeptide segment (bold lettering). The attachment site of retinal, Lys-296, and the counterion of the protonated Schiff base, Glu-113, are indicated with a (+) and (−), respectively. The membrane-embedded seven helical segments (A−G) are bordered approximately by the interrupted horizontal lines.

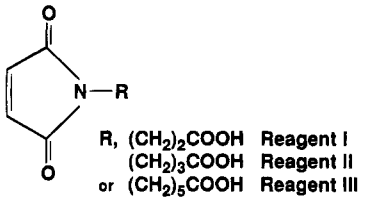


FIGURE 2: Structures of *N*-polymethylenecarboxymaleimide alkylating reagents.

## EXPERIMENTAL PROCEDURES

### *Materials*

*n*-Dodecyl β-D-maltoside (DM) was from Anatrace (Maumee, OH). *N*-Ethylmaleimide was from Sigma while maleimido-*N*-propionic acid (reagent I), maleimido-*N*-butanoic acid (reagent II), and maleimido-*N*-hexanoic acid (reagent III) (Figure 2) were from Fluka. Thermolysin was from Calbiochem. Sequenase (version 2.0) was from United States Biochemical, and the enhanced chemiluminescence detection system was from Amersham. Nitrocellulose filters (HAWP 02500) were from Millipore, and horseradish peroxidase-conjugated goat anti-mouse IgG was from Promega. Restriction endonucleases were from Boehringer Mannheim and New England Biolabs. 11-*cis*-Retinal was a gift of Dr. R. Crouch (Medical University of South Carolina and the National Eye Institute). Guanosine 5′-γ-[$^{35}$S]thiotriphosphate (1000 Ci/mmol), deoxyadenosine 5′-α-[$^{35}$S]-thiotriphosphate (500 Ci/mmol), *N*-[*ethyl*-2-$^{3}$H]ethylmaleimide, approximately 50 Ci/mmol, [1-$^{14}$C]iodoacetamide (25 mCi/mmol), and [$^{3}$H]iodoacetic acid (250 mCi/mmol) were from DuPont−New England Nuclear.

### *Methods*

*Construction of Mutant Opsin Genes.* The synthetic wild-type bovine opsin gene (Ferretti *et al.*, 1986) and the expression vector pMT4 containing this gene (Franke *et al.*, 1988) have been described. A mutant of the wild-type gene, which contains only the intradiscal cysteines (110, 185, 187) with the other seven cysteines having been replaced by serines, has also been described (Karnik *et al.*, 1988). This is designated the Cys-mutant. A derivative of this mutant in which the native Cys-140 is also retained was constructed by replacement of the *EcoR*I−*Aha*II restriction fragment with the corresponding fragment from a wild-type opsin gene modified to contain the *Rsr*II and *Spe*I restriction sites (Sakmar *et al.*, 1989). All the mutants carrying single cysteine substitutions at positions 136−139 and 141−150 (Figure 1) were derived from the latter mutant. These were constructed by replacing the *Spe*I−*Aha*II restriction fragment with two synthetic oligonucleotide duplexes containing the desired cysteine codon substitutions and a C140S substitution. All oligonucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer, purified by denaturing polyacrylamide electrophoresis, and characterized by 5′-end analysis (Ferretti *et al.*, 1986). The mutations were confirmed by dideoxynucleotide sequencing of CsCl-purified plasmid DNA (Sanger *et al.*, 1977).

*Expression and Purification of Rhodopsin Mutants.* The opsin genes were transiently expressed in COS-1 cells using the DEAE-dextran procedure as described (Oprian *et al.*, 1987; Karnik *et al.*, 1993). At 56−60 h posttransfection, the cells were harvested and washed with 10 mM $NaH_2PO_4$ (pH 7.0) containing 150 mM NaCl, and the rhodopsin chromophore was generated by addition of 5 μM 11-*cis*-retinal to the cell suspension in the dark. After 3 h at 4 °C,

the cells were solubilized in 1% dodecyl maltoside (DM) and the mutant pigments purified by immunoaffinity chromatography on 1D4-Sepharose (Oprian *et al.*, 1987). The resin was washed five times with 20 column volumes of (i) 20 mM Tris-HCl, pH 8.0, containing 2 mM ATP, 2 mM $MgCl_2$, 1 M NaCl, and 0.1% DM followed by (ii) 2 mM $NaH_2PO_4$, pH 6.0, containing 0.1% DM. The bound rhodopsin was eluted with 35 $\mu$M c'1-18 peptide in 2 mM $NaH_2PO_4$, pH 6.0, containing 0.1% DM or 10 mM Tris-HCl, pH 7.0, containing 150 mM NaCl and 0.1% DM.

*Spectral Characterization of the Rhodopsin Mutants.* Spectroscopic measurements were recorded with a Perkin-Elmer $\lambda$7 UV/visible (vis) spectrophotometer. Molar extinction coefficients were determined as described (Sakmar *et al.*, 1989; Bhattacharya *et al.*, 1992). The molar extinction coefficient of the wild-type rhodopsin chromophore was assumed to be 40 600 $M^{-1}$ $cm^{-1}$ (Wald & Brown, 1953). Pigments were illuminated for 10 s at 20 °C with a 150-W light source through a >495 nm long-pass filter.

*$G_T$ Activation by Rhodopsin Mutants.* $G_T$ was isolated from bovine rod outer segments (ROS) as previously described (Fung *et al.*, 1981). The ability of wild-type and mutant rhodopsins to activate $G_T$ was measured using the GTP−GDP exchange assay (Wessling-Resnick & Johnson, 1987). Briefly, the assay mixture consisted of 1 nM rhodopsin, 4 $\mu$M $G_T$, and 10 $\mu$M guanosine 5'-$\gamma$-[$^{35}$S]thiotriphosphate in 10 mM Tris-HCl, pH 7.5/100 mM NaCl/5 mM $MgCl_2$/2 mM dithiothreitol/0.012% DM. The assay mixture was illuminated (>495 nm) for 1 min at 20 °C and then allowed to remain in darkness for an additional 2 h. The extent of the exchange reaction was measured as described (Karnik *et al.*, 1993).

*Derivatization of Rhodopsin Mutants with Sulfhydryl Reagents.* [$^3$H]*N*-Ethylmaleimide ([$^3$H]NEM), as supplied by the manufacturer, was extracted into 50 mM Hepes, pH 7.6. The radioactive reagent was made up to 6 mM by the addition of unlabeled NEM (specific activity, 1.5 Ci/mmol). Similarly, the concentration of [$^3$H]iodoacetic acid was adjusted to 50 mM by mixing the appropriate amount of unlabeled iodoacetic acid in 0.1 M NaOH (specific activity, 50 mCi/mmol). [$^{14}$C]Iodoacetamide (4.7 mM in ethanol) was used without further dilution. The ROS, wild-type, and mutant rhodopsins were alkylated with a 100-fold excess of the sulfhydryl reagents in the dark while they were bound to 1D4-Sepharose (Resek *et al.*, 1993). In some cases, the pigments were first reacted with a 100-fold excess of the carboxylalkyl maleimide derivatives (reagents I, II, or III, Figure 2) prior to alkylation with [$^3$H]NEM. Typically, the resin was washed five times with 20 column volumes of 20 mM Tris-HCl, pH 8.0, containing 2 mM ATP, 2 mM $MgCl_2$, 1 M NaCl, and 0.1% DM followed by three washes with 20 column volumes of 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 2 mM EDTA, and 0.02% DM before addition of the sulfhydryl reagent. In some cases, the reaction buffer contained 1 M NaCl instead of 150 mM NaCl. The reaction volume was approximately 300 $\mu$L. After 6 or 16 h at 20 °C, the resin was extensively washed with 2 mM $NaH_2PO_4$, pH 6.0, containing 0.1% DM to remove excess reagent and the bound rhodopsin was eluted as described above. The extent of derivatization was determined from the absorption spectrum of the sample and counting the $^3$H or $^{14}$C radioactivity. Samples were also analyzed by nonreducing SDS−PAGE (Laemmli, 1970) with a 5% stacking and a 10% resolving gel and visualized by fluorography.

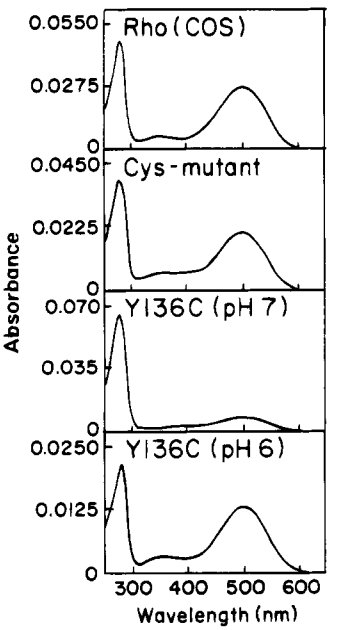


FIGURE 3: UV/vis absorption spectra of wild-type and cysteine mutant rhodopsins. Opsin and opsin mutants expressed in transiently transfected COS-1 cells were reconstituted with 11-*cis*-retinal, solubilized in DM, and immunopurified in pH 6.0 or 7.0 buffer (see Experimental Procedures). Spectra recorded at 20 °C in the dark are shown.

*Thermolysin Cleavage of Rhodopsin after Reaction with the Maleimides.* Procedures for the partial digestion of detergent solubilized rhodopsin with thermolysin were similar to those previously reported (Nakayama & Khorana, 1990). Briefly, 1 $\mu$g of maleimide-alkylated ROS and COS-1 cell wild-type rhodopsins were incubated with varying amounts of thermolysin (20:1 to 5:1, ratio of rhodopsin to enzyme by weight) in 20 mM Hepes, pH 6.8, containing 5 mM $CaCl_2$ and 0.015% DM for 16 h at 20 °C. The reaction was terminated by the addition of EDTA (10 mM), and the fragments were resolved by nonreducing SDS/Tris-Tricine PAGE using a 4% stacking and a 10% resolving gel. The protein bands were visualized by staining with Coomassie blue and fluorography.

## RESULTS

*Expression, Purification, and Spectral Characterization of Single Cysteine Substitution Mutants.* In general, the opsins from the cysteine substitution mutant genes were expressed at levels comparable to those of the wild-type and Cys-mutant genes. All of the opsins formed the 500-nm absorbing chromophore with 11-*cis*-retinal. Further, the correctly folded retinal-binding opsins were separated from the misfolded non-retinal-binding opsins by selective elution from the immunoaffinity matrix under conditions of low pH and ionic strength (Ridge *et al.*, 1995). UV/vis spectra demonstrating the effects of misfolded opsin removal on the purity of the Y136C[2] mutant are shown in Figure 3. All of the other cysteine substitution mutants were purified in a similar manner, and the $A_{280}/A_{500}$ ratio in all cases was

[2] Amino acid substitutions are designated by the wild-type amino acid residue, its position in the sequence, and the replacing amino acid residue. Thus, "Y136C" signifies the mutant in which the tyrosine at position 136 has been replaced by cysteine.

Table 1: $G_T$ Activation by and [$^3$H]*N*-Ethylmaleimide Incorporation into Wild-Type and Cysteine Substitution Mutant Rhodopsins

| pigment | GTP−GDP exchange (mol)[a] | [$^3$H]NEM incorporation (mol/mol Rho)[b] |
|---|---|---|
| WT | 316 ± 5 | 2.05 ± 0.24 |
| Cys-mutant | 218 ± 7 | 0.07 ± 0.03 |
| Y136C | 28 ± 1 | 1.05 ± 0.12 |
| V137C | 63 ± 1 | 0.98 ± 0.09 |
| V138C | 26 ± 1 | 0.95 ± 0.21 |
| V139C | 68 ± 4 | 0.92 ± 0.11 |
| C140 | 319 ± 4 | 0.85 ± 0.08 |
| K141C | 61 ± 3 | 0.94 ± 0.09 |
| P142C | 23 ± 1 | 1.08 ± 0.12 |
| M143C | 149 ± 2 | 0.88 ± 0.14 |
| S144C | 75 ± 4 | 0.64 ± 0.06 |
| N145C | 80 ± 3 | 0.94 ± 0.13 |
| F146C | 84 ± 3 | 1.06 ± 0.05 |
| R147C | 51 ± 3 | 0.67 ± 0.12 |
| F148C | 48 ± 1 | 1.11 ± 0.15 |
| G149C | 98 ± 5 | 1.05 ± 0.07 |
| E150C | 182 ± 7 | 0.89 ± 0.14 |

[a] The amount of pigment assayed was based on the molar extinction coefficient. The data shown are averages from two or three independent determinations. Experimental errors are shown after "±" in each result. [b] Stoichiometry of [$^3$H]NEM alkylation after a 16 h reaction. The data shown are averages from at least two independent determinations. Experimental errors are shown after "±" in each result.

between 1.6 and 1.8. The yields of the spectrally pure pigments with most of the mutants were lower than that obtained for the pure wild-type and Cys-mutant pigments (Figure 3). This was especially so for the Y136C, R147C, and G149C mutants where only 30−40% of the expressed opsins bound 11-*cis*-retinal. As the molar extinction of the chromophores in each of the mutants was similar to that of the Cys-mutant pigment (35 500−39 800 $M^{-1}$ $cm^{-1}$), it appears that the introduction of the cysteine residues in this region of the opsin did not affect the correct folding of these proteins.

All of the above mutants showed photobleaching behavior similar to that of the wild-type rhodopsin. Thus, upon illumination (>495 nm) for 10 s, all of the mutants formed the 380-nm absorbing metarhodopsin II (MII) species. The latter species, upon acidification, generated the 440-nm absorbing protonated retinyl-Schiff base (data not shown).

*$G_T$ Activation by the Single Cysteine Substitution Mutants.* Previously, Karnik *et al.* (1988) found that the Cys-mutant pigment showed reduced capacity to stimulate the GTPase activity of $T_\alpha$ in $G_T$ relative to wild-type rhodopsin. We now observed for this mutant similarly reduced GTP-GDP exchange when compared with the wild-type protein (Table 1). Interestingly, the presence of the native Cys-140 in the Cys-mutant (C140) restored the GTP−GDP exchange level to that of the wild-type rhodopsin (Table 1). Previously, we showed that replacement of Cys-140 by serine in the native rhodopsin, a naturally occurring mutation associated with retinitis pigmentosa (Macke *et al.*, 1993), resulted in a 15% decrease in the level of GTP−GDP exchange (Karnik *et al.*, 1993). This is apparently due to a decrease in the $V_{max}$ of the reaction with no significant effect on the $K_m$ for $G_T$. On the other hand, substitution of the cytoplasmic cysteines at positions 316, 322, or 323 with serine had virtually no effect on $G_T$ activation.

All of the other single amino acid to cysteine substitution mutants showed lower levels of GTP−GDP exchange than the Cys-mutant (Table 1). This was especially apparent with

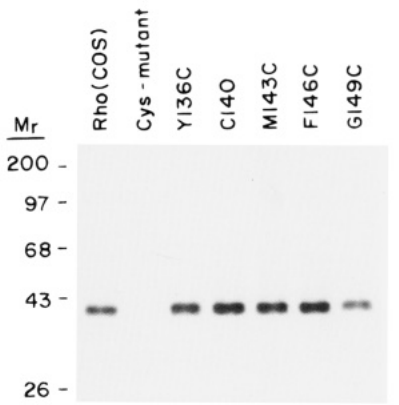


FIGURE 4: Fluorograph of wild-type and cysteine mutant rhodopsins alkylated with [$^3$H]*N*-ethylmaleimide. Lane 1, wild-type COS-1 cell rhodopsin [Rho(COS)]; lane 2, Cys-mutant rhodopsin; lanes 3−7, Y136C, C140, M143C, F146C, and G149C rhodopsins, respectively. The pigments were alkylated with [$^3$H]*N*-ethylmaleimide for 16 h at 20 °C in the dark while bound to 1D4-Sepharose (see Experimental Procedures) and eluted from the resin in pH 6.0 buffer, and equivalent amounts of protein were analyzed by SDS−PAGE and fluorography. Positions of molecular size standards are shown at the left in kilodaltons. The stoichiometry of alkylation is shown in Table 1.

the Y136C, V138C, and P142C mutants where only 10−15% of the activity of the Cys-mutant was observed. Previously, replacement of Arg-147 with Gln has been shown to have no affect on the GTPase activity of $G_T$ (Franke *et al.*, 1992). In this study, the R147C mutant showed about 22% the level of GTP−GDP exchange relative to the Cys-mutant (Table 1), suggesting that the cysteine substitution at this position has a more deleterious effect on the MII/$G_T$ interaction.

*Reactivity of the Sulfhydryl Group in the Cysteine Substitution Mutants with N-Ethylmaleimide.* *N*-Ethylmaleimide (NEM), a relatively membrane-permeant reagent, and its related nonpermeant derivatives (Griffiths *et al.*, 1981; Figure 2) are the reagents of choice for studies of the cysteine environment in the above mutants. To determine whether the introduced cysteine residues reacted with NEM, the mutant pigments were treated with [$^3$H]NEM in the dark while they were bound to 1D4-Sepharose. When wild-type COS cell rhodopsin was tested, only two cysteines reacted (Figure 4, lane 1, and Table 1). This is consistent with previous findings (DeGrip & Daemen, 1982; Ridge *et al.*, 1995) and the two cysteines that react have been identified as Cys-140 and Cys-316 (Findlay *et al.*, 1982). As expected (Ridge *et al.*, 1995), the Cys-mutant protein was inert to [$^3$H]-NEM under the above conditions (Figure 4, lane 2, and Table 1). In contrast, all the single cysteine substitution mutants showed reactivity with [$^3$H]NEM under the above standard conditions (Figure 4 and Table 1). With the exception of S144C and R147C, the mutants showed nearly stoichiometric alkylation after a 16 h reaction (Table 1). These results indicate that all of the newly introduced cysteines are accessible for reaction with NEM.

To detect differences in the reactivity and/or the accessibility of the different cysteine residues, the mutants were treated with [$^3$H]NEM for a shorter period (6 h). As is shown in Figure 5, strikingly different levels of alkylation were observed for the cysteine residues. While stoichiometric alkylation was apparent with the V137C, V138C, V139C, K141C, and G149C mutants, the C140, M143C, S144C, and

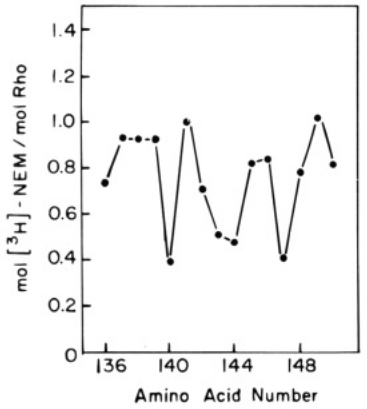


FIGURE 5: Stoichiometry of [$^3$H]*N*-ethylmaleimide alkylation in cysteine mutant rhodopsins after 6 h reaction. The pigments were alkylated with [$^3$H]*N*-ethylmaleimide for 6 h at 20 °C in the dark while bound to 1D4-Sepharose (see Experimental Procedures) and eluted from the resin in pH 6.0 buffer, and equivalent amounts of protein were assayed for $^3$H radioactivity by scintillation counting. The results of a single experiment are shown and are representative of two independent determinations.

R147C mutants were maximally alkylated to 0.4−0.5 mol of [$^3$H]NEM/mol of rhodopsin in this time range.

*Reactivity of the Sulfhydryl Group in the Cysteine Substitution Mutants with Polar and Charged Reagents.* The ability of polar and membrane-impermeant sulfhydryl modification reagents such a iodoacetic acid, iodoacetamide, and *N*-polymethylenecarboxymaleimides of various alkyl chain lengths (Figure 2) to react with the newly introduced cysteine residues was next investigated. These experiments were designed to distinguish whether the above described variations in the extent of reaction with NEM were due to intrinsic differences in the reactivity or accessibility of the sulfhydryl groups. Surprisingly, derivatization was not observed with iodoacetic acid or iodoacetamide under the reaction conditions used for NEM alkylation (data not shown). Further, increasing the ionic strength of the reaction buffer to reduce possible surface charge effects did not result in derivatization. Although both reagents reacted with wild-type rhodopsin under denaturing conditions, the level of incorporation was not stoichiometric (3.0−3.5 mol/mol rhodopsin). These results raised the possibility that these cysteine residues were located in an environment that was inaccessible to both iodoacetic acid or iodoacetamide.

This possibility was examined further by reacting selected cytoplasmic cysteine-containing mutants with the three different *N*-polymethylenecarboxymaleimides (Figure 2). Since these compounds are not radioactively labeled, the reactions were performed as follows. First, the cysteine mutant proteins were reacted with the carboxyalkyl maleimide derivatives or unlabeled NEM while bound to 1D4-Sepharose in the dark. After the excess reagent was washed away, the antibody bound pigments were then alkylated with [$^3$H]NEM. The amount of [$^3$H]NEM incorporated into the pigment was interpreted as residual labeling of the cysteine residue(s). As shown in Figure 6, neither ROS nor COS cell rhodopsin showed any [$^3$H]NEM labeling after prior reaction with unlabeled NEM, maleimido-*N*-butanoic acid (reagent II), or maleimido-*N*-hexanoic acid (reagent III). However, [$^3$H]NEM incorporation (0.34 ± 0.17 mol/mol rhodopsin) was observed after prior reaction with maleimido-*N*-propionic acid (reagent I).

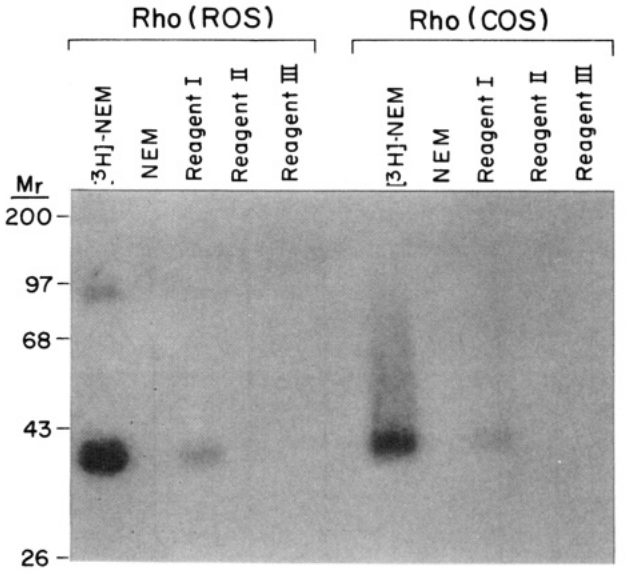


FIGURE 6: Fluorograph showing the effect of *N*-polymethylenecarboxymaleimides on [$^3$H]*N*-ethylmaleimide incorporation into ROS and wild-type COS-1 cell rhodopsins. The pigments were alkylated with unlabeled NEM or the different *N*-polymethylenecarboxymaleimides (Figure 2) for 16 h at 20 °C in the dark while bound to 1D4-Sepharose (see Experimental Procedures). After excess reagent was washed away, residual alkylation was determined by alkylating the samples with [$^3$H]*N*-ethylmaleimide for an additional 16 h at 20 °C. The pigments were eluted from the resin in pH 6.0 buffer, and equivalent amounts of protein were analyzed by SDS−PAGE and fluorography. Positions of molecular size standards are shown at the left in kilodaltons.

To determine the site(s) of [$^3$H]NEM labeling in these pigments, the alkylated rhodopsins were subjected to proteolytic digestion with thermolysin. Thermolysin cleaves rhodopsin between Ser-240 and Ala-241 in the third cytoplasmic loop and at Pro-327/Leu-328 in the COOH-terminal tail, yielding two large fragments designated F1 (amino acids 1−240) and F2 (amino acids 241−327) (Pober & Stryer, 1975; Hargrave *et al.*, 1987). Different ratios of rhodopsin to thermolysin were first tested in order to determine optimal conditions for proteolytic cleavage of the [$^3$H]NEM alkylated pigments. At a 5:1 ratio (rhodopsin/enzyme), essentially all of the alkylated ROS and COS-1 cell rhodopsin was converted into the two large fragments (Figure 7A,B). In the latter pigment the larger thermolytic fragment migrates as a diffuse band because of heterogeneity in the oligosaccharide chains. The presence of two radioactive fragments is consistent with earlier observations that the sites of sulfhydryl derivatization under native conditions are Cys-140 (F1 fragment) and Cys-316 (F2 fragment) (Findlay *et al.*, 1984). Notably, both ROS and COS-1 cell rhodopsin, after first reaction with maleimido-*N*-propionic acid (reagent I), showed radioactivity exclusively in the F1 fragment after thermolysin cleavage (Figure 7B; ROS rhodopsin not shown). These findings suggest that the site of residual [$^3$H]NEM labeling in these pigments was at Cys-140 and not Cys-316.

The above results prompted an investigation into the reaction of these carboxyalkyl maleimides with other cysteine residues in the CD loop. As shown in Figure 8, striking differences in the [$^3$H]NEM labeling after prior reaction with unlabeled NEM or the maleimide-*N*-polyethylene carboxylic acids were observed for the Y136C and E150C mutants. While [$^3$H]NEM incorporation into Y136C was observed after reaction with NEM and all the three maleimide

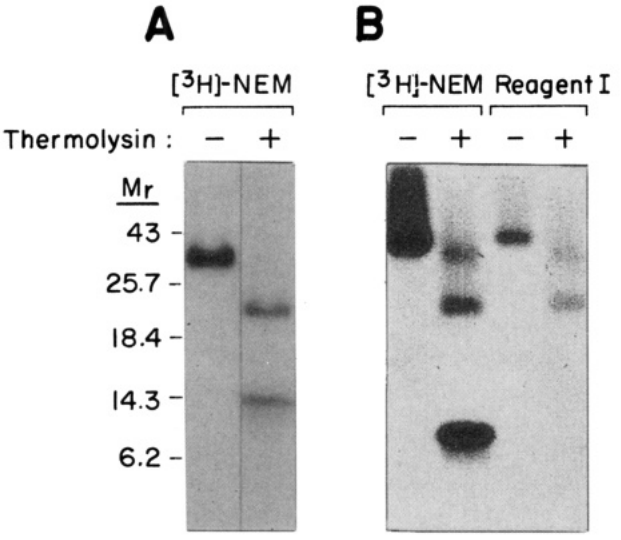


FIGURE 7: Fluorograph of [$^3$H]*N*-ethylmaleimide alkylated ROS and COS-1 cell rhodopsin after cleavage with thermolysin. (A) ROS rhodopsin was alkylated with [$^3$H]*N*-ethylmaleimide for 16 h at 20 °C in the dark while bound to 1D4-Sepharose and eluted from the resin in pH 6.0 buffer (see Experimental Procedures). The alkylated pigment was incubated in the absence or presence of thermolysin (5:1 rhodopsin/enzyme) for 16 h at 20 °C prior to analysis by SDS/Tris-Tricine PAGE. (B) Wild-type COS-1 cell rhodopsin was alkylated with [$^3$H]*N*-ethylmaleimide or reagent I (Figure 2) for 16 h at 20 °C in the dark while bound to 1D4-Sepharose. After excess reagent was washed away, the pigment first reacted with reagent I was alkylated with [$^3$H]*N*-ethylmaleimide for an additional 16 h at 20 °C (see Experimental Procedures). The alkylated pigments were incubated in the absence or presence of thermolysin using the conditions described above and analyzed by SDS/Tris-Tricine PAGE. Positions of molecular size standards are shown at the left in kilodaltons.

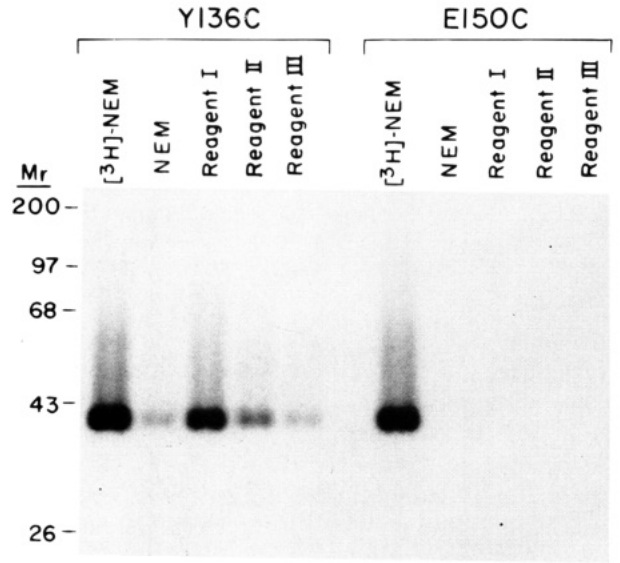


FIGURE 8: Fluorograph showing the effect of *N*-polymethylenecarboxymaleimides on [$^3$H]*N*-ethylmaleimide incorporation into the Y136C and E150C rhodopsins. The pigments were alkylated as described in the legend to Figure 6, and equivalent amounts of protein were analyzed by SDS−PAGE and fluorography. Positions of molecular size standards are shown at the left in kilodaltons.

derivatives, no incorporation was observed with the E150C mutant. This suggests that the cysteine residue in the latter is more exposed to the aqueous interface than that in the former mutant. Further, in the Y136C mutant, there was a clear correlation between the length of the hydrocarbon chain

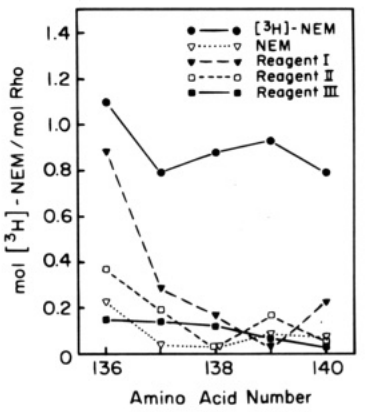


FIGURE 9: Effect of different *N*-polymethylenecarboxymaleimides on the stoichiometry of [$^3$H]*N*-ethylmaleimide alkylation into cysteine mutant rhodopsins. The pigments were alkylated as described in the legend to Figure 6, and equivalent amounts of protein were assayed for $^3$H radioactivity by scintillation counting. The results of a single experiment are shown and are representative of two independent determinations.

in the maleimide reagent and the amount of [$^3$H]NEM subsequently incorporated (Figure 9). Residual [$^3$H]NEM incorporation was apparent after reaction with all three carboxyalkyl maleimides in the V137C mutant while both reagent I and reagent III (Figure 2) resulted in 0.2 mol of [$^3$H]NEM/mol of rhodopsin at position 138 (Figure 9). The V139C and C140 mutants showed similar levels of [$^3$H]NEM incorporation after first reaction with reagent II and reagent I, respectively (Figure 9).

## DISCUSSION

Cysteine sulfhydryl groups in proteins provide a versatile handle for structural studies by attachment of suitable probes for chemical and biophysical investigations. Earlier work with cysteine-substitution mutants of the integral membrane protein bacteriorhodopsin proved rewarding in providing information on the membrane−aqueous boundary as well as the helical structure of the membrane-embedded polypeptide segments (Flitsch & Khorana, 1989; Altenbach *et al.*, 1989, 1990; Greenhalgh *et al.*, 1991). Similarly, cysteine substitution mutants of the bacterial aspartate receptor and the lactose permease have given a wealth of insight into the structure and function of these membrane proteins (Falke & Koshland, 1987; Milligan & Koshland, 1988; Falke *et al.*, 1988; Sahin-Toth & Kaback, 1993; Jung *et al.*, 1993; Dunten *et al.*, 1993). In rhodopsin, Resek *et al.* (1993) developed an approach to probe conformational changes in the cytoplasmic region by EPR studies of spin labels attached to strategically placed cysteine residues. In a complementary chemical approach, a photoactivatable group was attached to the cysteine sites for studies of protein−protein interactions (e.g., rhodopsin and $G_T$) as well as for intramolecular cross-linking within the rhodopsin molecule (Resek *et al.*, 1994; C.Z. and H.G.K., unpublished results).

We have now initiated a structural investigation of the cytoplasmic face of rhodopsin by using the single cysteine insertion approach to systematically probe the amino acids of the CD loop (Figure 1). This region forms an integral part of the photoreceptor structure involved in signal transduction processes. Thus, the CD loop is involved in the interaction between MII and $G_T$ (Konig *et al.*, 1989; Franke

*et al.*, 1990, 1992) and carries in its vicinity the conserved charged pair of amino acids, Glu-134 and Arg-135 (Sakmar *et al.*, 1989). We seek information on the following basic aspects of the rhodopsin structure in this region. First, the membrane−aqueous boundaries of the helices C and D. Second, the possibility of helical or other structures continuing from the membrane-embedded region into the aqueous domain. Third, there is probably a dark state tertiary structure in the cytoplasmic region of rhodopsin which, upon retinal isomerization, converts to a different structure. Can we obtain significant information by studying the changes in the reactivity of the newly introduced cysteine residues in the dark and light-activated states? Here we report a comparative study on the chemical reactivity of the CD loop single cysteine substitution mutants only in the dark state. Corresponding studies in the light-activated state remain to be done. However, EPR studies of the cysteine mutants following nitroxide spin-labeling have been done in both the dark and light-activated states and are reported in the accompanying paper (Farahbakhsh *et al.*, 1995).

As expected from earlier work (Franke *et al.*, 1992), all of the cysteine substitution mutants folded to form the characteristic rhodopsin chromophore with 11-*cis*-retinal (Figure 3) and, upon illumination, showed bleaching characteristics similar to that of wild-type rhodopsin (data not shown). Further, all of the mutants activated $G_T$ in a light-dependent manner, although there were dramatic variations in the extent of activation (Table 1). This result was expected in light of the previous extensive mutagenesis in this region of rhodopsin indicating the requirement of a specific polypeptide sequence for optimal MII/$G_T$ interactions (Franke *et al.*, 1990, 1992). However, there were clearly structural effects evident in the reactivity of the cysteine residues with the different sulfhydryl-specific reagents. Below, we comment on the possible reasons for the observed differences in sulfhydryl modification.

Every one of the single cysteine mutants was alkylated in the dark with the membrane-permeant reagent NEM (Figure 4 and Table 1). While 13 of the 15 mutants showed stoichiometric [$^3$H]NEM alkylation after 16 h (Table 1), the rates of alkylation for the different cysteine residues varied. When the reaction was carried out for 6 h, the 15 cysteine substitution mutants revealed a periodicity in the level of alkylation (Figure 5). In those cases where [$^3$H]NEM alkylation was not substantially different in the 6 h period (Y136C, V137C, V138C, V139C, K141C, N145C, F146C, F148C, G149C, and E150C), slight differences in the p$K_a$ of the introduced sulfhydryl group could account for these results. However, for the C140 and R147C mutants (Figure 5), differences in p$K_a$ are not likely to account for the sharp change in the extent of alkylation observed relative to the adjacent cysteine residues. Thus, other factors, presumably structural, influenced their accessibility to NEM and/or reactivity toward NEM. This may also account for the observed differences in NEM alkylation between P142C, M143C, and S144C and their adjacent cysteine residues where the variation in the extent of alkylation was not as marked as mentioned above.

The alkylation of ROS, COS-1 cell wild-type, and selected cysteine substitution rhodopsins with *N*-polymethylenecarboxymaleimides of various alkyl chain lengths (Figure 2) also revealed differences between the sulfhydryl groups. These tests involved, first, treatment of the mutant rhodopsins with the different reagents in Figure 2 followed by exposure to [$^3$H]NEM to ascertain the extents of the reactions in the first steps. With both ROS and COS-1 cell rhodopsins, alkylation with NEM, 4-maleimidobutanoic acid (reagent II), and 6-maleimidohexanoic acid (reagent III) (Figure 6) went to completion. However, alkylation with 3-maleimidopropionic acid (reagent I, Figure 2) was incomplete as shown by subsequent [$^3$H]NEM incorporation. On the basis of thermolysin digestion experiments (Figure 7B), the site of residual [$^3$H]NEM labeling in these rhodopsins appeared to be Cys-140. This finding is consistent with an earlier observation that Cys-140 is less solvent-exposed than Cys-316 (Resek *et al.*, 1993). Further experiments revealed differences in *N*-polymethylenecarboxymaleimide labeling for Y136C and E150C. While [$^3$H]NEM incorporation into the Y136C mutant was observed after alkylation with all of the compounds in Figure 2, no residual incorporation was observed with the E150C mutant (Figure 8). Clearly, the cysteine in the latter mutant is more exposed to the aqueous interface than in the Y136C mutant. This result is in agreement with the EPR studies reported in the accompanying paper (Farahbakhsh *et al.*, 1995) .

Further examination of the reactions between the carboxyalkyl maleimides and the mutants with cysteine residues in the proximal portion of the CD loop showed two distinct trends. For the Y136C and V137C mutants, the extent of reaction increased with increasing length of the alkyl chain in the reagents (Figure 9). These results suggest that longer hydrocarbon spacers allow the maleimide group to react more readily with these sulfhydryl groups in the hydrophobic milieu. On the other hand, at positions 138−140, the differences in reactivity with these maleimides were minor (Figure 9). These results suggest that the SH groups in these three mutants are relatively accessible to all the reagents. These findings are also consistent with the topographical location of these cysteines derived from EPR analysis (Farahbakhsh *et al.*, 1995).

Finally, none of the cysteine substitution mutants reacted with either iodoacetic acid or iodoacetamide under the conditions normally used for such reactions. Further, no reaction was observed even when the concentration of salt in the reaction buffers was raised up to 1 M to reduce possible surface charge effects. These results are the strongest indication of steric constraints in the sulfhydryl groups of the mutant proteins. The reactions of the latter groups with the above reagents are known to proceed by $SN_2$ mechanism (Means & Feeny, 1971) and therefore require specific stereochemical alignment of the two reaction components. Obviously, this arrangement is not attained in these mutants because of the structural constraints in the CD loop. These results are again supported by the EPR studies of spin labels attached to these same cysteine residues (Farahbakhsh *et al.*, 1995).

The results reported in this paper amply demonstrate the role of structure in the CD loop of rhodopsin in the reactivity of the successive cysteine residues. However, they do not allow a formulation of the structure. In forthcoming papers, we hope to extend these studies to (1) single cysteine substitution mutants in all regions of the cytoplasmic face of rhodopsin and (2) double cysteine mutants to develop proximity relationships in the cytoplasmic region.

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