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Structure and Function in Rhodopsin

REQUIREMENTS OF A SPECIFIC STRUCTURE FOR THE INTRADISCAL DOMAIN*

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We concluded previously from mutagenesis in the intradiscal domain of bovine rhodopsin that the formation of a tertiary structure comprising the N-terminal tail and the three polypeptide loops is essential to the *in vivo* assembly of the functional rhodopsin. We now report on more comprehensive mutagenic studies in the intradiscal domain to determine more precisely the requirement for the formation of the above-proposed tertiary structure. Three large deletions, two consisting of groups of 10 amino acids each, and the third of 34 amino acids, were carried out in the N-terminal loop. All the three mutant opsins only poorly formed the rhodopsin chromophore. In the BC loop, we carried out five 2 amino acid deletions, 2 single amino acid deletions, and three mutations in which short sequences in the loop were reversed. All the resulting mutant opsins had lost the ability to bind 11-*cis*-retinal. In the DE loop, where extensive mutagenesis had previously been carried out, we carried out 3 amino acid replacements (Asn, Thr, Tyr) at Cys^{187}. None of these mutants bound 11-*cis*-retinal. In loop FG, we carried out four 2 amino acid deletions, 1 single amino acid deletion, 3 amino acid replacements, and one mutation in which the sequence of the 7 amino acids was reversed. All the mutants in FG loop partially formed the rhodopsin chromophore. All the mutants now described appeared to be retained in the endoplasmic reticulum: several that were examined in detail were complexed with non-opsin proteins, the chaperonins. Treatment with ATP-$MgCl_2$ released the latter from the mutant rhodopsins. Our overall conclusion is that the formation of the specific structure in the intradiscal domain has highly stringent spatial requirements.

Rhodopsin, the dim light photoreceptor of the vertebrate rod cell, is an example *par excellance* of the receptors that couple to the guanine nucleotide-binding regulatory proteins (G proteins) in signal transduction (2). Structural models propose seven transmembrane helical segments as a common motif for this class of membrane receptors (3). The primary structure of rhodopsin has been determined both by protein (4, 5) and DNA sequencing (6). The protein contains a single polypeptide chain of 348 amino acids folded approximately to form the secondary structure pattern shown in Fig. 1. A molecule of 11-*cis* retinal is linked to the opsin by a protonated Schiff base at Lys^{296}. Upon illumination, the retinal isomerizes to the all-*trans* form and drives the protein through a series of transient photointermediates (7). The intermediate, designated metarhodopsin (Meta-II), binds to and activates the G protein, transducin. A cascade of biochemical reactions ensues that culminates in the closing of the cation conductance channel in the plasma membrane. A neural signal is thus generated (8, 9).

In structure-function studies, we have previously introduced by design a variety of deletions as well as point mutations in a synthetic rhodopsin gene (1, 10–17). Focusing, in particular, on the intradiscal domain, we observed three main types of phenotypes (14). Type I phenotype resembled the wild-type, in that it folded correctly and formed the normal chromophore with 11-*cis*-retinal. Type II mutants did not fold correctly. They remained in the endoplasmic reticulum and failed to bind 11-*cis*-retinal. Type III mutants were also defective in folding. They showed varying extents of chromophore regeneration with 11-*cis*-retinal but always less than quantitative. With the aim of defining more closely the requirements for the formation of the specific structure in the intradiscal domain, we now report on a more comprehensive set of mutagenic studies in this domain. The total mutations reported and characterized in this paper are shown in Fig. 1 and Table I. Thus, we carried out large deletions in the N-terminal tail. We introduced 1 and 2 amino acid deletions in the BC and FG loops and single amino acid replacements in the FG and DE loops. Further, to test the effect of substantial alterations in amino acid interactions without affecting the sizes of the loops BC and FG, we introduced mutations in which sequences of short peptide segments in the loops were reversed. The phenotypes of the different groups of mutations now described mostly paralleled the phenotypes encountered previously (14). The overall conclusion from the total mutagenesis performed in the intradiscal domain is that the stereochemical requirements of the intradiscal structure necessary for the assembly of the functional rhodopsin are highly stringent.

EXPERIMENTAL PROCEDURES

Materials

Dodecyl maltoside (DM)[1] was from Anatrace. [^{35}S]Methionine, deoxyadenosine 5′-[α-^{35}S]thiotriphosphate were from Dupont-New England Nuclear. Goat anti-mouse IgG conjugated to alkaline phosphatase, fluorescein-conjugated goat anti-mouse IgG, and rhodamine-conjugated goat anti-mouse IgG were from Boehringer Mannheim. The protease inhibitors, aprotinin, benzamidine-HCl, leupeptin and pepstatin, and ATP-agarose were from Sigma. Sequenase (version 2.0) was from United States Biochemicals Corp. 11-*cis*-Retinal was a gift of Dr. P. Sorter (Hoffman-La Roche). Sepharose 4B was purchased from Phar-

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[1] The abbreviations used are: DM, *n*-dodecyl β-D-maltoside; IgG, immunoglobulin G; rho-1D4, an antirhodopsin monoclonal antibody specific for the C-terminal tail of rhodopsin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PMSF, phenylmethylsulfonyl fluoride; BiP, immunoglobulin heavy chain binding protein; Grp, glucose-regulated protein; Hsp, heat shock protein; PAGE, polyacrylamide gel electrophoresis.

macia LKB Biotechnology Inc. and rho-1D4-Sepharose 4B was prepared as described (18). Anti-BiP antibody was a gift from Dr. David Bole (University of Michigan) while the antibodies to the chaperonins Hsp60 and Grp94 (19) were from Stress Genes, British Columbia.

Construction of the Rhodopsin Gene Mutants

The desired mutations in the synthetic rhodopsin gene (18) were introduced by the restriction fragment replacement method (20) by replacement of appropriate restriction fragments by synthetic DNA duplexes containing the required changed codons. All the oligonucleotides corresponding to the DNA fragments were synthesized on an Applied Biosystems 380B DNA synthesizer and purified as previously described (20). The amino acid replacements, the corresponding restriction fragments, and their positions in the gene are shown in Table I. All constructions were checked by sequencing the appropriate regions corresponding to insertions of the fragments containing the modified codons (21). The two N-terminal tail mutants, Δ1 and Δ2, were cloned between the restriction sites *Eco*RI and *Fsp*I and Δ3 was cloned between the restriction sites *Eco*RI and *Bcl*I in the vector pMT4. The BC loop mutants were cloned first in the cloning vector pOP3 (22) and subsequently the opsin gene (*Eco*RI-*Not*I fragment, 1060 base pairs) was transferred to the expression vector pMT4 (Table I). The mutant Δ4 was cloned between the restriction sites *Bgl*II and *Nco*I while Δ5-Δ10 were cloned between the restriction sites *Nco*I (nucleotide number 302) and *Xho*I (nucleotide number 339) (Table I) (20). The DE loop mutants, involving single amino acid replacements, were cloned between the restriction sites *Xba*I (nucleotide number 531) and *Cla*I (nucleotide number 571) in the vector pMT4. The FG loop deletion mutants (Δ11-Δ15) (Table I) and the triple amino acid replacement mutant, S281T/D282E/F283Y, were cloned between the *Nde*I and *Apa*I sites. The sequence reversal mutants, R-1 to R-3, in the BC loop (Table I) were cloned between the restriction sites *Nco*I and *Xho*I in the vector pMT4, while the mutant R-4 (FG loop) was cloned between the restriction sites *Nde*I and *Apa*I.

Expression, Chromophore Formation by the Expressed Opsins, and Purification of Rhodopsin Mutants

The wild-type rhodopsin gene and the rhodopsin mutant genes in the pMT4 vector were transiently expressed in COS-1 cells as described previously (18). COS cells were routinely harvested 3 days after transfection and were treated in one of the following different ways to reconstitute the opsins with 11-*cis*-retinal.

Treatment—They were treated in PBS (10 mM NaH_2PO_4, pH 7, 150 mM NaCl) buffer with 11-*cis*-retinal (3.5 μM) for 3 h in the dark. The cells were pelleted and treated with the solubilization buffer 1% (w/v) DM and PBS and 0.1 mM PMSF in the dark at 4 °C for 45 min to solubilize the rhodopsin and free opsin. The latter were then purified by immunoabsorption on rho-1D4-Sepharose 4B (18).

Chromophore Formation at Different Temperatures—The cells were incubated with 11-*cis*-retinal as above, but at 4, 20, or 37 °C. Solubilization and purification of the resulting rhodopsin was as above.

Chromophore Formation from the Lysed Cells—The cells were lysed in the hypotonic buffer (15 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$ + 1 mM dithiothreitol) in the presence of the protease inhibitors (10 μg/ml each of aprotinin, benzamidine-HCl, leupeptin and pepstatin, and 0.1 mM PMSF). Lysis was monitored by phase contrast microscopy, and the suspension was passed through a 25 gauge needle five times to homogenize the lysed cell suspension. Formation of the chromophore and purification of the rhodopsin formed were as above.

Chromophore Formation from the Membrane Fraction—The cells were lysed as described above. The suspension was pelleted by centrifugation, and the pellet was homogenized and layered on a 20%/50% sucrose gradient. The membrane band at the gradient interface was resuspended in PBS, and chromophore formation was carried out with 11-*cis*-retinal for 3 h. After solubilization, rhodopsin and opsin were purified as described above.

Urea Wash of the Membranes—The membrane fraction was prepared from transfected COS cells as above. The membrane pellet was resuspended in the PBS buffer containing 5 M urea and the suspension incubated for 10 min at 4 °C. The pellet was collected by centrifugation, and the step was repeated once and then the pellet was washed in PBS buffer twice. The membrane pellet was resuspended in PBS, and the chromophore was generated with 11-*cis*-retinal as described above. Solubilization and immunopurification of the mutant rhodopsins were as above.

Immunoblotting

The opsins after SDS-PAGE were visualized by immunoblotting with the monoclonal antibody rho-1D4. For immunoblotting with anti-chaperonin antibodies, the total eluates from the rho-1D4 Sepharose 4B were concentrated using Millipore ultra filters (10,000 normal molecular weight limit filters) and subjected to gel electrophoresis.

Expression of Rhodopsin Mutants in the Presence of [^{35}S]Methionine

Rhodopsin mutant genes were transiently expressed in COS cells for 48 h. [^{35}S]Methionine, final concentration, 65 mCi/ml in the medium, was then added for 30 min. The cells were harvested and solubilized in the solubilization buffer in the presence of protease inhibitors aprotinin, benzamidine-HCl, leupeptin and pepstatin (10 μg/ml of each), and 1 mM PMSF and 10 units/ml of apyrase. The insoluble fraction was removed by centrifugation and rhodopsin was purified from the fraction by absorption to rho-1D4 Sepharose 4B.

Opsin-bound Chaperonins and Treatments for Their Dissociation

Urea Wash—Mutant rhodopsins and opsins were prepared from COS cells as above and adsorbed to rho-1D4-Sepharose 4B. The resin was washed with 50 resin bed volumes of PBS containing 1 M urea and then with 200 volumes of the wash buffer (10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.1% (w/v) DM). The proteins were then eluted with the antibody-antipeptide as described previously (18).

Removal of Non-opsin Proteins by ATP + $MgCl_2$—Protocol 1: the total proteins (opsins and non-opsin proteins) were bound to rho-1D4-Sepharose. The resin was washed at room temperature with 200 volumes of the wash buffer, pH 6 (see above) containing 1 mM ATP and 3 mM $MgCl_2$. The non-opsin proteins thus eluted were examined by SDS-PAGE. The opsins were then eluted with the antibody-antipeptide in the wash buffer, pH 7.0. Protocol 2: the COS cells were harvested 3 days after transfection, the chromophore was generated with 11-*cis*-retinal for 3 h, and the cells were treated with the solubilization buffer, pH 6, containing 5 mM ATP + 5 mM $MgCl_2$ at room temperature for 45 min to dissociate the non-opsin proteins from the mutant rhodopsins. The latter were purified by immunoadsorption as described. Protocol 3: COS cells were harvested 3 days after transfection. They were suspended in PBS, and 11-*cis*-retinal was added for 3 h. The medium was supplemented with DM (0.1% final) and PMSF (0.1 mM final). After 45 min, the insoluble fraction was removed by centrifugation, and the supernatant fraction was treated with ATP-agarose for 2 h at 4 °C. The supernatant solution was used to purify the mutant rhodopsins by immunoabsorption.

Spectral Characterization of Mutant Rhodopsins

UV-Vis absorption spectra of the wild-type and mutant rhodopsins were measured in the dark with a Perkin-Elmer λ-7 spectrophotometer at 20 °C. Samples were in TBS (10 mM Tris-HCl, pH 7.0, 150 mM NaCl) containing 0.1% (w/v) DM. Irradiation of the samples was with a 300 watt fiber optic light source with a <495 nm cut-off filter at 20 °C for the specified times. For absorption spectra at acidic pH, the samples were acidified with 2 N H_2SO_4 to pH 1.9. Stability of the chromophore toward hydroxylamine in the dark was examined by adding a solution of neutral hydroxylamine hydrochloride (final concentration, 100 mM).

Immunofluorescence

COS cells were grown on glass coverslips in 10-cm plates for 16 h, transfected as described, and further grown for 72 h. Cells were washed with cold PBS at 4 °C three times, permeabilized in methanol (−20 °C) for 5 min, and then fixed in acetone (−20 °C) for 5 min. After air-drying in the hood, the cells were rehydrated with PBS + 0.02% NaN_3. After 1 h of incubation with the antibody rho-1D4 (10 μg/ml), cells were washed three times with PBS and then incubated with anti-mouse goat IgG (10 μg/ml) conjugated with either fluorescein or rhodamine for 1 h. The cells were washed with PBS and mounted on microscope glass slides. The stained cells were observed by confocal laser scanning microscope and by immunofluorescence microscope. The stained cells were scanned at 0.5-μm thickness using Bio-Rad MRC600 confocal laser scanning microscope, and the collected images were integrated.

RESULTS

Mutants in the N-terminal Tail

Previously, Doi *et al.* (14) reported four deletions in the N-terminal tail. These eliminated, respectively, the amino acids

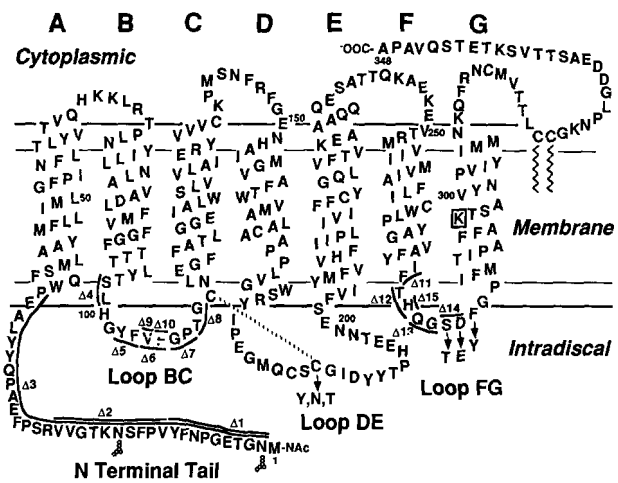

FIG. 1. **Point mutations and deletions made in the intradiscal domain of bovine rhodopsin.** Shown is a secondary structure model for rhodopsin with the seven helical segments marked *A-G*. The *wiggly lines* connected with C322 and C323 indicate palmitoyl group. K296, the site of 11-*cis*-retinal attachment, is *boxed*. Single and multiple amino acid deletions are shown by *underlines* and *numbered* Δ*1-*Δ*15*. They are shown systematically according to the polypeptide segments where they occur in Table I.

7–10, 11–14, 18–20, and 21–29. The resulting mutant opsins formed the rhodopsin-like chromophore with 11-*cis*-retinal to the extent of only 10–20% (type III mutations). Of the mutations now constructed, Δ1-Δ3 (Fig. 1, Table I), Δ1 removes the first *N*-glycosylation site while Δ2 deletes the second *N*-glycosylation site. Δ3 is devoid of both glycosylation sites. The opsins expressed from Δ1 and Δ2 mutants formed the rhodopsin-like chromophore to less than 20% of the wild-type opsin. The result with Δ1 opsin is shown in Fig. 2*A*. The opsin from Δ3 formed very little 500-nm absorbing chromophore which, as expected, underwent shift to 380 nm (presumably Meta II) on irradiation. In addition, a chromophore absorbing at 380 nm in the dark was also formed (Fig. 2*B*). The nature of this species is not clear. The bleaching behavior of the reconstituted chromophores from mutant opsins Δ1 and Δ2 was as expected (Fig. 2*A*) while the slight increase at 380 nm on irradiation of Δ3 chromophore was consistent with the formation of a small amount of the 500 nm absorbing chromophore by this mutant opsin.

The bands seen by immunoblotting after SDS-PAGE of the proteins expressed from Δ1-Δ3 (Fig. 3) were as expected. Thus, the wild-type, Δ1 and Δ2 mutants showed bands corresponding in molecular weight to both the glycosylated and non-glycosylated species, while the mutant Δ3 opsins showed only the unglycosylated species.

Mutants in BC Loop

Previously, only one rather large deletion (amino acids 101–108) mutant was studied in this loop (14). The resulting opsin did not bind 11-*cis*-retinal (type II mutation). We have now made a number of short deletions, single amino acid replacements as well as sequence-reversal mutations in this loop (Fig. 1, Table I). Five deletion mutants, Δ4-Δ8, each involving deletion of 2 amino acids (Fig. 1), were first prepared. None of the opsins from these mutants formed any chromophore with 11-*cis*-retinal (type II mutations), as shown for the Δ4 mutant in Fig. 4*A* (*SDS-PAGE* in Fig. 4*B*). Two single amino acid deletions (Δ9 and Δ10; Fig. 1, Table I) were then performed. The opsins from these mutants also failed to bind 11-*cis*-retinal. The possibility was considered that any shortening of the loop was deleterious to the packing of the helices and, therefore, to the correct folding of the intradiscal domain. Therefore, mutations involving only reversals of short sequences were constructed. Thus, in R1 (Table I) the sequence of 7 amino acids was reversed while in R2 and R3 only 2 amino acids were reversed. None of the three mutants with the reversed amino acid sequences formed opsins that were able to bind 11-*cis*-retinal.

The Mutants in DE Loop

Extensive mutagenesis has previously been carried out in this loop (14). The phenotypes observed for the mutants were mainly of the type II when deletion mutations involved the conserved region (amino acids 174–190) and type I when the mutations were in the non-conserved region (amino acids 191–202). We now tested replacement of Cys^{187}, 1 of the 2 disulfide-forming cysteines, by 3 amino acids (Thr, Tyr and Gln). None of the opsins expressed by these mutants formed 500-nm absorbing chromophore with 11-*cis*-retinal. Replacements at Cys^{187} have been studied in detail by Davidson and Khorana (17).[2]

The Mutants in FG Loop

1 and 2 Amino Acid Deletions—One 4 amino acid deletion was carried out previously in this loop (14). The resulting opsin failed to bind 11-*cis*-retinal. The current secondary structure model (Fig. 1) indicates a small size for this loop and it seemed possible that the above 4 amino acid deletion affected packing of the helices to form a functional seven helical cluster. We now carried out four 2 amino acid deletions (Δ11-Δ14) and one single amino acid deletion (Δ15) (Fig. 1, Table I). The phenotype of the opsins from all the five mutants was that of type III. Thus, the opsins from the mutants Δ11 to Δ14 formed the 500-nm absorbing chromophore to the extent of 50–60% of the wild-type while the single amino acid deletion mutant (Δ15) formed the chromophore in somewhat higher yield (~~70%). The levels of chromophore formation by these mutants are, in general, much higher than those reported in earlier work (14), but are well below quantitative. The spectral properties (spectra in dark, light, and in acid) illustrated in Fig. 4*A* for Δ11 mutant as well as SDS-PAGE pattern in Fig. 4*B* are typical of the mutants in loop FG.

The Triple Amino Acid Replacement Mutant S281T/D282E/F283Y in Loop FG—The amino acid replacements in this mutant retained the general character of the native amino acids, but they tested further the steric requirements for the tertiary structure in the intradiscal loop. The opsin expressed from this mutant formed the chromophore to about 90%, essentially like the wild-type. The reconstituted mutant rhodopsin showed photobleaching behavior like the wild-type (data not shown).

The Sequence Reversal Mutant (R-4) (Table I) (Amino Acid Sequence 280–283, FDSG)—This mutant would not be expected to affect the packing of the helices but may show major changes in interactions between amino acids in the intradiscal tertiary structure. The opsin produced from this mutant formed the 500-nm absorbing chromophore to the extent of about 50% of the wild-type. The result is in agreement with the general phenotype (type III) of the mutants in the FG loop.

Stability of the Mutant Rhodopsins to Hydroxylamine

The chromophores formed by the deletion mutants in the FG loop were inert, like the wild-type, toward hydroxylamine in the dark.

Chromophore Formation from the Deletion Mutants in FG Loop under Different Conditions

In general, type III mutants appear to form mixtures of correctly folded (retinal-binding) and misfolded opsins. In particular, the FG loop deletion mutants showed variation in the extent of chromophore formation. We attempted to see the

[2] A. Anukanth and H. G. Khorana, unpublished work.

TABLE I

Amino acid replacements, deletions, and other changes made in the intradiscal domain of rhodopsin

For location of the mutations, see Fig. 1.

Mutations		Restriction fragments replaced in the gene	Nucleotide nos. of the synthetic duplexes with altered codons
Deletion no.	Amino acids deleted		
N-terminal tail			
Δ1	2–10	*Eco*RI-*Fsp*I	~5–66
Δ2	11–20	*Eco*RI-*Fsp*I	~5–66
Δ3	2–34	*Eco*RI-*Bcl*I	~5–146
BC loop: deletions			
Δ4	98–99	*Bgl*II-*Nco*I	251–302
Δ5	102–103	*Nco*I-*Xho*I	302–339
Δ6	104–105	*Nco*I-*Xho*I	302–339
Δ7	106–107	*Nco*I-*Xho*I	302–339
Δ8	108–109	*Nco*I-*Xho*I	302–339
Δ9	104	*Nco*I-*Xho*I	302–339
Δ10	105	*Nco*I-*Xho*I	302–339
DE loop: amino acid replacements			
C187 → Y		*Xba*I-*Cla*I	531–571
C187 → N		*Xba*I-*Cla*I	531–571
C187 → T		*Xba*I-*Cla*I	531–571
Loop FG: deletions			
Δ11	275–276	*Nde*I-*Apa*I	806–859
Δ12	277–278	*Nde*I-*Apa*I	806–859
Δ13	279–280	*Nde*I-*Apa*I	806–859
Δ14	281–282	*Nde*I-*Apa*I	806–859
Δ15	278	*Nde*I-*Apa*I	806–859
Amino acid replacements			
Triple mutant, S281T/D282E/F283Y		*Nde*I-*Apa*I	806–859

Mutants in which the native sequences were reversed (not shown in Fig. 1)

Native sequence	Mutant no.	Reversed sequence in mutant	Restriction fragments replaced in the gene	Nucleotide nos. of the synthetic duplexes with altered codons
Loop BC				
YFVFGPT	R-1	TPGFVFY	*Nco*I-*Xho*I	302–339
102 → 108		102 → 108		
GC	R-2	CG	*Nco*I-*Xho*I	302–339
109 → 110		109 → 110		
CN	R-3	NC	*Nco*I-*Xho*I	302–339
110 → 111		110 → 111		
Loop FG				
GSDF	R-4	FDSG	*Nde*I-*Apa*I	806–859
280 → 283		280 → 283		

influence of different conditions on the extent of correct folding and consequent 500-nm absorbing chromophore formation.

Effect of Temperature

COS cells following expression of the mutants (Δ11-Δ14) were treated with 11-*cis*-retinal at 4, 20, or 37 °C under the standard conditions. No significant difference in the extent of chromophore formation was found.

Retinal Addition after Lysis of Cells and to Purified Membranes

The cells were lysed and membranes were prepared as described under "Experimental Procedures." 11-*cis*-Retinal was then added. Neither of these two variations in the method of addition of 11-*cis*-retinal affected the extent of chromophore formation.

Behavior of Wild-type and Mutant Opsins in COS Cell Membranes to Extraction with Urea

Membranes were prepared from COS cells that had expressed either the wild-type opsin or the mutant opsins (Δ11-Δ14). All the membrane preparations were washed with 5 M urea as described under "Experimental Procedures." 11-*cis*-Retinal was added under standard conditions and the membranes solubilized with the solubilization buffer (see above).

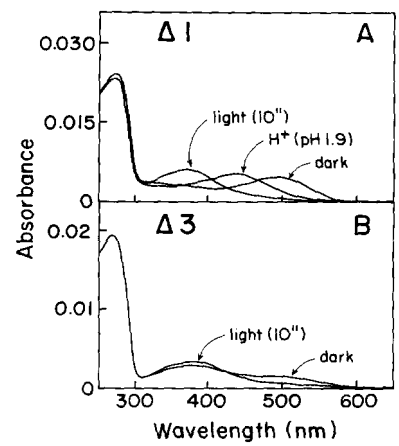

FIG. 2. **UV-Vis absorption spectra (in the dark and after illumination) of regenerated mutant rhodopsins from the mutants Δ1 (*A*) and Δ3 (*B*).** The mutant genes were expressed in COS cells, the opsins purified, and the chromophores regenerated with 11-*cis*-retinal as described under "Experimental Procedures." Note that the Δ3 mutant rhodopsin showed low chromophore formation with absorbance at 500 nm and, in addition, showed an absorbance peak at 380 nm even in the dark. Upon acidification, the latter peak shifted to 440 nm.

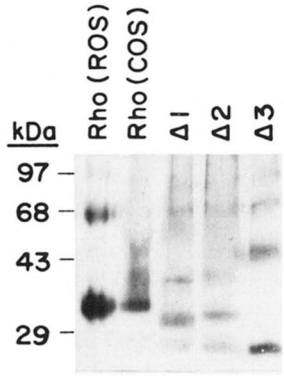

FIG. 3. **Immunoblotting, with the antibody rho-1D4, of the opsins expressed from mutants Δ1 to Δ3 after SDS-PAGE.** Wild-type rhodopsins from ROS and from COS cell are included as reference markers. Δ1 mutant lacks the Asn^2 glycosylation site while Δ2 lacks the Asn^{15} glycosylation site. Δ3 is devoid of both glycosylation sites.

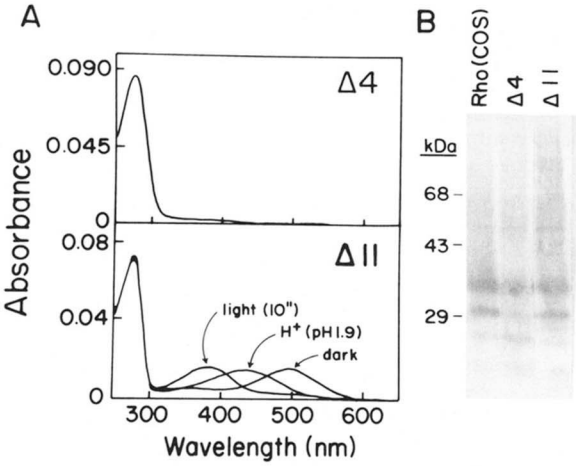

FIG. 4. *A*, UV-Vis absorption spectra of opsins from mutants Δ4 and Δ11 after chromophore generation with 11-*cis* retinal. The conditions for the different spectra in the panel for Δ11 are shown. *B*, immunoblots (with the antibody rho-1D4) of the opsins from Δ4 and Δ11 mutants after SDS-PAGE.

Purification by immunoabsorption showed normal formation of the 500-nm absorbing chromophore from the wild-type opsin. However, no opsin (280 or 500 nm absorbing) was recovered from the mutants. Thus, the misfolded mutant opsins were amenable to extraction from the membranes by 5 M urea.

Localization of the Mutant Opsins in COS Cells by Immunofluorescence

Immunofluorescence carried as out described under "Experimental Procedures" showed the results for selected mutants given in Fig. 5. The *arrows* show the location of the opsins. As is seen, wild-type opsin was present on the cell surface. In contrast, opsins from the mutants Δ4 and Δ11 were not transported to the cell surface but instead were in the endoplasmic reticulum. Similar results were obtained for all the 2-amino-acid deletion mutants in the BC and FG loops that were examined by this method (data not shown).

Characterization of Non-opsin Proteins (Chaperonins) Complexed with Mutant Opsins

Electron microscopy showed that the intradiscal opsin mutants were not transported to the cell surface (14). Immunofluorescence further showed that these mutants probably were retained in the endoplasmic reticulum (1, present work). It seemed likely that the mutant opsins were associated with certain chaperonins in the endoplasmic reticulum. Evidence

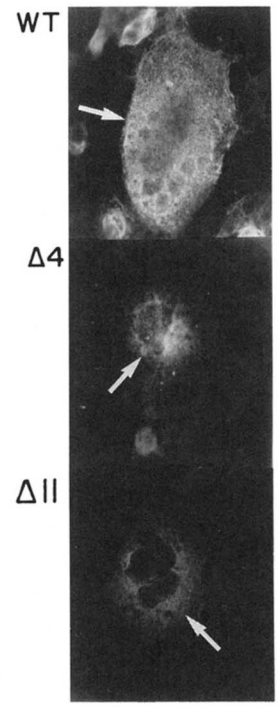

FIG. 5. **Visualization of the location of the mutant opsins in COS cells by immunofluorescence.** Shown are the fluorescence patterns of wild-type, Δ4, and Δ11 opsins. The *arrows* point to the opsin locations as shown by fluorescence. Details of the procedure are in text.

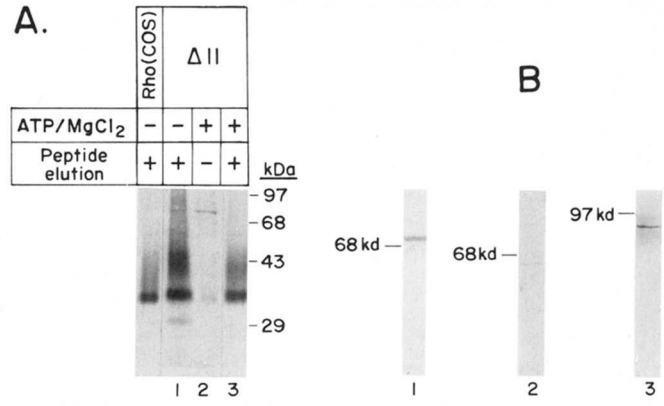

FIG. 6. **The presence of chaperonins in the mutant opsins.** *A*, silver staining of SDS-PAGE gels. Wild-type rhodopsin: total proteins eluted with antibody antipeptide without ATP/$MgCl_2$ wash. *Lanes 1–3*, Δ11 mutant opsin: ATP/$MgCl_2$ and/or peptide elution were as indicated. *B*, immunoblots with anti-chaperonin antibodies. Mutant rhodopsins were prepared and eluted from rho-1D4-Sepharose 4B as described under "Experimental Procedures." After SDS-PAGE the proteins were immunoblotted with the anti-chaperonin antibodies; *lane 1*, with anti-Grp78 (BiP) antibody; *lane 2*, with anti-Hsp60 antibody; *lane 3*, with anti-Grp94 antibody.

was obtained for this conclusion by SDS-PAGE of the eluates from the rho-1D4-Sepharose 4B of the opsins from the mutants Δ4 and Δ11. Silver staining (Fig. 6*A*) showed that at least four proteins, additional to the opsin bands, in the molecular mass range of 60–94 kDa were present. Immunoblotting with antibodies specific to Grp78 (BiP) (Fig. 6*B*, *lane 1*), to Hsp60 (Fig. 6*B*, *lane 2*), and to Grp94 (Fig. 6*B*, *lane 3*) showed binding to the specific protein bands.

Some of the chaperonins are known to be released from their complexes with the misfolded proteins by ATP + $Mg^{2+}Cl_2$. The opsins from Δ4 and Δ11 were expressed in COS cells in the presence of [^{35}S]methionine. The isotopically labeled opsins and the labeled non-opsin proteins were examined by SDS-PAGE in two separate protocols (Fig. 7). In protocol 1, the complexes of

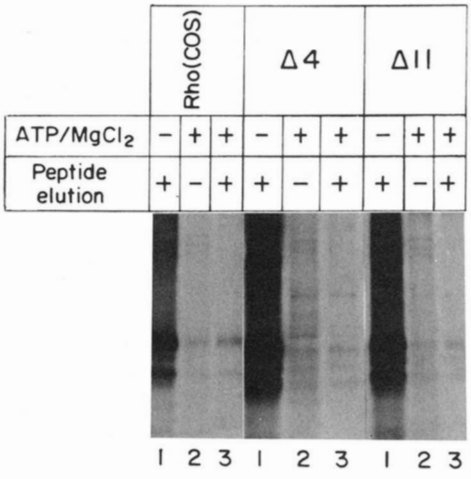

FIG. 7. **Electrophoretic patterns of wild-type opsin and opsins from the mutants Δ4 and Δ11 expressed in the presence of [^{35}S]methionine.** The opsins were adsorbed onto the rho-1D4-Sepharose 4B and eluted with the antibody antipeptide C_1-C_{18} before and after ATP-$MgCl_2$ washes in TBS. Shown are three sets of three lanes each. The *first lane* in each set contained the polypeptides eluted without ATP/$MgCl_2$ wash; *lane 2* in each set contained polypeptides eluted with ATP/$MgCl_2$, while *lane 3* in each set contained the polypeptides eluted with C_1-C_{18} peptide after ATP-$MgCl_2$ wash.

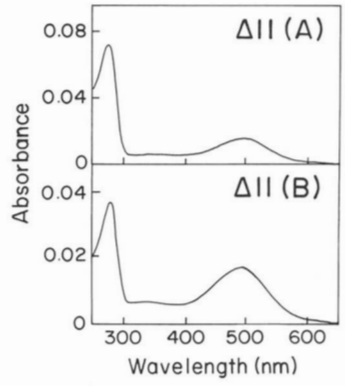

FIG. 8. **UV-Vis absorption spectra of the opsin from Δ11 mutant purified by immunoaffinity chromatography.** *A*, without ATP/$MgCl_2$ wash; *B*, after ATP/$MgCl_2$ wash.

mutant rhodopsins with rho-1D4-Sepharose 4B were incubated with ATP + $MgCl_2$ as described under "Experimental Procedures." Protocol 2: ATP + $MgCl_2$ was added to the solubilized membranes in the solubilization buffer itself. In both cases, UV visible absorption ratio A_{280}/A_{500} improved from 3 to 3.5 to about 2.1 for the FG loop mutants. This is illustrated in Fig. 8 for the mutant Δ11. Since some of the chaperonins are ATP-binding proteins, mutant Δ11, in the solubilization buffer, was first applied to ATP-agarose to remove the ATP-binding proteins. The eluate from this step was used to purify mutant rhodopsins by immunoadsorption to rho-1D4-Sepharose 4B. The mutant rhodopsin from Δ11 eluted with the antibody-antipeptide showed A_{280}/A_{500} ratio of 1.9.

DISCUSSION

We have previously proposed that there is a defined sequence in which the three domains in rhodopsin assemble *in vivo* to form the functional photoreceptor. The critical initiative rests with the intradiscal domain which must form a specific tertiary structure. Because of the obvious importance of this postulate we have now extended our previous mutagenic studies and carried out essentially comprehensive mutagenesis in the intradiscal domain. Thus, in this paper, we have now reported a variety of mutations especially in the BC and FG loops as well as additional mutations in the N-terminal tail and the DE loop. These mutations were chosen to systematically test our previous conclusion regarding the stringent requirements for the effective formation of the intradiscal structure. The present results uniformly support the above conclusion, namely that a structure involving all the segments, the N-terminal tail and the three loops in the intradiscal domain, is essential to the correct folding and assembly of the functional photoreceptor.

Commenting on the choices made in the design of mutations, we previously reported on four substantial deletions in the N-terminal tail, namely, amino acids 7–10, 11–14, 18–20, and 21–29 (14). All the four mutants formed the rhodopsin chromophore poorly, and because they remained in the ER the glycosylation in them, presumably, was of the "high mannose" type. We have now constructed three large deletion mutants that involve the glycosylation regions and, in fact, all of the N-terminal tail. Thus, Δ1, Δ2, and Δ3 (Fig. 1, Table I) deleted, respectively, the amino acids 2–10, 11–20, and 2–34. The resulting opsins formed the chromophore very poorly. Judging from the molecular weight of the opsins formed, both of the expected monoglycosylated and the unglycosylated species were produced on expression of the Δ1-Δ3 mutants.

A single large deletion, deletion of the amino acids 101–108, was previously reported in the BC loop (14). This resulted in the type II phenotype, which did not bind retinal. We have now studied in great detail the effects of alterations in this loop. We carried out deletions, one at a time, of groups of 2 amino acids (Δ4 to Δ8) or of a single amino acid (Δ9, Δ10). All gave opsins that did not bind retinal. Considering the possibility that deletions in this loop, whose approximate size is only presumed, might affect the packing of the helices, we next carried out mutations in which the sequences of the amino acids involved (Table I) were reversed. None of the three short or long sequence reversal mutants formed opsins that folded to bind 11-*cis*-retinal. It should be noted that the amino acids constituting loop BC are largely conserved within the opsin families (23–25). In particular Gly^{106} appears to be conserved most (4–6). Its replacement by Arg or Trp has been found in patients with the inherited genetic disorder disease autosomal dominant retinitis pigmentosa (25).

The loop DE was studied quite exhaustively in the previous work (14). In the present work, the 3 single amino acid substitution mutants, C187Y, C187N, and C187T, all resulted in opsins that failed to bind 11-*cis*-retinal. Mutations affecting cysteines 110 and 187, the components of a conserved disulfide bond, have been studied in detail separately (17).

Earlier a 4-amino-acid deletion in the FG loop resulted in an opsin that was unable to bind 11-*cis*-retinal (14). In the present work, we constructed four 2-amino-acid deletion mutants, 1 single amino acid deletion mutant and a triple mutant with the amino acid replacements S281T, D282E, and F283Y. Again, with the aim of not altering the size of the loop, a sequence reversal mutant was designed. In this the polar and nonpolar amino acids as well as the charged and uncharged amino acids were all interchanged relative to the wild-type. All the above mutants showed the general phenotype which previously was classified as type III (14). Thus, in this group, the correctly folded retinal-binding structure and the misfolded structure appear to compete. In general, the above mutants formed the rhodopsin chromophore in the range of 50–70% of the wild-type. The mutations in the N-terminal tail also show a similar type III phenotype. Thus, the consequence of mutations in the two regions, N-terminal tail and the FG loop, is not as severe as that from the mutations in the BC loop and in the conserved part of the DE loop.

Recently, a general method has been developed for the sepa-

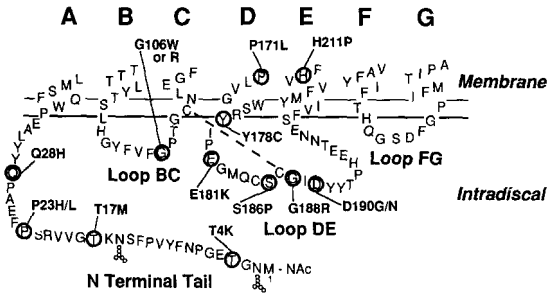

FIG. 9. **Natural point mutations in the intradiscal domain of human rhodopsin in retinitis pigmentosa.**

ration of the correctly folded retinal binding and the misfolded opsins.[3] This method was successfully used for the preparation of the above type III mutant rhodopsins with wild-type $\epsilon_{280}/\epsilon_{500}$ ratio from several of the above (type III) mutants. The purified mutant rhodopsin showed quantitative shifting of the 500-nm absorption peak to the 380 nm peak on photobleaching. They also showed the presence of the protonated Schiff base (λ_{max}, 440 nm) on treatment of the bleached rhodopsins with acid. Finally, like the wild-type rhodopsin, the purified type III rhodopsins were inert to hydroxylamine in the dark. Therefore, in all these characteristics the correctly folded mutant rhodopsin, purified form the type III mutants, were similar to the wild-type rhodopsin.

A common property of all of the mutant opsins (that have been examined for their localization in the COS cells) is that they remain in the endoplasmic reticulum. Since these mutants are very likely misfolded, the possibility was considered that they are present in the endoplasmic reticulum as complexes with non-opsin proteins such as chaperonins. This has been demonstrated for a variety of mutants. The results are illustrated in Figs. 6 and 7 for the mutants Δ4 and Δ11. The association of several other opsin mutants with chaperonins has also been clearly established.[4] At least some of the chaperonin proteins involved in these complexes have been identified by their mobilities as well as their immunoreactivity against the corresponding antibodies. These are Grp78, Hsp60, and Grp94. In another study evidence has been obtained for specific complexation of a mutant opsin with peptide disulfide isomerase.[5] These results are all in agreement with the wealth of data that has accumulated for association of malfolded proteins with chaperonins.

[3] K. D. Ridge, and H. G. Khorana, manuscript in preparation.
[4] Z. Lu, K. D. Ridge, and H. G. Khorana, unpublished work.
[5] A. S. M. Krishnarao and H. G. Khorana, unpublished work.

Recently, there has been a surge of information on the nature of mutations in the human rhodopsin gene that cause autosomal dominant retinitis pigmentosa. These have been identified mostly as point mutations occurring in all the three structural domains (Fig. 1). We illustrate in Fig. 9 the naturally occurring mutations in the intradiscal domain, for comparison with the mutants that we have reported previously (14) and in this paper. There is a close parallel between the phenotypes of our mutations and those that occur naturally (1).

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REFERENCES

1. Kaushal, S., and Khorana, H. G. (1994) *Biochemistry* **33,** 6121–6128
2. Khorana, H. G. (1992) *J. Biol. Chem.* **267,** 1–4
3. Dohlman, H. G., Caron, M. G., and Leftkowitz, R. J. (1987) *Biochemistry* **26,** 2657–2664
4. Ovchinnikov, Y. A., Abdulaev, N. G., Feigina, M. Y., Artamonov, I. D., Zolotarev, A. S., Kostina, M. B., Bogachuck, A. S., Moroshinikov, A. I., Martinov, V. I., and Kudelin, A. B. (1982) *Bioorg. Khim.* **8,** 1011–1014
5. Hargrave, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fong, S.-L., Rao, J. K. M., and Argos, P. (1983) *Biophys. Struct. Mechn.* **9,** 235–244
6. Nathans, J., and Hogness, D. S. (1983) *Cell* **34,** 807–814
7. Wald, G. (1968) *Nature* **219,** 800–807
8. Chabre, M., and Deterre, P. (1989) *Eur. J. Biochem.* **179,** 255–266
9. Stryer, L. (1986) *Annu. Rev. Neurosci.* **9,** 87–119
10. Karnik, S. S., Sakmar, T. P., Chen, H.-B., and Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8459–8463
11. Nakayama, T. A., and Khorana, H. G. (1990) *J. Org. Chem.* **55,** 4953–4956
12. Sakmar, T. P., Franke, R. R., and Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 8309–8313
13. Sakmar, T. P., Franke, R. R., and Khorana, H. G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3079–3083
14. Doi, T., Molday, R. S. and Khorana, H. G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4991–4995
15. Franke, R. R., Sakmar, T. P., Graham, R. M., and Khorana, H. G. (1992) *J. Biol. Chem.* **267,** 14767–14774
16. Karnik, S. S., Ridge, K. D., Bhattacharya, S., and Khorana, H. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 40–44
17. Davidson, F. F., Loewen, P. C., and Khorana, H. G. (1994) *Proc. Natl. Acad. Sci. U. S. A.,* **91,** 4029–4033
18. Oprian, D. D., Molday, R. S., Kaufman, R. J., and Khorana, H. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8874–8878
19. Pelham, R. B. (1986) *Cell* **46,** 959–961
20. Ferretti, L., Karnik, S. S., Khorana, H. G., Nassal, M., and Oprian, D. D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 599–603
21. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
22. Khorana, H. G., Knox, B. E., Nasi, E., Swanson, R., and Thompson, D. A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7917–7921
23. Applebury, M. L., and Hargrave, P. A. (1986) *Vision Res.* **26,** 1881–1895
24. Yokoyama, R., and Yokoyama, S. (1990) *Vision Res.* **30,** 807–816
25. Fishman, G. A., Stone, E. M., Gilbert, L. D. and Sheffield, V. C. (1992) *Arch. Ophthalmol.* **110,** 646–653