Structure and Function in Rhodopsin. 7. Point Mutations Associated with Autosomal Dominant Retinitis Pigmentosa[†,‡]

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ABSTRACT: Autosomal dominant retinitis pigmentosa (ADRP) is a hereditary form of retinitis pigmentosa which accounts for about 15% of all types of the latter disease. Recently, close to 50 mutations, mostly point mutations, have been identified in the rhodopsin gene in ADRP patients. We have introduced these mutations in the synthetic bovine rhodopsin gene and herein report on the expression of the mutant genes in COS-1 cells and studies *in vitro* of the properties of the expressed opsins. The mutant phenotypes fall into three classes: Class I mutants are expressed in COS-1 cells at wild-type levels, form the normal rhodopsin chromophore with 11-*cis*-retinal, and are transported to the cell surface. However, on illumination, they activate transducin inefficiently. Class II mutants remain in the endoplasmic reticulum and do not bind 11-*cis*-retinal to form the chromophore. Class III mutants are expressed at low levels and form rhodopsin chromophore only poorly. They also remain in the endoplasmic reticulum and, as expected, show high mannose glycosylation. Nearly all of the mutants studied show abnormal sensitivity to light compared to the wild type, and they activate transducin less efficiently. We conclude that the majority of the ADRP mutants have folding defects.

Rhodopsin, the dim light photoreceptor of the vertebrate rod cell, is an example *par excellence* of the receptors that couple to the guanine nucleotide binding regulatory proteins (G proteins) in signal transduction. Structural models propose seven transmembrane helical segments as a common motif for this class of membrane receptors (Dohlman et al., 1991). The primary structure of rhodopsin has been determined by both protein (Ovchinnikov et al., 1982; Hargrave et al., 1983) and DNA sequencing (Nathans & Hogness, 1983). The protein contains a single polypeptide chain of 348 amino acids folded approximately to form the secondary structure pattern shown in Figure 1. 11-*cis*-Retinal serves as the chromophore, and it 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 (Wald, 1968). 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 (Chabre, 1985; Stryer, 1986).

Retinitis pigmentosa (RP) is a disease which causes retinal degeneration, and it has been observed clinically in many different forms. The symptoms include progressive night blindness, rod cell degeneration frequently accompanied by cone loss, and progressive decrease in electroretinogram potentials. The result is the loss of overall retinal function. RP is a hereditary disease which can be transmitted as an X-linked, autosomal recessive or autosomal dominant (ADRP) trait (Heckenlively, 1988). The clinical course and severity of the disease are variable, depending on the type of RP.

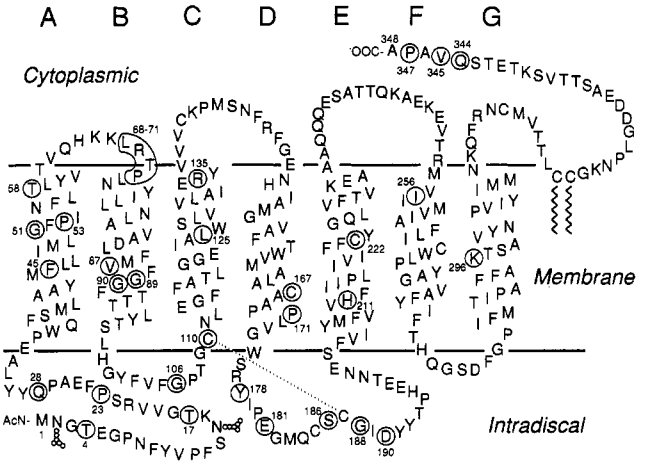

FIGURE 1: A secondary structure model of bovine rhodopsin. The putative seven membrane-embedded helical segments are indicated by the letters A–G. Approximate boundaries between the membrane-embedded domain and aqueous phase are shown by the horizontal lines. Cys-110 and Cys-187 form a disulfide bond (dashed line). Cys-322 and Cys-323 are palmitoylated (wiggly lines). The rhodopsin mutants associated with ADRP are indicated by circles around the amino acids (numbered) that are mutated. The actual mutations are systematically shown in Table 1.

Dryja and co-workers (1990) were the first to identify a point mutation, P23H, as the single change in the human rhodopsin gene associated with ADRP. Since then, well over 40 point mutations and a few deletions in the rhodopsin gene of different families of ADRP patients have been discovered by a number of research groups (Dryja et al., 1991; Sung et al., 1991; Sheffield et al., 1991; Bhattacharya et al., 1991). There is great variability in the clinical course of the disease depending on the nature of the mutation in the rhodopsin gene associated with ADRP (Dryja, 1992). The positions of the total point and deletion mutations that have been identified

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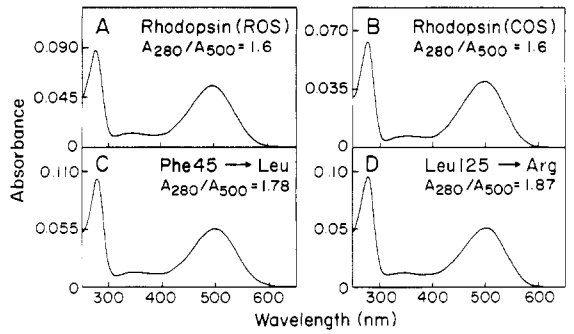

FIGURE 2: UV/vis absorption spectra in the dark of wild-type and class I ADRP mutants: wild-type rhodopsin (ROS) (panel A), rhodopsin (COS) (panel B), Phe-45 → Leu (panel C), and Leu-125 → Arg (panel D). The mutants in panels C and D are examples of class I (see text) ADRP mutants which are most like wild-type rhodopsin, except for transducin activation (see below). Methods for expression of the mutant genes in COS cells, reconstitution of the expressed opsins with 11-*cis*-retinal and immunoaffinity purification, are described in the Materials and Methods.

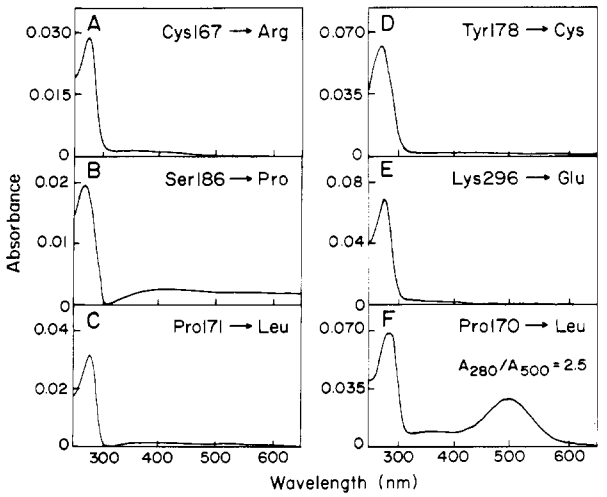

FIGURE 3: UV/vis absorption spectra in the dark of class II ADRP mutants: Cys-167 → Arg (panel A), Ser-186 → Pro (panel B), Pro-171 → Leu (panel C), Tyr-178 → Cys (panel D), Lys-296 → Glu (panel E). These are examples of class II ADRP mutants which essentially fail to regenerate the chromophore. Panel F (Pro-170 → Leu) shows the spectrum of the non-RP mutant in which the proline at position 170 was replaced by Leu, in place of the natural replacement at 171 (panel C). Methods for expression and purification of the expressed opsins are in the Materials and Methods.

are indicated in the protein secondary structure model in Figure 1, and the actual amino acid changes are shown in Table 1. Thus, the mutations are found in all three intradiscal, transmembrane, and cytoplasmic regions.

In structure–function studies, we have previously introduced by design a variety of deletions as well as point mutations in a synthetic rhodopsin gene (Nakayama & Khorana, 1990; Sakmar et al., 1989; Doi et al., 1990; Franke et al., 1992; Karnik et al., 1993; Kaushal et al., 1994; Davidson et al., 1994). Focusing, in particular, on the intradiscal domain, we observed three main types of phenotypes (Doi et al., 1990). The 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 (ER)[1] 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 this background, we have now studied most of the currently known ADRP mutations. We have cloned these mutations into a synthetic opsin gene (Ferretti et al., 1986; Oprian et al., 1987), expressed the mutant genes in COS-1 cells, purified the mutant proteins, and studied their properties. The results documented here for all of the ADRP mutants show characteristics that have parallels with the phenotypes of our previously described mutations. Recently, Min et al. (1993) have reported on some ADRP mutations that affect transducin activation.

[1] Abbreviations: DM, dodecyl maltoside; PVDF, poly(vinyl difluoride); PBS, phosphate-buffered saline; ER, endoplasmic reticulum; PMSF, phenylmethanesulfonyl fluoride; DMEM, Delbecco's modified Eagle's medium.

Table 1: Naturally Occurring Mutations (Single Amino Acid Replacements or Deletions) Associated with ADRP[a]

amino acid no.	amino acid change	amino acid no.	amino acid change
4	T → K	135	R → G
17	T → M	167	C → R
23	P → H	171	P → L
28	Q → H	178	Y → C
45	F → L	181	E → K
51	G → R, V	186	S → P
53	P → R	188	G → R
58	T → R	190	D → G, N, Y
68–71	ΔL, R, T, P	211	H → P
87	V → D	256	ΔI
89	G → D	296	K → E
90	G → D	344	Q → stop
106	G → R, W	345	V → M
110	C → Y	347	P → L, S
125	L → R		

[a] The location of the individual mutants is shown in Figure 1.

MATERIALS AND METHODS

Materials. Bovine retinas used for the preparation of rhodopsin were from J. A. Lawson Co. (Lincoln, NE). Deoxyadenosine 5′-[α-^{35}S]triphosphate (500 Ci/mmol) and guanosine 5′-[γ-^{35}S]triphosphate (1000 Ci/mmol) were from NEN. GTPγS, endonuclease H, and Triton X-114 were from Boehringer Mannheim. Dodecyl maltoside was from Anatrace. Brefeldin A and cyanogen bromide activated Sepharose 4B were from Sigma. Sequenase (version 2.0) was from United States Biochemicals Corp. DNA purification kits were from Qiagen. Nitrocellulose filters (HAWP25) and PVDF membranes were from Millipore. 11-*cis*-Retinal was a generous gift from Dr. Peter Sorter (Hoffman-LaRoche) and Dr. R. Crouch (Medical University of South Carolina and the National Eye Institute). The monoclonal antibody cell lines for 1D4 (epitope, C-terminal octapeptide sequence) and 4D2 (epitope, N-terminal amino acid sequence, 8–18) were kind gifts from Dr. R. Molday (Molday, 1988).

Construction of Rhodopsin Gene Mutants. ADRP mutations (Table 1) were introduced into the synthetic opsin gene (Ferretti et al., 1986) one at a time 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 (Ferretti et al., 1986). The amino acid replacements, the corresponding restriction fragments, and their positions in the gene are shown in Table 2. All constructions were checked by sequencing the appropriate regions corresponding to insertions of the fragments containing the modified codons.

Table 2: Restriction Fragment Replacements in the Construction of Rhodopsin Gene Mutants with Altered Codons

ADRP mutants	restriction fragment replaced in the gene	nucleotide numbers of the synthetic duplexes with altered codons
T4K, T17M	*Kpn*I–*Fsp*I	16–66
P23H, P23L	*Fsp*I–*Ban*II	66–106
Q28H, F45L	*Fsp*I–*Bcl*I	66–145
G51R, G51V, P53R, T58R	*Bcl*I–*Hin*dIII	145–204
Δ68–71	*Hin*dIII–*Bgl*II	204–251
V87D, G89D, G90D	*Bgl*II–*Nco*I	251–302
G106R, G106W, C110Y	*Nco*I–*Xho*I	302–339
L125R	*Xho*I–*Pvu*I	339–403
R13L	*Pvu*I–*Aha*II	403–472
C167R	*Aha*II–*Xba*I	472–531
P170L, P171L, Δ170–171	*Sfi*I–*Cla*I	507–571
Y178C, E181K, S186P, G188R	*Xba*I–*Cla*I	531–571
D190G, D190N, D190Y	*Cla*I–*Ava*II	571–632
H211P	*AvaII*–*Msc*I	632–676
I256Δ, Δ255–256	*Sal*I–*Not*I	1004–1054

The mutant opsin genes corresponding to V87D, G89D, and G90D (Table 2) were constructed in the expression/cloning vector designated pSK, which was constructed as follows. The opsin gene, as the *Eco*RI–*Bam*HI fragment from the vector pMT4 (Franke et al., 1992), was cloned downstream from the CMV promoter in the vector pCMV5 (Andersson et al., 1989). The latter vector contains the 5′-untranslated region of the alfalfa mosaic virus 4 RNA, a sequence that acts as a translational enhancer by decreasing the requirements for initiation factors in protein synthesis. The vector pSK now constructed offers the advantage that a greater number of the restriction sites in the synthetic gene are now unique. The expression levels of the wild-type opsin gene in COS-1 cells were similar to those of the pMT4 and pSK vectors.

The mutant opsin genes for G106R, G106W, and C110Y were first cloned into the vector pOP3 (Khorana et al., 1988). The mutant opsin gene was excised from this vector as an *Eco*RI–*Not*I fragment and ligated into the *Eco*RI–*Not*I large fragment of pMT4. Before transfecting COS-1 cells with this vector, the presence of the mutation in the mutant gene was confirmed by sequencing.

Expression and Purification of Mutants. The procedures for the culturing and transfection of COS-1 cells have been previously described (Oprian et al., 1987). The methods of immunoaffinity purification using the monoclonal antibody 1D4 bound to Sepharose 4B have also been described (Oprian et al., 1987). A typical procedure used in the present work was as follows: COS-1 cells were plated at a density of (1.25–1.5) × 10^7 cells per 150 × 25 mm culture dish and transfected 10–14 h later with 12.5 μg of purified plasmid DNA. The cells were harvested 70–80 h after transfection, washed with 10 mM NaH_2PO_4 (pH 7.0) containing 150 mM NaCl (PBS), and incubated with 5 μM 11-*cis*-retinal for 3 h at 4 °C in the dark. After solubilization in 1% DM, the pigments were purified by immunoaffinity adsorption on 1D4–Sepharose (Karnik et al., 1993). The resin was thoroughly washed with either (1) 10 mM Tris–HCl (pH 7.0) containing 150 mM NaCl and 0.1% DM or (2) 2 mM NaH_2PO_4 (pH 6.0) containing 0.1% DM. The bound rhodopsin was eluted with the peptide corresponding to the carboxyl-terminal 18 amino acids of rhodopsin (35 μM) in the appropriate buffer. Bovine rhodopsin from ROS was purified by the same procedure. Rhodopsin preparations typically had absorbance ratios (A_{280}/A_{500}) of 1.6–2.0.

Expression of Rhodopsin in the Presence of Brefeldin A. Brefeldin A has been found to block protein transport from the ER to the Golgi by disrupting the Golgi membrane structure (Lippincott-Schwartz et al., 1988). Our aim therefore was to see if the presence of Brefeldin A during the expression of opsin would still permit the accessibility of 11-*cis*-retinal to the ER membrane in intact whole cell chromophore regeneration studies. Brefeldin A was added to the pMT4-transfected cells immediately after the cloroquine incubation at a concentration of 3.0 μg/mL.

Bleaching Properties of the ADRP Mutants. The mutant opsins that formed the rhodopsin-like chromophores with 11-*cis*-retinal were studied for their bleaching properties. They were irradiated (495 nm except for G90D which was illuminated with white light) for varying lengths of time at 20 °C and the solutions then acidified by the addition of 2 N H_2SO_4 to a final pH of 1.9. The absorption peak at 440 nm obtained on acidification reflected the amount of the retinal intact in Schiff base linkage with lysine-296.

Binding and Activation of Transducin. Transducin was prepared by the method of Fung et al. (1981). The GTPγS exchange assays were done as previously described (Nakayama & Khorana, 1991).

Immunofluorescence Microscopy. COS-1 cells were plated onto microscope glass coverslips and transfected. After 72 h, the cells were washed twice with cold PBS and then incubated for 5 min with ice-cold methanol and then for 5 min with ice-cold acetone to fix the cells. The fixed cells were then washed twice with PBS and incubated for 2 h with 4D2 antibody (5 μg/mL) and 1% BSA in PBS at room temperature. The cells were then washed with 1% BSA in PBS twice and incubated for 1 h with a 1:200 dilution of rhodamine-conjugated antimouse antibody. The unbound antibody was then removed by washing with PBS, and coverslips were mounted on glass slides.

RESULTS

A. Mutants in the Intradiscal Domain

a. The N-Terminal Tail: Mutants T4K, T17M, P23H, P23L, and Q28H. Two mutants, T4K and T17M, expressed the proteins in similar amounts, being comparable to the wild type. These mutations occur within the two separate (N-2, N-15) consensus tripeptide sequences required for N-linked glycosylation (14). From immunoblots, both proteins were present in the mature form, correlating with the conclusion that both proteins were transported to the cell surface. As expected the opsins from both mutants had slightly smaller molecular weights (M_r = 37 000) than wild-type COS opsin. The opsins from these mutants regenerated the chromophores only partially. Thus, when purified by immunoaffinity chromatography and elution at pH 7.0, they gave absorption spectra with A_{280}/A_{500} ratios of 2.5–4.0. This result is similar to those obtained with the type III mutants previously described (Doi et al., 1990) and shows that there is a fraction of the purified opsins which is unable to bind 11-*cis*-retinal. However, when the partially regenerated mutants were eluted at pH 6.0, the spectral ratios between 1.6 and 1.8 were obtained. Thus, under these conditions pure chromophore pigments were selectively eluted (K. Ridge, unpublished results). The bleaching and transducin activation assays reported below were carried out with the pure mutant rhodopsins eluted at pH 6.

The mutants P23H, P23L, and Q28H yielded much less chromophore with 11-*cis*-retinal than the wild type. The $A_{280}/$

A_{500} absorbance ratio of chromophores formed was between 6 and 7. The opsins expressed by these mutants showed three discrete bands on gel electrophoresis as probed by immunoblotting (e.g., lane 6, fig 5). These phenotypic characteristics are as those observed with the glycosylation mutants (e.g., N15Q) and deletion mutants (e.g., Δ18–21) at the N-terminus (Doi et al., 1990). The mutant opsins which show this multiband pattern on electrophoresis show little transport of opsin to the cell surface. Instead most of the protein was found in the endoplasmic reticulum as seen by immunofluorescence microscopy (Figure 6).

b. Mutants in the Intradiscal Loops: G106R, G106W, C110Y (Loop BC), Y178C, E181K, S186P, G188R, D190G, D190N, and D190Y (Loop DE). The three mutants G106R, G106W, and C110Y in the BC loop formed chromophore with 11-*cis*-retinal to a lesser extent than the wild type as shown by the poor 280/500-nm absorbance ratios. The mutant C110Y disrupts the conserved Cys-110–Cys-187 disulfide bond; however, the opsin expressed from this mutant formed the chromophore at albeit very low levels (A_{280}/A_{500} ratio 10–12).

The point mutations in the DE loop were primarily in the conserved region of the loop (179–189) (Bhattacharya et al., 1991). The proteins formed from the mutants P171L, Y178C, E181K, S186P, and G188R did not bind 11-*cis*-retinal to form the rhodopsin chromophore to any significant extent. The average amounts of purified opsins obtained were approximately one-third of the wild-type opsin obtained from COS cells. All the opsins showed a multiband pattern on gel electrophoresis like the opsin from the mutant P23H. None of these proteins was expressed at the cell surface as observed by immunofluorescence.

The mutants D190G and D190N located in the nonconserved region of the DE loop showed wild-type levels of expression, and the opsins formed normal levels of the chromophore. These two mutant opsins also showed smears on electrophoresis characteristic of wild-type glycosylation. The mutant D190Y, on the other hand, behaved like the mutants in the conserved region in that it did not form the chromophore at all.

B. Mutations in the Transmembrane Domain

a. Helix A: Mutants F45L, G51V, G51R, P53R, and T58R. Of these, F45L and G51V represent relatively conservative hydrophobic replacements. Both mutants expressed in COS cells normally, and the opsins formed the characteristic chromophore. On electrophoresis, they showed one band with a normal glycosylation pattern. Of the mutants G51R, P53R, and T58R, which represent replacements by the positively charged arginine, T58R expressed like the wild type, and the opsin regenerated the visual chromophore. However, the mutants G51R and P53R expressed the opsins at low levels, and the latter regenerated the chromophore poorly (<1/3 of the wild type). These mutant opsins also showed the multiband patterns on gel electrophoresis characteristic of mutants that remain in the ER.

b. Helix B: Mutants Δ68–71, V87D, G89D, and G90D.[2] The deletion mutant Δ68–71 expressed the protein at a very low level, and the opsin formed very little chromophore. The three point mutants V87D, G89D, and G90D are all well within the transmembrane domain and involve insertion of the charged aspartic acid residue. The three mutants

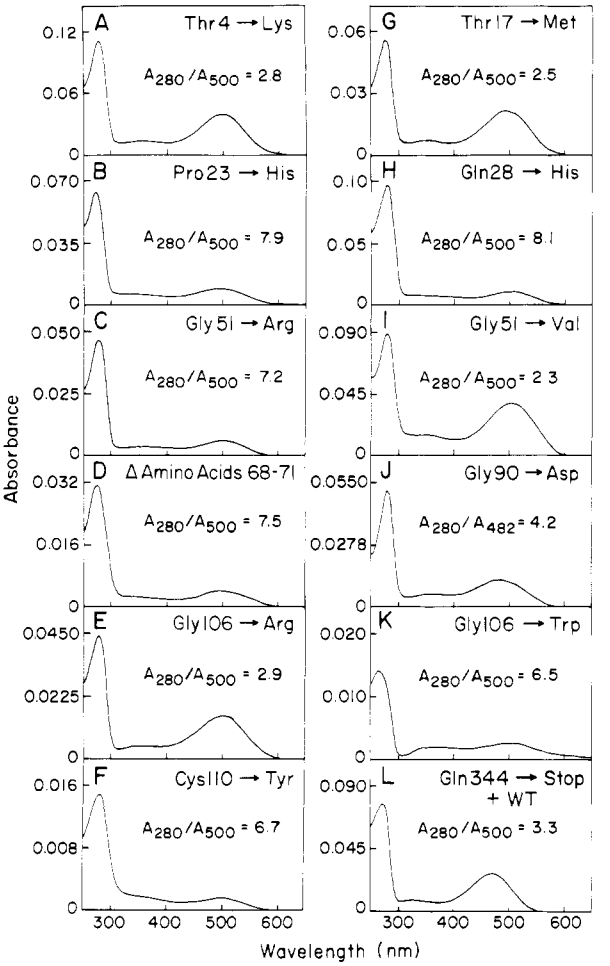

FIGURE 4: UV/vis absorption spectra in the dark of ADRP mutants (class III) that show variable but low chromophore formation with 11-*cis*-retinal. The 12 examples chosen are (panel A) Thr-4 → Lys, (panel B) Pro-23 → His, (panel C) Gly-51 → Arg, (panel D) deletion of amino acids 68–71, (panel E) Gly-106 → Arg, (panel F) Cys-110 → Tyr, (panel G) Thr-17 → Met, (panel H) Gln-28 → His, (panel I) Gly-51 → Val, (panel J) Gly-90 → Asp, (panel K) Gly-106 → Trp, and (panel L) Gln-344 → stop codon and the wild type transfected together. Class III mutants showed variable 280/500-nm spectral ratios, ranging between 2.3 and 8.1. The spectra shown were from mutants eluted from 1D4–Sepharose at pH 7.0 in the presence of 150 mM NaCl.

regenerated the chromophore albeit poorly. The mutants V87D and G89D yielded less chromophore than did G90D. Among the mutants, G90D showed a marked blue shift in the absorption spectrum (λ_{max} 480 nm). The three mutants also showed multiband patterns on immunoblots.

c. Helix C: Mutants L125R and R135L. Neither of these mutations which would be expected to change the environment around the substituting amino acids affected the level of expression or chromophore formation. As a consequence, the mutant opsins folded correctly and, on electrophoretic gels, showed normal glycosylation patterns.

d. Helix D: Mutants C167R and P171L. Neither of these mutants expressed at normal levels nor did they fold correctly as indicated by the absence of chromophore formation. The UV/vis spectrum for P171L is shown in Figure 3, panel C.

e. Helix E: Mutants H211P and C222R. Both of the mutants represent striking changes in the amino acids. Expression of the mutants was about one-third to one-quarter of the wild type, and no regeneration of the chromophore with 11-*cis*-retinal was observed.

f. Helix F: Deletion of the Amino Acid Ile-256. The single as well as double deletion of the two Ile residues expressed

[2] G90D is not strictly an ADRP mutant. It was discovered in a Michigan family with congenital nyctalopia (Sieving et al., 1992).

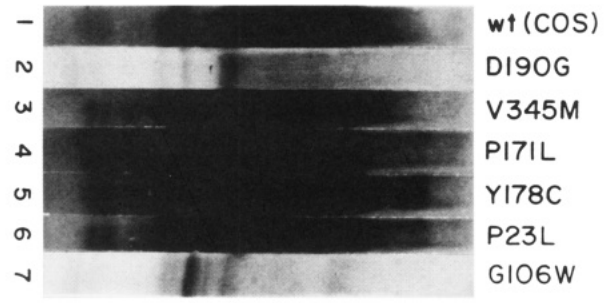

FIGURE 5: Characterization of the three types of ADRP mutants by gel electrophoresis. Examples of class I–III mutants are given. Equivalent amounts of purified proteins, as determined by the absorption at 280 nm, were analyzed by SDS–PAGE (12% separating gel) and then immunoblotted with the monoclonal antibody 1D4. Lane 1 represents wild-type COS rhodopsin; lanes 2 and 3 contain D190G and V345M, respectively (class I mutants). Lanes 4 and 5 show the class II mutants P171L and Y178C, respectively, while lanes 6 and 7 show the class III mutants P23L and G106W, respectively.

and regenerated the chromophore at very low levels.

g. Helix G: The Mutant K296E. K296E represents a substitution at the lysine residue that normally forms a Schiff base with 11-*cis*-retinal. Two additional mutants were made at this site, K296D and K296R. All three mutants expressed opsins at about three-fourths of the wild-type level. All three opsins showed the phenotype of mature opsin (glycosylation smear) on immunoblots.

C. Mutations in the Cytoplasmic Domain

Mutations at the C-Terminus: Q344stop, V345M, P347L, and P347S. Like the N-terminus, the C-terminal tail of rhodopsin is believed to be exposed to the aqueous phase (Figure 1). The mutant V345M was the only mutant that formed an opsin which could be successfully purified on 1D4–Sepharose 4B. The opsin regenerated like wild-type rhodopsin and had a similar glycosylation smear. The opsins from the mutants Q344stop, P347L, and P347S were expressed and formed the chromophores at the normal levels. This was determined from the light/dark difference spectra of whole cell solubilized extracts. The expressed opsins on electrophoresis showed single bands corresponding to the wild type. These preliminary results with the above three mutants agree with the findings that the purified proteins show wild type-like spectral characteristics and gel electrophoretic patterns (unpublished work of F. Davidson and H. G. Khorana).

D. Expression of the Mutant Q344stop in the Presence of the Wild-Type Opsin Gene

ADRP patients are heterogenous for the mutant allele; i.e., they have one copy of the mutant gene and one copy of the wild-type gene. To mimic the *in vivo* genotype, COS-1 cells were cotransfected with both the wild type and Q344stop. Since the Q344stop product does not bind to the 1D4 antibody, the effect of this mutant gene on wild-type expression could be differentiated. As seen in Figure 4 (panel L), the level of expression and the amount of chromophore were not substantially changed in the presence of Q344stop.

E. The Mutant P170L

The location of every amino acid in each helix is unique in that its orientation allows specific interactions with amino acids in the neighboring helices. We reasoned that the opsin formed from the natural mutant P171L did not allow correct folding to form the 11-*cis*-retinal binding pocket. Therefore, the position of the mutation was shifted and the mutant P170L was prepared. As shown in Figure 3 (panel F), the opsin formed generated the chromophore in reasonable amount (A_{280}/A_{500} ratio 2.5).

F. Bleaching Properties of ADRP Mutants That Regenerate the Rhodopsin Chromophore

Of the large number of purified ADRP opsins and regenerated chromophores, we show the irradiation behavior of mutants selected to illustrate the different patterns observed (Figure 7). The mutants F45L (panel E), G106R, R135L, D190G, and D190N (data not shown) showed Meta-II formation (curve 2, panel E) and a 440-nm spectrum (curve 3, panel E) like that of the wild type. In the mutants T4K (panel A) and T17M (panel B) the bleaching was somewhat abnormal although the spectrum on acidification showed normal presence of the Schiff base. In the mutant T58R (panel H) a large fraction was converted to a 380-nm species although bleaching was slower than in the wild type and absorption between 450 and 500 nm persisted. The bleaching behaviors of the mutants P23H (panel C), Q28H (panel D), and G51R (panel F) to form Meta-II were all very abnormal. The mutant G51V (panel G) formed a 480-nm-absorbing species, consistent with Meta-I formation, that only slowly converted to a 380-nm species. Subsequent acidification showed the formation of a Schiff base-like spectrum (430-nm peak). Finally, the mutant G90D, which showed the blue-shifted chromophore (480 nm) in the dark, bleached rapidly at room temperature (panel I) and, upon acidification, did not show any species absorbing between 420 and 440 nm. The entire 380-nm species seemed to represent free *all-trans*-retinal. When bleaching was carried out at 4 °C (panel J), the formation of an intermediate absorbing at 460 nm was first observed, and this slowly converted to a 380-nm species upon further illumination.

G. Transducin Activation by ADRP Mutants Following Illumination

Figure 8A shows transducin activation as measured by the GTPγ^{35}S assay by wild-type rhodopsin and the mutants T4K and T17M as a function of the concentration of the rhodopsins. As is seen the light-activated mutants coupled poorly to transducin activation. Similarly, Figure 8B shows a comparison of the wild-type rhodopsin and the mutants F45L and L125R. In general, the low efficiency of coupling of the mutants to transducin activation was paralleled by the instability of the meta-II intermediate as evidenced by the faster release of free *all-trans*-retinal.

DISCUSSION

The overall functions that typify a sensory system such as vision are (1) the light transduction signal which brings about the *cis-* → *trans*-retinal isomerization, (2) transduction of this signal to the cytoplasmic domain where the ensuing cascade of biochemical reactions results in amplification of the signal, (3) adaptation to the signal, and, finally, (4) shutoff of the signal. We are interested in understanding the mechanisms involved in all of these functions. By extension, progress toward these goals should provide insights into the mechanisms of the G-protein-coupled seven helical receptors, in general. Our first goal is to understand the specific functions that the individual domains in rhodopsin (Figure 1) perform. We can then hope to understand how these functions are interregulated within the three domains. In our current work,

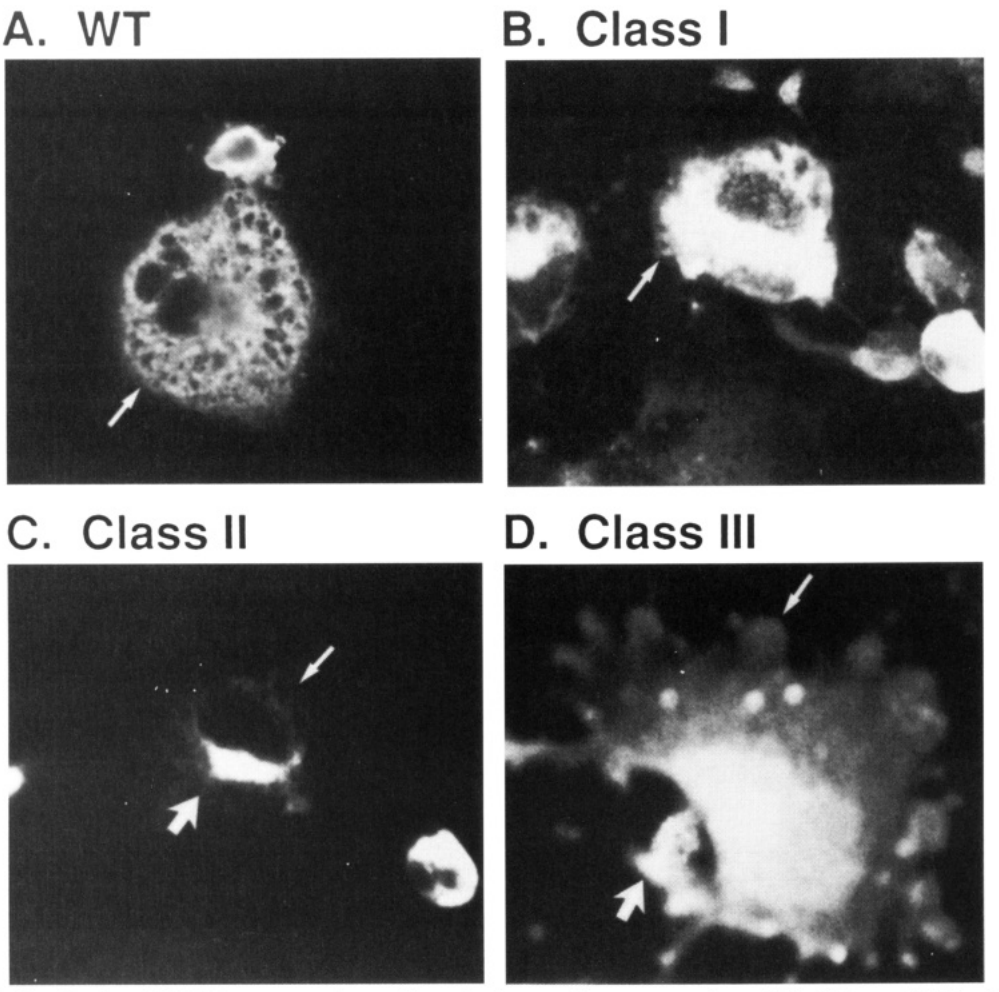

FIGURE 6: Cellular localization of ADRP mutant opsins as shown by immunofluorescence: (panel A) wild-type COS-1 cell opsin, (panel B) the mutant P347S (class I), (panel C) the mutant D190Y (class II), (panel D) the mutant P23H (class III). The thin arrow represents the cell surface for all four panels. The thick arrows in panels C and D represent perinuclear staining consistent with localization in the endoplasmic reticulum. Table 3 shows the percentage of live cells that also expressed opsin at the cell surface. The primary antibody was 1D4 in all cases except Q344stop and P347S; in those two instances the N-terminal antibody 4D2 was used.

a principal interest is in understanding the *in vivo* assembly and folding of the nascent opsin polypeptide chain to the correct functional molecule.

Recently, we have obtained valuable insights into structure–function relationships in rhodopsin by designed mutagenesis in every one of the three domains. In particular, focusing on the intradiscal domain, we concluded that the formation of a precise tertiary structure comprising the N-terminal tail and all the loops in the intradiscal domain is an early requirement for rhodopsin assembly. The alignment of the seven transmembrane helices to form a cluster with specific interhelical interactions and, consequently, a retinal-binding pocket spanning the helices is a consequence of the above intradiscal structure. We further infer that the organization of the cytoplasmic domain to a specific tertiary structure automatically follows the above-described assembly of the transmembrane domain. Thus, the primary responsibility for the eventual formation of fully functional rhodopsin rests with the intradiscal domain. Consistent with this conclusion is the general finding that, in contrast with the critical folding requirements in the intradiscal domain, mutations in the cytoplasmic loops do not affect the folding of the receptor unless they affect the packing of the helices (Franke et al., 1992).

Clearly large questions concerning the mechanisms in signal transduction remain unanswered. The large number of natural mutations in rhodopsin now identified with ADRP represent a great boon to further structure–function studies of rhodopsin. Because these mutations are mostly single amino acid changes found in all of the three domains, they provide a great wealth of definitive opportunities for structural studies. Therefore, we have initiated a program of systematic studies upon them. In this initial report we have described the cloning of essentially all of the known mutations in our synthetic opsin gene, expression of the mutant genes in COS-1 cells, and characterization *in vitro* of the opsins obtained in regard to their chemical and biochemical properties. We comment below on the results obtained according to the domains where the mutations occur (Table 1). The mutations are classified in Table 3 according to their phenotypes.

Point mutations in the intradiscal domain are distributed in several conserved regions (P23H, a frequently found mutation, was the first ADRP mutation to be identified). Their phenotypes parallel those previously described for the intradiscal mutants (Doi et al., 1990; Table 3). However, the ADRP mutations are single amino acid replacements while most of the earlier mutations (Doi et al., 1990) represented deletions, albeit short. The fact that single amino acid replacements can cause phenotypes similar to those found previously emphasizes the stringent requirements for the tertiary structure in this domain. The mutation C110Y is particularly interesting in that it prevents the formation of the conserved Cys-110–Cys-187 disulfide bond. A separate study has shown that certain substitutions of these cysteines

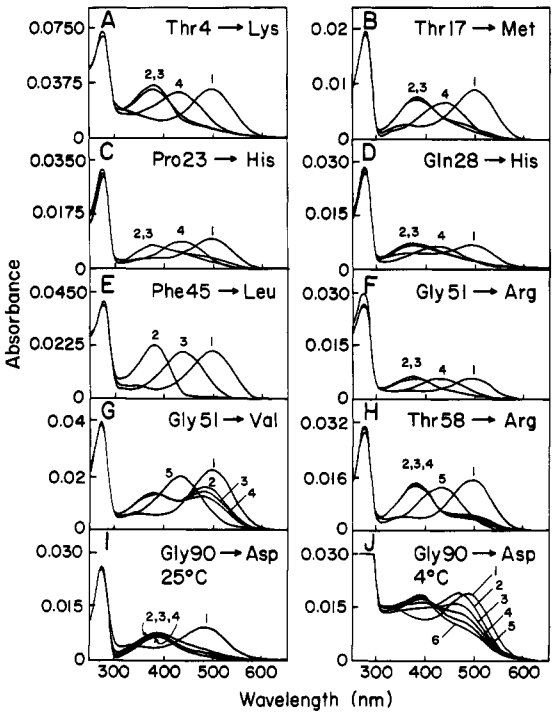

FIGURE 7: Absorption spectral shifts as observed on bleaching of different ADRP mutants: (panel A) Thr-4 → Lys, (panel B) Thr-17 → Met, (panel C) Pro-23 → His, (panel D) Gln-28 → His, (panel E) Phr-45 → Leu, (panel F) Gly-51 → Arg, (panel G) Gly-51 → Val, (panel H) Thr-58 → Arg, (panel I) Gly-90 → Asp (25 °C), and (panel J) Gly-90 → Asp (4 °C). In all cases, the absorption spectra in the dark are labeled 1, and each subsequent number represents a 10-s illumination with a fiber optic light source with a 495-nm cutoff filter. In the case of Gly-90 → Asp no cutoff filter was used. The spectrum with the largest number in each panel represents the chromophore on acidification.

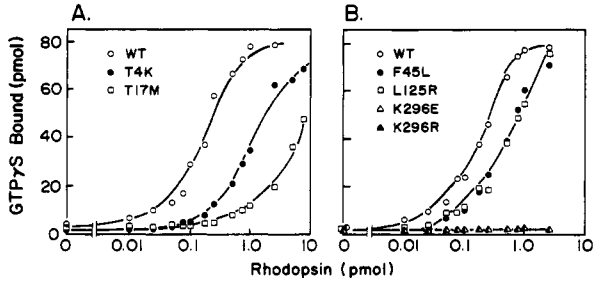

FIGURE 8: Transducin activation by light-activated ADRP mutants. The binding and activation of transducin as a function of rhodopsin concentration were measured as described under the Materials and Methods. The ADRP mutants used in the assay were immunopurified using 1D4–Sepharose, elutions being carried out with 2 mM NaH_2PO_4, pH 6.0, in 0.1% DM in the absence of any salt. The amounts of the mutant rhodopsins used in the assay were based on the molar extinction coefficient.

do allow correct folding and chromophore formation but affect the Meta-II state (Davidson et al., 1994).

The transmembrane domain is the site of primary response to the light-induced chromophore isomerization. To understand the dynamics of coupling of the *cis* → *trans* switch to the structural change in the helical cluster leading to presumed conformational changes in the cytoplasmic domain, we need to understand the orientations of the seven helices relative to one another. Specific interhelical interactions stabilize the helical cluster and lead to the precise formation of the retinal-binding pocket. We also note that the extensive experience obtained with the helical bundle in bacteriorhodopsin (Khorana, 1993) provides general guidelines for the study of the helical bundle in rhodopsin. The fundamental principles

Table 3: ADRP Mutants Classified According to Their Phenotypes[a]

class I	class II	class III
F45L	C167R	T4K
T58R	P171L	T17M
L125R	Y178C	P23H
R135G	E181K	Q28H
D190G	S186P	G51R
D190N	G188R	G51V
Q344stop	D190Y	P53R
V345M	H211P	Δ68–71
P347L	C222R	V87D
P347S	K296E	G89D
		G90D
		G106R
		G106W
		C110Y
		I256Δ

[a] Class I closely resembles wild-type rhodopsin expressed in COS cells. Thus, these mutants fold correctly to bind 11-*cis*-retinal and form the characteristic rhodopsin chromophore. Class II mutants are defective in folding and stay in the endoplasmic reticulum. They do not bind 11-*cis*-retinal. Class III mutants also remain in the endoplasmic reticulum. They bind 11-*cis*-retinal and regenerate the 500-nm-absorbing chromophore only partially.

governing movement of helices following light transduction would clearly be similar in the two proteins. Thus, as in bacteriorhodopsin, the protonation and deprotonation states of the Schiff base in rhodopsin including interaction of the latter with the glutamate-113 counterion in helix C will be important facets of the signal transduction process (Matthews et al., 1963; Hofmann, 1986; Cohen et al., 1992). Thus, the ADRP point mutations in the transmembrane domain should provide important clues to the helix–helix interactions and, by inference, the orientations of different helices, indicating the amino acids required for cluster formation and those facing the periphery of the bundle and into the boundary phospholipids.

As seen by their effects on ground-state folding of the opsin to form rhodopsin-like chromophore with 11-*cis*-retinal, three general and expected phenotypes were observed for the mutants in the transmembrane domain. Thus, in helix A, the mutations F45L and G51V are examples of replacements of a hydrophobic amino acid by another. Both the mutants formed opsins that formed the wild-type chromophore. However, many of the amino acid replacements in the transmembrane domain (G51R, P53R, T58R, V87D, G89D, G90D, L125R, C167R) are replacements by charged amino acids. Aspartic acid, a frequent replacement, may not cause any perturbation within the bundle by being uncharged (protonated). However, the substitutions V87D and G89D caused poor formation of the chromophore, while G90D was particularly interesting in that it caused a marked blue shift (to 480 nm) in the chromophore. Substitution by arginine would cause serious perturbation without a counterion, unless its side chain projects out to the external face of the bundle. Presumably, the latter is the case in T58R, which formed the chromophore like the wild type.

In all these amino acid replacements the actual location of the side chain of an amino acid around the helix would be specifically important. An illustrative example was provided by shifting the mutation P171L (panel C, Figure 3), which did not fold to regenerate the chromophore at all, to the adjacent position. The mutant P170L (panel F, Figure 3) folded and regenerated the chromophore very well.

Dramatic effects of single amino acid insertions and deletions in helices were demonstrated previously in bacteriorhodopsin (Marti et al., 1992). Examples of these in rhodopsin are provided by the deletion 68–71 and by the deletions Δ256 and

Δ255–256 (Figure 1). These mutants were expressed poorly and formed chromophores at very low levels. Clearly, the packing of the helices was affected.

Finally, the cytoplasmic domain represents the culmination of the signal transduction and is responsible for all the biochemistry that is encountered in the visual sensory system. Thus, all the protein–protein interactions and, consequently, regulation of all the sensory functions occur in this domain. Little is known at the molecular level regarding the ground-state structure of this domain and the nature of the conformational change(s) that occur as a result of the impact from the transmembrane domain. However, a significant recent insight has been that all three structural domains in rhodopsin are dynamically involved in the attainment of the Meta-II state that efficiently couples to transducin activation (Kaushal et al., 1994; Davidson et al., 1994). From the present studies, it emerges as an important conclusion that a large number of ADRP mutants couple inefficiently to transducin activation (examples in Figure 8), although they can form the correctly folded ground-state structures.

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ADDED IN PROOF

We have noted the recent publication by C.-H. Sung, C. M. Davenport, and J. Nathans [(1993) *J. Biol. Chem. 268*, 26645–26649] entitled "Rhodopsin Mutations Responsible for Autosomal Dominant Retinitis Pigmentosa."

REFERENCES

Adamus, G., Zam, Z. S., Arendt, A., Palczewski, K., McDowell, J. H., & Hargrave, P. A. (1991) *Vision Res. 31*, 17–31.

Andersson, S., Davis, D. N., Dahlback, H., Jornvall, H., & Russell, D. W. (1989) *J. Biol. Chem. 264*, 8222–8229.

Bhattacharya, S. S., Inglehearn, C. F., Reen, J., Lester, D., Bashir, R., Jay, M., & Bird, A. C. (1991) *Invest. Ophthalmol. Visual Sci. 32* (suppl.), 890.

Bordier, C. (1981) *J. Biol. Chem. 256*, 1604–1607.

Chabre, M. (1985) *Annu. Rev. Biophys. Chem. 14*, 331–360.

Cohen, G. B., Oprian, D. D., & Robinson, P. R. (1992) *Biochemistry 31*, 12592–12601.

Davidson, F. F., Loewen, P. C., & Khorana, H. G. (1994) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Dohlman, H. G., Thorner, J., Caron, M. G., & Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653–688.

Doi, T., Molday, R. S., & Khorana, H. G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4991–4995.

Dryja, T. P. (1992) *Eye 6*, 1–10.

Dryja, T. P., McGee, T. L., Reichel, E., Hahn, L. B., Cowley, G. S., Yandell, D. W., Sandberg, M. A., & Berson, E. L. (1990) *Nature (London) 343*, 364–366.

Dryja, T. P., Hahn, L. B., Cowley, G. S., McGee, T. L., & Berson, E. L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9370–9374.

Ferretti, L., Karnik, S. S., Khorana, H. G., Nassal, M., & Oprian, D. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 599–603.

Franke, R. R., Sakmar, T. P., Graham, R. M., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 14767–14774.

Fung, B. K. K., Hurley, J. B., & Stryer, L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 152–156.

Hargrave, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fong, S.-L., Mohana Rao, J. K., & Argos, P. (1983) *Biophys. Struct. Mech. 9*, 235–244.

Heckenlively, J. R. (1988) *Retinitis Pigmentosa*, Lippencott, Philadelphia, PA.

Hofmann, K. P. (1986) *Photobiol. Photophys. 13*, 309–327.

Karnik, S. S., Ridge, K. D., Bhattacharya, S., & Khorana, H. G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 40–44.

Kaushal, S., Ridge, K. D., & Khorana, H. G. (1994) *Proc. Natl. Acad. U.S.A.* (in press).

Khorana, H. G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1166–1171.

Khorana, H. G., Knox, B. E., Nasi, E., Swanson, R., & Thompson, D. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7917–7921.

Lippincott-Schwartz, J., Bonifacino, J. S., Yuan, L. C., & Klausner, R. D. (1988) *Cell 54*, 209–220.

Marti, T., Otto, H., Rosselet, S. J., Heyn, M. P., & Khorana, H. G. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1219–1223.

Matthews, R. G., Hubbard, R., Brown, P. K., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215–240.

Min, K. C., Zvyaga, T. A., Cypess, A. M., & Sakmar, T. P. (1993) *J. Biol. Chem. 268*, 9400–9404.

Molday, R. S. (1988) in *Progress in Retinal Research* (Osborne, N. N., & Chader, G. J., Eds.) Vol. 8, Pergamon Press, London.

Nakayama, T. A., & Khorana, H. G. (1991) *J. Biol. Chem. 266*, 4269–4275.

Nathans, J., & Hogness, D. S. (1983) *Cell 34*, 807–814.

Oprian, D. D., Molday, R. S., Kaufman, R. J., & Khorana, H. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8874–8878.

Ovchinnikov, Y. A., Abdulaev, N. G., Feigina, M. Y., Artamonov, I. D., Zolotarev, A. S., Kostina, M. B., Bogachuk, A. S., Miroshnikov, A. I., Martynov, V. I., & Kudelin, A. B. (1982) *Bioorg. Khim. 8*, 1011–1014.

Sakmar, T. P., Franke, R. R., & Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8309–8313.

Sheffield, V. C., Fishman, G. A., Beck, J. S., Kimura, A. E., & Stone, E. M. (1991) *Am. J. Hum. Genet. 49*, 699–706.

Shin, J., Lee, S., & Strominger, J. L. (1993) *Science 159*, 1901–1904.

Sieving, P. A., Richards, J. E., Bingham, E. L., & Naarendrop, F. (1992) *Invest. Ophthal. Vis. Sci.* (abstr. suppl.) 1397.

Stryer, L. (1986) *Annu. Rev. Neurosci. 9*, 87–119.

Sung, C. H., Davenport, C. M., Hennessey, J. C., Maumenee, I. H., Jacobson, S. G., Heckenlively, J. R., Nowakowski, R., Fishman, G., Gouras, P., & Nathans, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6481–6485.

Wald, G. (1968) *Nature (London) 219*, 800–807.