*Proc. Natl. Acad. Sci. USA*
Vol. 90, pp. 1166–1171, February 1993
Colloquium Paper

***This paper was presented at a colloquium entitled "Molecular Recognition," organized by Ronald Breslow, held September 10 and 11, 1992, at the National Academy of Sciences, Washington, DC.***

# Two light-transducing membrane proteins: Bacteriorhodopsin and the mammalian rhodopsin

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**ABSTRACT** **Site-directed mutagenesis has provided insight into the mechanisms of action of bacteriorhodopsin and rhodopsin. These studies are summarized here.**

Bacteriorhodopsin was discovered and identified as a light-dependent proton pump in the early 1970s (1), whereas the discovery of rhodopsin, the dim-light vision photoreceptor, is well over 100 years old (2). The latter serves as an example *par excellance* of the superfamily of seven-helix, G protein-coupled receptors. Rhodopsin and the color vision receptors as well as bacteriorhodopsin and related proteins in *Halobacterium halobium* all form a group that uses retinal as the chromophore. The chromophore is invariably linked to the ε-amino group of a lysine residue as a Schiff base. The action of light involves specific isomerization of a double bond in the chromophore (Fig. 1) (light transduction). This isomerization couples to specific conformational changes in the proteins (signal transduction).

## BACTERIORHODOPSIN

Electron diffraction work (3, 4) suggested a seven-helix structural motif for bacteriorhodopsin. This finding had a profound impact on our thinking about polytopic integral membrane proteins that were subsequently discovered. In the 1970s and 1980s, the photocycle in bacteriorhodopsin received a great deal of attention in order to understand the features of proton translocation (5).

In our work on bacteriorhodopsin the initial aim was to understand its protein chemistry, orientation in the membrane, delipidation, and reconstitution of the proton pumping function in vesicles. During these studies the remarkable discovery was made that bacteriorhodopsin could be completely denatured under a variety of conditions (e.g., treatment with trifluoroacetic acid) and it would refold essentially quantitatively to native functional structure (6). This finding proved to be critically important in subsequent structure–function studies. We focused on isolation and characterization of the bacterioopsin gene, developing systems for expression of the gene, site-specific mutagenesis, and, subsequently, understanding how the light-dependent proton transport worked.

**Single Amino Acid Replacements in Bacteriorhodopsin.** A number of physical scientists (M. Polanyi, T. Shedlovsky, J. Griffith, L. Onsager, M. Eigen) proposed the idea of an extended hydrogen-bonded system for facilitated proton conduction in ice (Fig. 2). This was extended by Nagle and Morowitz (7) to proton transfers in proteins.

The first aim in our work was to test the above idea in bacteriorhodopsin by interrupting possible hydrogen bond formation in the membrane-embedded helices. This was done by replacing amino acids that could form hydrogen bonds by those that could not. Further, the acidic amino acids that could function in protonated/deprotonated states were replaced by corresponding neutral amino acids (e.g., Asp → Asn). In addition, as far as possible, the substituting amino acids were smaller than the corresponding ones in the native protein. Table 1 shows the main amino acid replacements that were made, although a considerable number of additional replacements have since been made. All of these replacements were point mutations; i.e., one amino acid was replaced at a time (8–13).

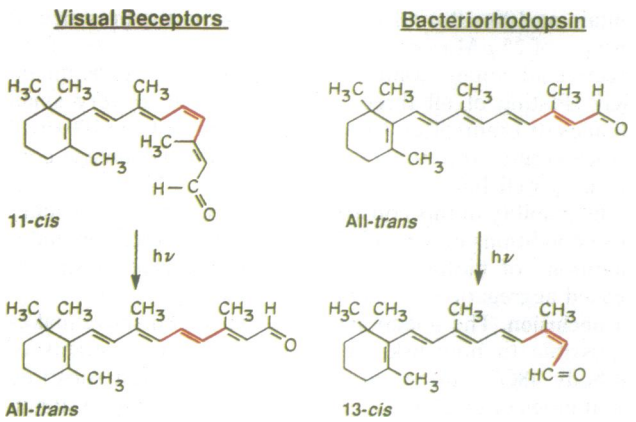


FIG. 1. The retinal chromophores and their light-induced isomerizations in visual receptors and bacteriorhodopsin.

The heterologous expression system in most of the mutant work used *Escherichia coli*, although more recently, efficient expression of the bacterioopsin gene and mutants has been developed by transformation in *H. halobium* (14). Bacteriorhodopsin and mutants do not fold correctly in *E. coli*; hence the mutants are purified (a two-step procedure) in denatured form and these are then folded and reconstituted by the standard procedure (Fig. 3). The experiment is doubly informative. First, it shows the rate of folding of the protein as reflected by the formation of the chromophore. These rates usually vary widely from that of the wild-type opsin. Second, if there are shifts in the UV/visible spectra from the wild type, these are indicated by the above procedure.

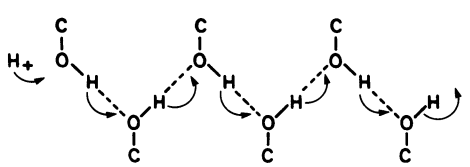

FIG. 2. Hydrogen-bonded water molecules forming a proton conductance system.

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Table 1. Replacements of individual amino acids in bacteriorhodopsin

| Wild type | No. of residues | Substitution(s) |
|---|---|---|
| Ser | 13 | Ala |
| Thr | 18 | Ala, Val |
| Asp | 9 | Asn, Glu, Ala |
| Glu | 9 | Asp, Gln, Ala |
| Tyr | 11 | Phe |
| Pro | 11* | Leu, Ala, Gly |
| Arg | 7 | Gln |
| Trp | 6 | Phe |

The amino acids in each set in the wild type were individually replaced as indicated above (8–13).
*Only the three in helices were mutated.

Early efforts at site-specific mutagenesis by oligonucleotide-directed mutagenesis were unsuccessful. A highly efficient and successful strategy was later developed which was based on the total synthesis of the opsin gene containing unique restriction sites placed at fairly uniform intervals. Mutagenesis then involved replacement of DNA fragments by synthetic counterparts containing the desired codon changes.

All of the mutants containing single amino acid replacements folded in lipid/detergent/retinal mixtures and formed bacteriorhodopsin-like chromophores. Studies of their spectral properties and proton pumping showed three types of mutants. Type 1 mutants showed no effect on the UV/visible spectrum or on proton pumping. Type 2 mutants showed spectral shifts but no marked effect on proton pumping. The spectral shifts, usually to the blue, arise from the fact that the amino acids being altered normally interact with the chromophore. About 20 amino acids thus identified are presumed to line the binding pocket of retinal (6). A similar conclusion has been drawn by Henderson *et al.* (4) from their structural studies. Type 3 mutants, constituting a small group of amino acids, severely affected proton pumping in the steady state (Table 2). Of these, the mutation Asp-85 → Asn completely abolished proton pumping. Thus the main players in proton transport by bacteriorhodopsin are as shown in Fig. 4. Replacement of several additional amino acids has also been shown to affect proton pumping, but further work is necessary to assess the significance of these results.

While the above findings provided critical information regarding the amino acids mainly involved in proton transfers during the photocycle, their specific roles needed to be determined. These were studied in detail by a variety of

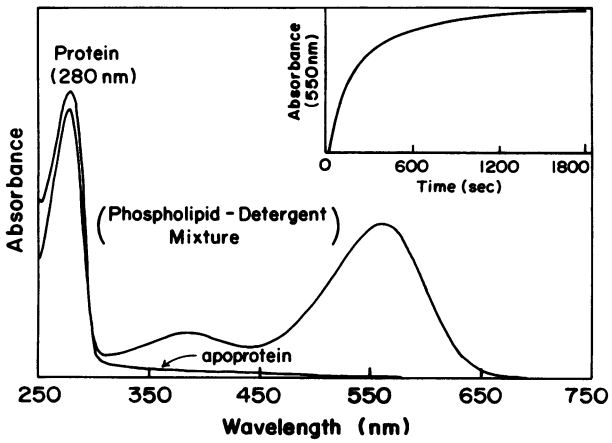


FIG. 3. Bacteriorhodopsin chromophore formation from bacterioopsin and retinal in a phospholipid/detergent mixture. (*Inset*) Rate of folding and chromophore formation.

Table 2. Amino acids whose replacement seriously affects proton pumping by bacteriorhodopsin in steady state

| Mutation | $H^+$ pumping, % |
|---|---|
| None | 100 |
| Asp-85 → Ala | 0 |
| Asp-85 → Asn | 0 |
| Asp-85 → Glu | 39 |
| Asp-96 → Ala | 2 |
| Asp-96 → Asn | 3 |
| Asp-96 → Glu | 82 |
| Asp-212 → Ala | ND |
| Asp-212 → Asn | 15 |
| Asp-212 → Glu | 6 |
| Arg-82 → Ala | 30 |
| Arg-82 → Gln | 66 |

ND, not determined.

biophysical methods—e.g., time-resolved spectroscopy in collaboration with different groups [Maarten Heyn (Freie University, Berlin), Ken Rothschild (Boston University), Vladimir Skulachev (State University of Moscow), and Tim Marinetti (Rockefeller University) and their colleagues]. Thus, Heyn and Skulachev and their coworkers studied the Asp-96 → Asn change and showed slowing of both the decay of the M intermediate and the associated proton movement (15). They also showed that Asp-96 is the internal proton donor in the reprotonation of the Schiff base (16). Further, Asp-85, Asp-212, and Arg-82 were shown to affect the proton release in the early phase of the photocycle (17). Fourier transform IR spectroscopy by Rothschild and coworkers (18) proved valuable in ascertaining protonation/deprotonation states of the aspartic acids in bacteriorhodopsin. These results and those of other groups—e.g., Osterheldt and coworkers (19, 20)—showed that Asp-85 is the acceptor of the proton from the protonated Schiff base in the early phase of the photocycle (Fig. 5). In the second half of the photocycle, the intermediate M is reprotonated internally by protonated Asp-96. Asp-96 is then reprotonated from the cytoplasmic side between the N and O intermediates (21).

Fig. 6 shows a model of the proton pump which is consistent with the present data.

The results summarized above are consistent with the established facts that the pump transports $H^+$ ions from the

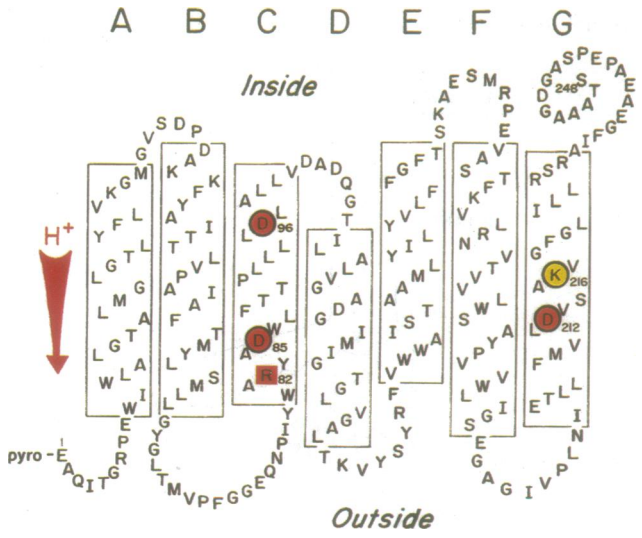


FIG. 4. A secondary structure model of bacteriorhodopsin showing the amino acids involved in proton translocation. Retinal is linked to Lys-216 (K-216) as a Schiff base. The arrow indicates the direction of proton transport.

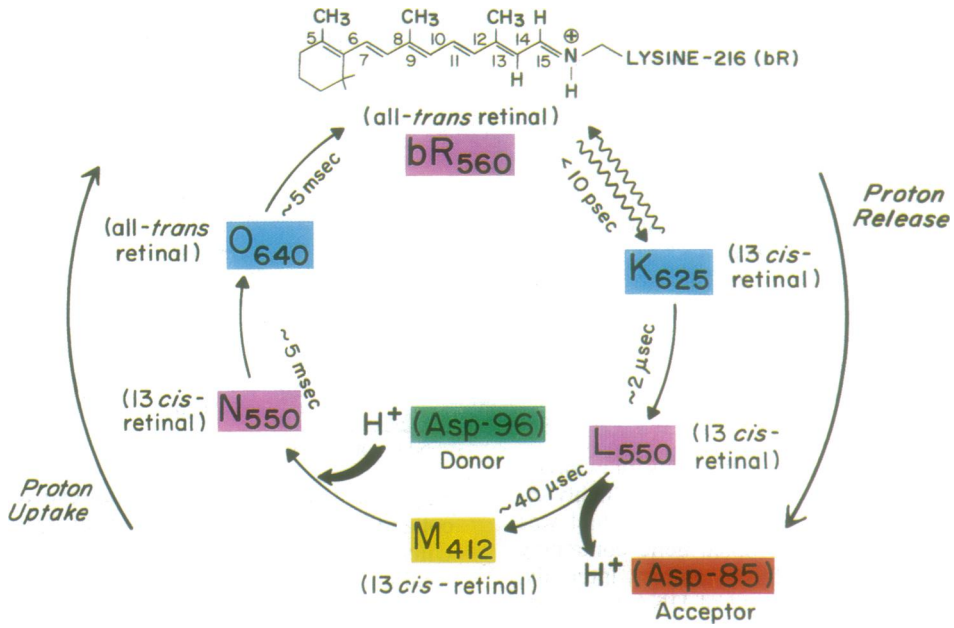


FIG. 5. Intermediates in the bacteriorhodopsin (bR) photocycle. In the first half of the cycle, a proton is released from the protonated Schiff base and transferred to Asp-85. M, the resulting unprotonated Schiff base intermediate, is reprotonated by Asp-96, which, in turn, is protonated from the cytoplasm at the N/O step.

cytoplasm to the extracellular space with a stoichiometry of one proton per cycle. The transmembrane proton translocation involves two major electrogenic steps, which are closely coupled to the formation and decay of the M intermediate of the photochemical cycle: (*i*) proton ejection from the protein interior into the periplasm and (*ii*) proton rebinding from the cytoplasmic side of the membrane. Fig. 6 also shows the apposition of helix C and helix G. Asp-85 is now close to the Schiff base and it serves as a counterion to the Schiff base as well as a proton acceptor (22).

**What We Do Not Know About the Proton Pump.** (*a*) When and how the transfer of the proton occurs from Asp-85 to the exterior of the cell remain unknown. Nor is the mechanism of proton transfer from the Schiff base to Asp-85 established (23). (*b*) The mechanism of transfer of the proton from Asp-96 to the unprotonated Schiff base is not known. The distance is large (about 12 Å) and direct transfer is excluded unless a conformational change at the appropriate time draws the groups much closer. (*c*) Does water participate in the photocycle and proton transfer? Light-dependent bleaching of bacteriorhodopsin by hydroxylamine suggests that water enters at specific steps (probably the L intermediate) in the photocycle (24). How long does water stay in and what does it do? Is water participation consistent with the fundamentals of a proton pump? (*d*) Finally, the most important and fundamental question for bacteriorhodopsin and other light-driven retinal proteins is: How do the geometrical changes in retinal couple to the structural changes in the protein?

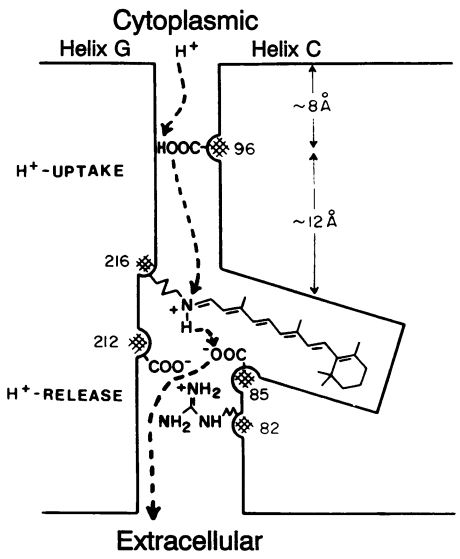


FIG. 6. A proton translocation model for bacteriorhodopsin.

## VERTEBRATE RHODOPSIN

Rhodopsin accounts for more than 90% of the protein in retinal rod cells. It consists of a single polypeptide chain of 348 amino acids of which about 50% is in the membrane-embedded domain, while the other half is distributed about equally between the cytoplasmic and the intradiscal domains. A secondary structure model for bovine rhodopsin is shown in Fig. 7. The three domains—cytoplasmic, membrane, and intradiscal—are characteristic of visual and other G protein-coupled receptors. Each domain is endowed with specific functions. Thus, these receptors work very differently from bacteriorhodopsin, which also contains seven helices but carries out proton transport mainly or exclusively through the membrane-embedded domain.

The chromophore of the visual receptors in the rods and cones of vertebrate and invertebrate retinas universally is 11-*cis*-retinal (Fig. 1), or occasionally dehydroretinal (vitamin $A_2$). 11-*cis*-Retinal is linked as a Schiff base to the ε-amino group of Lys-296 (helix G, Fig. 7). The protonated Schiff base is stabilized by the carboxylate counterion of Glu-113 (25–27).

**Signal Transduction and the Ensuing Biochemical Cascade.** Light-catalyzed isomerization of 11-*cis*-retinal to all-*trans*-retinal (Fig. 1) in rhodopsin initiates the successive formation of a number of photointermediates: rhodopsin → photorhodopsin → bathorhodopsin → lumirhodopsin → metarhodopsin I → metarhodopsin II → metarhodopsin III → opsin plus all-*trans*-retinal.

Metarhodopsin II containing unprotonated Schiff base is the active species (28) that allows binding and activation of transducin. This is the first event in the biochemical cascade

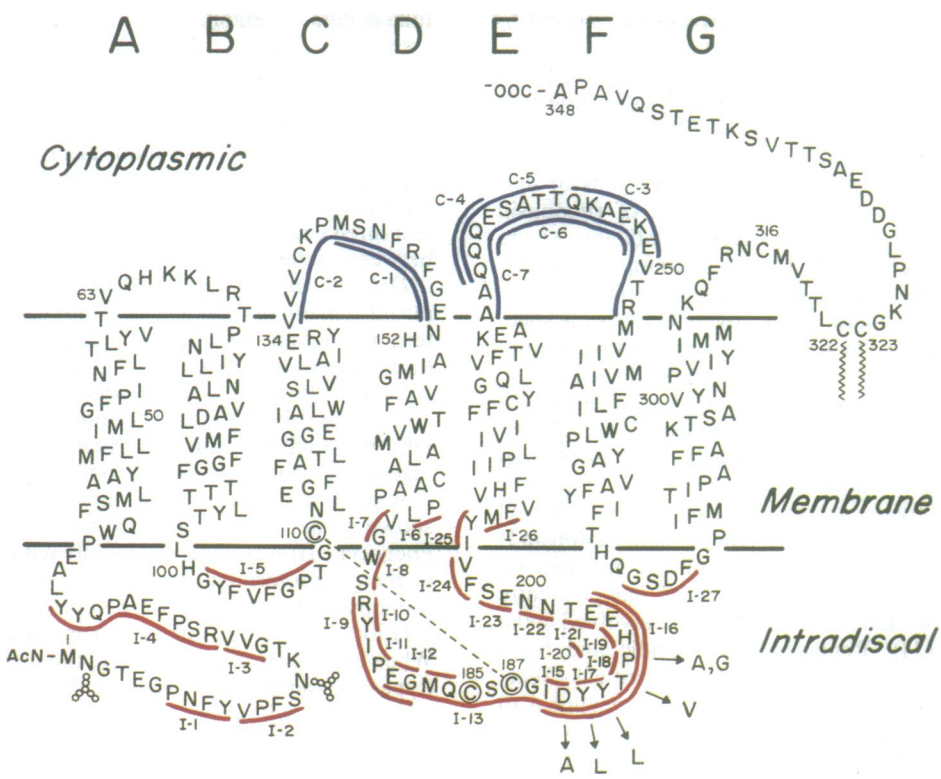


FIG. 7. A secondary structure model of bovine rhodopsin. The deletion mutations in the cytoplasmic domain and in the intradiscal domain are shown. The seven transmembrane helices are labeled A–G.

(Fig. 8) that finally leads to the closing of the cGMP-dependent cation channels in the plasma membrane and results in the hyperpolarization of the rod cell.

The nature of the conformational change(s) in the cytoplasmic domain that accompany metarhodopsin II formation is not known (see below). However, one molecule of metarhodopsin II may activate many hundreds of transducin molecules. There is also a second catalytic step in the latter part of the cascade (Fig. 8) involving cGMP phosphodiesterase. The cGMP-dependent cation conductance channel in the rod cell plasma membrane is extremely sensitive to cGMP concentration. A decrease in cGMP concentration leads to the closing of the channels. Thus, the phosphodiesterase activated as in Fig. 8 is a powerful catalyst in signal amplification.

**Signal Quenching and Adaptation.** The hallmarks of sensory systems are sensitization (amplification of the signal), desensitization (quenching and adaptation), and shutoff. The spatial organization of the reactions is remarkable. Thus, all biochemical reactions occur on the cytoplasmic face of the molecule, which is in contact with the cytoplasmic space between the discs. The principal chemical mechanism for turning off the interaction between the G protein transducin and metarhodopsin II is multiple phosphorylation by rhodopsin kinase of serines and threonines near the carboxyl terminus (29). Arrestin, a 48-kDa protein, then binds to the polyphosphorylated metarhodopsin II (30). The activation of transducin is thus terminated. Many aspects of the process of multiple phosphorylation and the chemistry of interaction between arrestin and phosphorylated rhodopsin are not understood at present.

**Structure–Function Relationships in Rhodopsin.** *Expression of a synthetic rhodopsin gene, characterization of the expressed protein, and site-specific mutagenesis.* The total

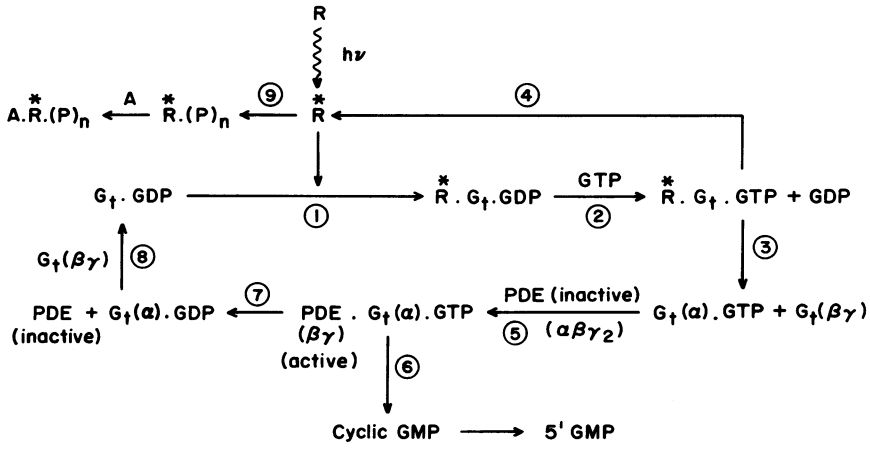


FIG. 8. Biochemical cascade on the cytoplasmic face following signal transduction in rhodopsin (R). $G_t$, transducin; $R.(P)_n$, polyphosphorylated rhodopsin; A, arrestin; PDE, phosphodiesterase.

synthesis of a rhodopsin gene (1192 bp) was carried out to facilitate mutagenesis by fragment replacement as with bacteriorhodopsin above. It contained appropriately placed unique restriction sites (31). For expression, the gene was placed in the vector shown in Fig. 9. Expression was in COS-1 cells that were transiently transfected. After 50–72 hr, 11-*cis*-retinal was added either into the intact cells or in a crude membrane preparation. Regeneration of rhodopsin chromophore before isolation enhanced the stability of the receptor. After detergent solubilization, rhodopsin was purified in one step by an immunoaffinity method (32). The purified rhodopsin was characterized by its UV/visible spectrum and by a GTPase assay following transducin activation.

*The nature of interaction between metarhodopsin II and transducin.* Previously, peptides containing sequences corresponding to the cytoplasmic loops were found to inhibit the binding of transducin to metarhodopsin II. The results suggested that as many as three cytoplasmic loops—CD, EF, and the loop formed by the membrane anchoring of palmitoyl groups—may be involved in the transducin–metarhodopsin II interaction (33) (Fig. 7). Extensive site-specific mutagenesis of the cytoplasmic loops now reported (Fig. 7) independently shows that at least two loops, CD and EF, are involved in the above interaction (34). Replacement of acidic and basic groups in loop EF showed significant but not critical effects on transducin binding and activation. Thus, there appear to be no critical electrostatic interactions between the loop EF and transducin. In contrast, the charge pair Glu-134/Arg-135 (helix C), which is conserved in all the G protein-coupled receptors, is clearly required for binding of transducin to metarhodopsin II. Thus, reversal of the charge pair abolished transducin binding and, in general, substitutions of the charged amino acids by neutral amino acids seriously affected transducin activation (34).

Of the deletions in loop CD (Fig. 7), C-1, a deletion of 8 amino acids, allowed binding of 11-*cis*-retinal but did not activate transducin. The deletion mutant C-2 failed to bind retinal. However, on replacement of the 13 deleted amino acids in C-2 with an unrelated sequence of the same length (amino-terminal residues 2–14 in rhodopsin; Fig. 7), the mutant bound retinal and transducin but did not activate the latter.

Of the five deletion mutations in loop EF (Fig. 1), C-4, containing a 4-amino acid deletion, showed about 50% of the normal activation of transducin, while none of the other mutants (C-3, C-5, C-6, C-7) showed any activity. The results suggest that the C-3 part of the EF loop, closer to the carboxyl terminus, is more important for interaction with transducin

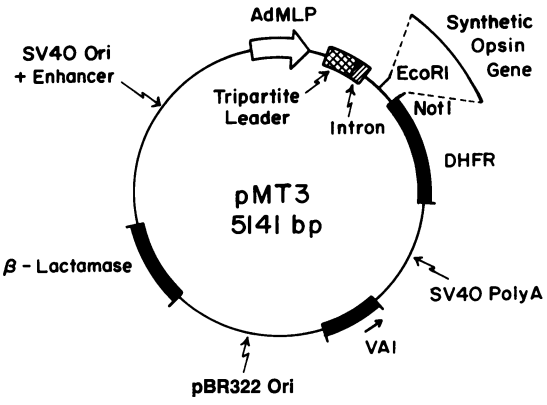


FIG. 9. The vector used in transient transfection and expression of the rhodopsin gene in COS cells. SV40, simian virus 40; Ori, origin of replication; AdMLP, adenovirus major late promoter; DHFR, dihydrofolate reductase gene; PolyA, polyadenylylation signal; VAI, viral-associated gene.

than is the C-4 region. The mutant C-6 bound transducin but showed no GTPase activity. Thus, the most significant conclusions from mutagenesis regarding transducin–metarhodopsin II interactions are as follows: (*i*) most of the deletion mutants, except those that may not allow proper packing of the helices, bind 11-*cis*-retinal; (*ii*) the metarhodopsin II intermediates formed from these mutants may bind transducin, but they may not allow its activation (34); and three CD and EF loops are both required for interaction between metarhodopsin II and transducin.

*Deletion mutagenesis indicates a structural role for the intradiscal domain.* Single deletions carried out in the amino-terminal tail and in loops BC, DE, and FG (Fig. 7) showed three groups of phenotypes (35). Phenotype I resembled wild-type rhodopsin, and it was restricted to deletions and amino acid substitutions in the nonconserved part of the loop DE, proximal to helix E. Phenotype II (Fig. 7) did not regenerate rhodopsin-like chromophore, and the glycosylation pattern was markedly different. This phenotype was shown by deletions I-5 to I-13, I-15 to I-17, and I-24 to I-27. Phenotype III was observed mainly with the deletion mutants in the amino-terminal tail. These mutants regenerated the rhodopsin chromophore only very poorly, and their glycosylation pattern was different from those of types I and II. When expressed in COS-1 cells, the majority of the mutants (types II and III) remained in the endoplasmic reticulum whereas the wild type opsin was in the plasma membrane (35). This is, presumably, because the mutant proteins are structurally defective. The fact that rhodopsin mutants of types II and III can arise by a single relatively short deletion in any one of the loops BC, DE, or FG or the amino-terminal tail suggests that these intradiscal peptide segments cooperatively form a folded structure on the intradiscal face and that this structure is stabilized by the disulfide bond between Cys-110 and Cys-187 (36). Previously, these two cysteines were shown to be required for the formation of functional rhodopsin (37). The deletion mutants presumably cannot form the tertiary structure and, therefore, the disulfide bond. One mutation (Cys-187 → Arg) which may result in the lack of the disulfide bond formation in a color vision pigment has been shown to cause the loss of red and green sensitivity (38).

**Comments.** The intradiscal mutagenesis results provide important clues to the assembly of the receptor molecule. We assume that the assembly of the molecule begins with the entry of the nascent rhodopsin polypeptide chain into the endoplasmic lumen. Following high mannose glycosylation, the molecule folds, and this includes the formation and insertion of the seven helices into the membrane and raises the first important question that needs to be answered: When do the helices form and insert into the membrane? Is their entry to form the bundle coordinated? It would seem that in the mutants which do not bind retinal and therefore have not formed a binding pocket, the helical segments in the endoplasmic reticulum must not be coordinated. We further postulate that the important next step in rhodopsin assembly is the formation of an intradiscal tertiary structure that involves amino-terminal tail and loops BC, DE, and FG. The formation of this structure is followed by the formation of the disulfide bond between Cys-110 and Cys-187. The structure leads to the alignment of the even helices in the membrane and establishment of maximal helix–helix interactions and the formation of the retinal binding pocket and, concomitantly, in the formation of a specific tertiary structure for the cytoplasmic domain. The principles for the assembly of the G protein-coupled seven-helix receptors and for their function are probably similar. There is, for example, a disulfide bond located in positions similar to that described above in rhodopsin (Fig. 7) in large numbers of receptors.

Finally, signal transduction in rhodopsin following retinal isomerization must be understood in terms of specific move-

ments in the helices. These movements then bring about coordinated structural changes in the cytoplasmic domain formed from interactions between the cytoplasmic loops. Thus, the functioning of the receptor molecule involves the functioning and coordination of the three domains in characteristic tertiary structures.


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