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Rhodopsin is a member of a family of receptors that contain seven transmembrane helices and are coupled to G proteins. The nature of the interactions between rhodopsin mutants and the G protein, transducin (G_t), was investigated by flash photolysis in order to monitor directly G_t binding and dissociation. Three mutant opsins with alterations in their cytoplasmic loops bound 11-*cis*-retinal to yield pigments with native rhodopsin absorption spectra, but they failed to stimulate the guanosine triphosphatase activity of G_t. The opsin mutations included reversal of a charged pair conserved in all G protein–coupled receptors at the cytoplasmic border of the third transmembrane helix (mutant CD1), replacement of 13 amino acids in the second cytoplasmic loop (mutant CD2), and deletion of 13 amino acids from the third cytoplasmic loop (mutant EF1). Whereas mutant CD1 failed to bind G_t, mutants CD2 and EF1 showed normal G_t binding but failed to release G_t in the presence of guanosine triphosphate. Therefore, it appears that at least the second and third cytoplasmic loops of rhodopsin are required for activation of bound G_t.

PHOTOACTIVATED RHODOPSIN, METArhodopsin II (MII), binds to G_t and catalyzes guanosine diphosphate (GDP)–guanosine triphosphate (GTP) exchange (*1*). The GTP-bound form of the α subunit of G_t then dissociates from MII and interacts with a guanosine 3′,5′-monophosphate (cyclic GMP) phosphodiesterase, a process that ultimately results in the generation of a neural signal (*2*). Evidence suggests that the cytoplasmic surface of rhodopsin (Fig. 1A) participates in G_t binding and activation. The sites of rhodopsin that interact with G_t have been proposed to include loop CD (*3*), loop EF (*4, 5*), or a combination of these loops, as well as a third loop formed by the COOH-terminal tail (Fig. 1A) (*6*). To further study the specificity of interaction between rhodopsin and G_t, we prepared rhodopsin mutants in which cytoplasmic loops CD or EF were altered by amino acid replacements or deletions (Fig. 1B). In mutant CD1, a charged pair ($Glu^{134}Arg^{135}$) was mutated to $Arg^{134}Glu^{135}$ (*7*). A GluArg or AspArg charged pair is conserved at the cytoplasmic border of helix C in all known G protein–coupled receptors. In mutant CD2, a segment of 13 amino acids (residues 140 to 152) in loop CD was replaced by an unrelated sequence of equal length. A 14–amino acid segment (residues 137 to 150) was deleted from loop CD to form mutant CD3, and a 13–amino acid segment (residues 237 to 249) was deleted from loop EF to form mutant EF1. The mutant opsin genes were expressed in COS-1 cells, regenerated with 11-*cis*-retinal, and purified in detergent solution by an immunoaffinity procedure (*4, 8*).

Ultraviolet (UV)-visible spectroscopy and in vitro guanosine triphosphatase (GTPase) assays were performed on these opsin mutants (Table 1). Mutant CD3 did not bind

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11-*cis*-retinal and, thus, did not yield a chromophore. Mutants CD1, CD2, and EF1 formed pigments with absorption spectra indistinguishable from that of native rhodopsin [wavelength of maximal absorption (λ_{max}) = 500 nm] and showed the characteristic shift in λ_{max} to 380 nm (λ_{max} of MII) on illumination. However, these mutants did not activate G_t, as assayed by light-dependent inorganic phosphate (P_i) release. A mutation resulting in a loss of light-induced GTPase activity in this assay might be attributable to a defect in MII formation or to the formation of an MII-like species that either failed to bind G_t or that bound G_t but failed to induce the guanine nucleotide exchange necessary for the activation process.

To identify the specific defect that prevented light-induced activation of transducin, we studied the rhodopsin mutants by flash photolysis under conditions that allowed the distinct monitoring of MII formation, G_t binding, and G_t activation and release (*9, 10*) (Fig. 2). Mutant CD1 formed an MII species that failed to bind G_t. Mutants CD2 and EF1 formed an additional amount of MII species ("extra MII") in the presence of G_t to the same degree as wild-type rhodopsin, indicating unaltered binding to G_t. However, neither CD2 nor EF1 allowed dissociation of the MII-G_t complex in the presence of GTP, indicating that a defect existed in the G_t activation pathway. Thus, G_t binding was uncoupled from G_t release in mutants CD2 and EF1.

Thus, the failure of mutant CD1 to bind G_t may indicate that a direct interaction exists between the $Glu^{134}Arg^{135}$ charged pair and G_t. However, this charged pair may help other cytoplasmic sites to form the G_t binding domain. Because mutations in loop CD or EF allowed a stable MII-G_t complex to form but prevented G_t activation, it is likely that these two loops are part of a G_t activation site on the cytoplasmic surface of rhodopsin. Our results are consistent with peptide competition studies on native rhodopsin showing that any two of three cytoplasmic loops (loop CD, loop EF, or the loop consisting of the proximal portion of the COOH-terminal tail) were sufficient to bind G_t (*6*). This implies that at least one interaction site exists on each of these loops. An indirect effect of mutagenesis that causes a conformational perturbation or alteration of a posttranslational modification cannot be excluded. However, formation of MII is extremely sensitive to changes in the molecular environment of rhodopsin (*10, 11*) and should therefore act as an indicator of overall rhodopsin structure. Thus, a significant conformational perturbation is improbable because each mutant displayed a normal λ_{max} and formed a normal amount of MII with the same kinetics. We conclude that (i) binding of G_t and stabilization of MII is not sufficient for G_t activation and (ii) activation of bound G_t requires at least two sites (loops CD and EF) on the cytoplasmic surface of rhodopsin.

The state of the nucleotide binding site of

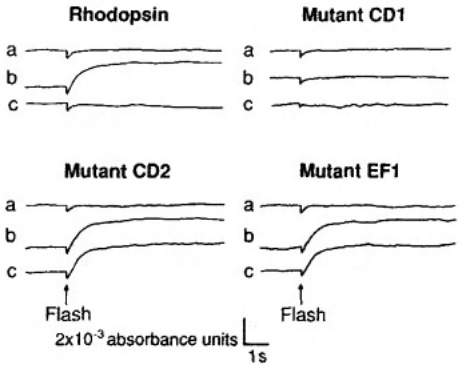

Fig. 2. Flash-induced formation of MII and G_t-dependent stabilization of MII. Flash photolysis experiments were performed according to a dual-wavelength spectrophotometric method (*6, 27*). Results are for purified COS cell rhodopsin, CD1, CD2, and EF1. Three traces are shown for each pigment: a, pigment alone; b, pigment plus G_t; and c, pigment plus G_t plus GTP. COS cell rhodopsin produced a small amount of MII on illumination. In the presence of G_t, the amount of MII increased because G_t bound MII to shift the MI-MII equilibrium toward MII. In the presence of G_t and GTP, activated G_t dissociated from MII so that the MI-MII equilibrium was unaffected and no additional MII was observed. Mutant CD1 formed a spectrally normal MII species that did not bind to G_t to stabilize MII. Mutants CD2 and EF1 both formed MII species that were stabilized by G_t binding so that extra MII signals were observed. However, GTP did not cause the release of G_t and the extra MII signal persisted. Traces show the formation of MII in arbitrary absorbance units as a function of time. The small negative deflection in the trace at the flash is due to formation of early light-induced products including MI (*28*).

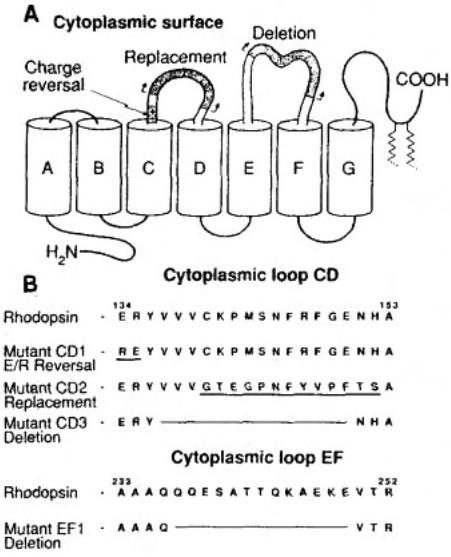

Fig. 1. (**A**) Structural model of rhodopsin (*20*). The seven putative transmembrane helices (A through G) are represented by cylinders. Cytoplasmic loops linking the successive helices are designated AB, CD, and EF. A fourth putative loop is formed by a portion of the COOH-terminal tail between helix G and a pair of palmitoylated cysteines (*21*). The cytoplasmic surface of rhodopsin interacts with soluble G_t. The charged pair $Glu^{134}Arg^{135}$ has been proposed to form the cytoplasmic border of transmembrane helix C (*7, 22*). (**B**) Amino acid sequences of loops CD and EF in bovine opsin (*23*) and the corresponding mutants. In mutant CD1, the charged pair $Glu^{134}Arg^{135}$ was reversed. Because mutant CD3, a deletion of 14 amino acids (residues 137 to 150), failed to bind 11-*cis*-retinal and yield a chromophore, mutant CD2 was prepared in which 13 amino acids (residues 140 to 152) were replaced by a sequence derived from the NH_2-terminal tail region (residues 2 to 14) of rhodopsin. Mutant EF1 contained a deletion of 13 amino acids (residues 237 to 249). Site-directed mutagenesis was performed on a synthetic opsin gene (*24*) cloned into a modified version of the mammalian expression vector, pMT-2 (*4*). All mutants were prepared by restriction fragment replacement ("cassette") mutagenesis (*25*). Cloned synthetic DNA sequences were confirmed by the dideoxy chain termination method of DNA sequencing (*26*). Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Table 1. Characteristics of mutant rhodopsins. Opsin apoproteins were expressed in transiently transfected COS cells (*8*). Harvested cells were incubated with 11-*cis*-retinal (10 μM) to regenerate pigments. The pigments were solubilized in dodecyl maltoside, purified according to an immunoaffinity procedure (*8, 18*), and subjected to UV-visible spectroscopy and transducin activation assays (*7*). The λ_{max} was determined in 0.1% dodecyl maltoside (pH 7.1) at 20°C. On illumination with light of greater than 495-nm wavelength, each of the pigments was converted to an MII species absorbing at 380 nm. However, none of the mutant rhodopsins stimulated the GTPase activity of G_t. GTPase activity refers to the light-dependent release of Pi in a system consisting of purified pigment, purified bovine G_t, and GTP (SE ≤ 10.7%, n = 20) (*7*). The values used for extinction coefficients of rhodopsin and the mutant pigments were 42,700 M^{-1} cm^{-1} (*19*). The specific activity of rhodopsin was 61.6 ± 20.4 pmol of Pi released per picomole of pigment per minute (mean ± SE, n = 6). The GTPase activity values are normalized to the activity of rhodopsin purified from COS cells in parallel with the mutant pigments. Mutant opsin CD3 did not bind 11-*cis*-retinal to yield a chromophore.

Rhodopsin	Pigment λ_{max} (nm)	GTPase activity (normalized)
Wild type	500	1.00
EF1	500	0.004
CD1	500	0.009
CD2	500	0.012

G_t, which binds to mutants CD2 and EF1, is not measured directly in these experiments. Hypothetically, the G_t nucleotide binding site may be unoccupied or may contain GDP or GTP. We find it likely that the GDP form of G_t binds to photoactivated rhodopsin, and that the release of GDP then allows MII stabilization (*12*). The activation step that is blocked in mutants CD2 and EF1 may be the signal that induces formation of the GTP binding pocket in G_t. Thus, we speculate that mutants CD2 and EF1 bind to G_t that is nucleotide free.

Many receptors that couple to G proteins have been identified, and deciphering the mechanism of G protein activation is central to an understanding of G protein–mediated signal transduction. Mutants of the β_2-adrenergic receptor (β-AR), which is structurally related to rhodopsin (*13*), have been studied in whole-membrane preparations with steady-state agonist binding assays. A 34–amino acid deletion from the third cytoplasmic loop (EF) of β-AR produced a receptor with a single high-affinity agonist state, which did not activate adenylate cyclase (*14, 15*). The interpretation of this result was that the mutant receptor was uncoupled from the G protein, G_s. A seven–amino acid deletion in the same loop (EF) of β-AR results in a moderate impairment of the adenylate cyclase response and a single high-affinity agonist state (*16*). It was concluded that this receptor domain in loop EF might participate in the transmission of an agonist-induced stimulatory signal to G_s. Our direct demonstration of an inactive mutant rhodopsin-G_t complex supports the interpretation that these deletion mutants of β-AR bound G_s to form a complex with impaired activity. Furthermore, the combined results of our and other studies (*14–17*) suggest that the G_s-induced high-affinity agonist state of β-AR is analogous to the G_t-induced stabilization of MII. The correlation between rhodopsin and β-AR might be tested further by purifying mutant β-ARs and studying agonist binding affinity and the stimulation of G_s in artificial vesicles. Thus, the study of rhodopsin-G_t interactions may provide general information regarding transmembrane signaling, and our direct spectroscopic assay of purified rhodopsin mutants should allow a more detailed analysis of discrete steps in the G protein activation pathway.

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9. Solubilization of rhodopsin in steroid-containing micelles allows MII formation, G_t binding, and G_t activation and release to be monitored separately (*1, 10*). The light-induced intermediates, metarhodopsin I (MI) and metarhodopsin II (MII), exist in equilibrium, and binding of G_t to MII shifts the equilibrium toward MII. The amount of MII-G_t complex and, therefore, the binding of G_t to MII can be quantitated by measuring the G_t-dependent shift from MI (λ_{max} = 480 nm) to MII (λ_{max} = 380 nm). The resulting MII-G_t complex remains stable for minutes in the absence of GTP and the kinetics of its formation and steady-state concentration can be determined. However, in the presence of GTP, MII stimulates the exchange of GDP for GTP on G_t. The GTP-bound form of the α subunit of G_t then dissociates from MII and the equilibrium shifts back to MI [D. Emeis and K. P. Hofmann, *FEBS Lett.* **136**, 201 (1981)].
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28. Transiently transfected COS-1 cells (~10^8) treated with 11-*cis*-retinal were solubilized in buffer A [7 mM deoxylysolecithin, 5 mM cortisone, 10 mM Hepes (pH 8), and 1 mM $CaCl_2$] supplemented with 1 mM phenylmethylsulfonyl fluoride at room temperature for 1 hour. Insoluble material was separated by centrifugation at 100,000*g* for 30 min at 20°C, and pigment was purified by an immunoaffinity procedure (*8*), with the exception that washing and peptide elution were performed in buffer A. UV-visible spectra of rhodopsin and mutants CD1, CD2, and EF1 were identical (visible λ_{max} = 500 nm). In the flash photolysis experiments, the effective path length was 0.7 cm on 0.8 ml of sample that contained pigment (200 nM), G_t (40 nM), and GTP (20 μM) in buffer A. Each light flash (520 ± 15 nm) bleached 5% of the pigment present. The formation of MII was measured as the difference between absorbances at 380 nm and 417 nm (the isosbestic point between MI and MII). Experiments were performed at 4°C, but for each sample, a separate experiment was performed with pigment alone at 20°C. At the higher temperature, the normal extent (60%) of conversion to an MII species was observed for each mutant tested. COS cell rhodopsin was identical to both washed disk membrane and purified solubilized bovine rhodopsin with respect to MII formation, extra MII formation in the presence of G_t, and the absence of the extra MII signal in the presence of G_t and GTP (R. R. Franke, B. König, T. P. Sakmar, H. G. Khorana, K. P. Hofmann, unpublished data).

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