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Structure and Function in Rhodopsin

STUDIES OF THE INTERACTION BETWEEN THE RHODOPSIN CYTOPLASMIC DOMAIN AND TRANSDUCIN*

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Structural requirements for the activation of transducin by rhodopsin have been studied by site-specific mutagenesis of bovine rhodopsin. A variety of single amino acid replacements and amino acid insertions and deletions of varying sizes were carried out in the two cytoplasmic loops CD (amino acids 134–151) and EF (amino acids 231–252). Except for deletion mutant Δ137–150, all the mutants bound 11-*cis*-retinal and displayed normal spectral characteristics. Deletion mutant Δ236–239 in loop EF caused a 50% reduction of transducin activation, whereas deletion mutant Δ244–249 and the larger deletions in loop EF abolished transducin activation. An 8-amino acid deletion in the cytoplasmic loop CD as well as a replacement of 13 amino acids with an unrelated sequence showed no transducin activation. Several single amino acid substitutions also caused significant reduction in transducin activation. The conserved charged pair Glu-134/Arg-135 in the cytoplasmic loop CD was required for transducin activation; its reversal or neutralization abolished transducin activation. Three amino acid replacements in loop EF (S240A, T243V, and K248L) resulted in significant reduction in transducin activation. We conclude that 1) both the cytoplasmic loops CD and EF are required for transducin activation, and 2) effective functional interaction between rhodopsin and transducin involves relatively large peptide sequences in the cytoplasmic loops.

Rhodopsin is the photoreceptor in the retinal rod cell. Its primary structure has been established through both peptide and DNA sequencing (1–3). The apoprotein consists of a single polypeptide chain of 348 amino acids. Hydropathy plots, proteolysis experiments, and binding studies with monoclonal antibodies suggest that rhodopsin contains seven transmembrane segments (A–G) with water-exposed polypeptide domains on the cytoplasmic and intradiscal sides (2, 4–7). A secondary structure model identifying the seven putative α-helical transmembrane segments is shown in Fig. 1. The chromophore 11-*cis*-retinal, covalently linked to Lys-296 as a Schiff base, is embedded in the hydrophobic core of rhodopsin (8). Absorption of light by rhodopsin causes isomerization of the chromophore from 11-*cis*- to all-*trans*-retinal and drives rhodopsin through a series of structural changes leading to the photointermediate metarhodopsin II (MII).[1] MII activates transducin and also mediates interactions with other proteins on the cytoplasmic surface. The interaction between MII and transducin is an important early step in signal transduction in the visual process. Because of its fundamental importance, we are interested in studying this interaction at the molecular level. Insights into rhodopsin-transducin interaction should be of general significance for the study of the superfamily of receptors that are coupled to G proteins.

Some information on the rhodopsin-transducin interaction has been obtained from previous studies. Proteolysis of rhodopsin suggested the involvement of loop EF in transducin binding (9). In another approach, antipeptide antibodies directed against specific sites on the cytoplasmic domain of rhodopsin were tested for their ability to block transducin binding (10). More recently, peptides corresponding to portions of the cytoplasmic loops in rhodopsin were used to test for inhibition of rhodopsin-transducin interaction (11). It was concluded that as many as three cytoplasmic loops of rhodopsin may be involved in interaction with transducin. However, specific questions regarding the binding and activation of transducin by rhodopsin remain unanswered. What is the nature of the structural interaction between transducin and rhodopsin? What are the conformational changes in the two molecules during this interaction? What are the mechanisms of transducin-mediated GTP-GDP exchange, the subsequent release of Tα-GTP, and the separation of the β-γ subunits as a complex that occur on the cytoplasmic face of rhodopsin?

Recently, we reported on rhodopsin mutants with deletions in the cytoplasmic loops (12). These mutants bound but failed to activate transducin. A conserved charge pair (Glu-134/Arg-135) in rhodopsin was suggested to be a part of the transducin-binding site. Furthermore, deletion and replacements of large peptides showed that substantial portions of loops CD and EF were necessary for functional interaction of rhodopsin with transducin. We have now continued to investigate rhodopsin-transducin interaction by extensive application of site-specific mutagenesis in the cytoplasmic loops CD and EF in rhodopsin (Fig. 1). The mutations carried out include deletions of peptide sequences of varying sizes (Table I) and single amino acid replacements as well as amino acid insertions (Tables II and III). Our results indicate the importance of specific peptide sequences in rhodopsin in the above interaction. Thus, whereas a mutant with a deletion of an 8-amino acid sequence from loop CD showed a normal UV-visible spectrum, it failed to activate transducin. Another loop CD

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[1] The abbreviations used are: MII, metarhodopsin II; G protein, guanine nucleotide-binding regulatory protein; dATPγS, deoxyadenosine 5′-*O*-(thiotriphosphate).

mutant with 13 amino acids replaced by an unrelated amino acid sequence formed a normal chromophore, but also failed to activate transducin. A deletion of 14 amino acids in the same loop caused loss of retinal binding capability, presumably because of structural constraints introduced by the deletion. In loop EF, the carboxyl-terminal region (amino acids 244–249) and the region containing potential phosphorylation sites near the center of the loop (amino acids 236–243) are evidently important for transducin activation. Of the presumed 22 amino acids in loop EF, 19 could be deleted without affecting retinal binding, although transducin activation was lost. Finally, systematic analysis of single and multiple amino acid substitutions in loop EF showed that 3 amino acids (Ser-240, Thr-243, and Lys-248) were particularly important. We conclude that rhodopsin (meta-rhodopsin II)-transducin interaction involves both loops CD and EF in rhodopsin and that the interaction involves large portions of these loops.

EXPERIMENTAL PROCEDURES

Materials—The expression vector pMT2, a β-lactamase derivative of p91023 (13), was generously provided by Dr. R. J. Kaufman (Genetics Institute, Inc., Cambridge, MA). 11-*cis*-Retinal was a generous gift of Drs. P. Sorter and V. Toome (Hoffmann-La Roche). Dodecyl maltoside was purchased from Sigma, and [γ-^{32}P]GTP and [^{35}S]dATPαS were from Du Pont-New England Nuclear. Sources of COS-1 cells, bovine retinas, and Sepharose 2B and the preparation of the buffers and media have been described (14, 15).

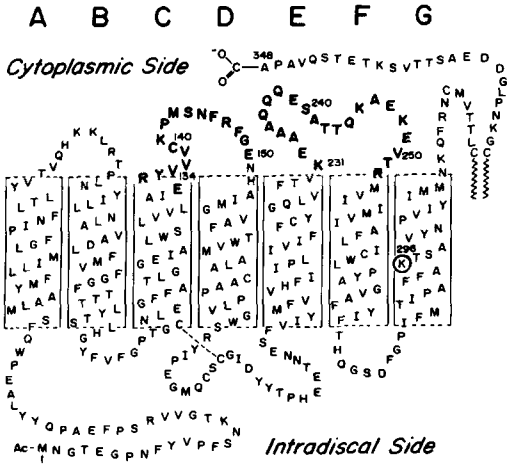

FIG. 1. **Secondary structure model of bovine rhodopsin.** The seven putative transmembrane helical segments are in *boxes A–G*. One-letter abbreviations are used for amino acids. The *dashed line* between Cys-110 and Cys-187 on the intradiscal side shows a disulfide bond. Cys-322 and Cys-323 on the cytoplasmic side are palmitoylated. The amino acids in the cytoplasmic loops CD and EF that are the focus of this work are in *boldface type*.

Monoclonal Antibody—The 1D4 hybridoma cell line was generously provided by Dr. R. S. Molday (16). The hybridomas were grown in large scale in the tissue culture facilities of the Massachusetts Institute of Technology Cancer Center. 1D4 antibodies were harvested from the hybridoma media by $(NH_4)_2SO_4$ precipitation. The coupling of the antibody to Sepharose 2B has been described (14). The octadecapeptide (positions −1′ to −18′ from the carboxyl-terminal end of rhodopsin) used to elute rhodopsin from the immunoaffinity resin was the gift of Dr. P. Kim.

Preparation of Oligonucleotides—Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA synthesizer. The purification and characterization of the oligonucleotides were performed according to Ferretti *et al.* (17).

Construction of Rhodopsin Genes with Mutations in Loop CD: Mutants CD-1 to CD-5—The mutant opsin genes were constructed by restriction fragment replacement in the synthetic rhodopsin gene (17). For the construction of the mutants targeting the charged pair Glu-134/Arg-135 (mutants CD-1 to CD-5) (Table II), two new unique restriction sites (*Rsr*II and *Spe*I) were introduced into the synthetic gene in the expression plasmid (18). After digestion with *Rsr*II and *Spe*I, the large fragment was separated on an agarose gel and was ligated with the synthetic DNA duplexes with the desired codon alteration(s).

Deletion Mutations in Loop CD—The two deletion mutations CD-Δ1 and CD-Δ2 and the loop replacement mutation CD-7 were introduced between the restriction sites *Pvu*I and *Aha*II (Fig. 2). Because these sites were not unique within the plasmid, digestions with *Apa*I and *Avr*II were used to generate a fragment in which *Pvu*I and *Aha*II sites were unique. The purified *Apa*I-*Avr*II fragment was digested with *Pvu*I and *Aha*II to generate three fragments. The fragments were separated by agarose gel electrophoresis, and the two large fragments were purified. Because the ligation of *Pvu*I cleavage sites is sensitive to deoxyadenosine methylation, the plasmid used for *Pvu*I digests was isolated from a dam$^-$ strain of *Escherichia coli*. The large fragment from the *Apa*I-*Avr*II digestion was prepared separately from a plasmid isolated from *E. coli* strain DH1. Fragments I–III and the synthetic DNA duplex were ligated (Fig. 2). Ligation mixtures were used to transform $CaCl_2$-treated *E. coli* strain DH1. Plasmid DNA was prepared from ampicillin-resistant colonies.

Rhodopsin Genes with Mutations in Loop EF—Deletion mutants EF-Δ1 to EF-Δ4 (Table I) and substitution mutants EF-2 to EF-15 (Table III) were assembled in two-component ligations using the large *Mlu*I-*Pst*I restriction fragment and synthetic duplexes containing the desired codon alteration(s).

Mutant EF-1 (Table III)—Mutant EF-1 was constructed in a three-component ligation using an additional *Ava*II-*Pst*I restriction fragment from the synthetic gene. For the preparation of mutant EF-Δ5, the pMT4 vector was digested with *Mlu*I and *Pst*I. The large restriction fragment was purified from an agarose gel, and the single-stranded overhangs were removed by digestion with mungbean nuclease. The blunt ends were ligated to recircularize the plasmid and to yield an in-frame deletion. Ligation mixtures were used to transform $CaCl_2$-treated *E. coli* strain DH1. Plasmid DNA was prepared from ampicillin-resistant colonies. A small restriction fragment containing the mutation was subcloned into pMT4 to minimize the chance of spurious mutations caused by nuclease treatment. The ligation mixture consisted of an *Eco*RI-*Hin*fI fragment (613 base

TABLE I

Deletion mutants constructed in cytoplasmic loops CD and EF

The solid lines indicate the sequences deleted in the loop sequence.

Mutant	Amino acid sequence	Amino acids deleted (inclusive)
Loop CD		
wt[a]	134 ERYVVVCKPMSNFRFGENHA 153	
CD-Δ1	ERYVVVCKP_____NHA	143–150
CD-Δ2	ERY_____NHA	137–150
Loop EF		
wt	231 KEAAAQQQESATTQKAEKEVTR 252	
EF-Δ1	KEAAAQQQESATT_____VTR	244–249
EF-Δ2	KEAAA____SATTQKAEKEVTR	236–239
EF-Δ3	KEAAA_____QKAEKEVTR	236–243
EF-Δ4	KEAAAQ_____VTR	237–249
EF-Δ5	KEA_____	234–252

[a] wt, wild-type bovine rhodopsin amino acid sequence (1–3).

TABLE II

Amino acid replacements in loop CD

The amino acids substituted in different mutants are underlined.

Mutant	Amino acid sequence
wt[a]	134 ERYVVVCKPMSNFRFGENHA 153
CD-1	DRYVVVCKPMSNFRFGENHA
CD-2	QRYVVVCKPMSNFRFGENHA
CD-3	EQYVVVCKPMSNFRFGENHA
CD-4	AAYVVVCKPMSNFRFGENHA
CD-5	REYVVVCKPMSNFRFGENHA
CD-6	ERYVVVCKPMSNFQFGENHA
CD-7	ERYVVVGTEGPNFYVPFTSA

[a] wt, bovine rhodopsin amino acid sequence (1–3).

TABLE III

Amino acid replacements in loop EF

The amino acid substitutions in the different mutants are underlined.

Mutant	Amino acid sequence
wt[a]	231 KEAAAQQQES ATTQKAEKEVTR 252
EF-1	TQAAAQQQES ATTQKAEKEVTR
EF-2	KEAAAQQQQS ATTQKAEKEVTR
EF-3	KEAAAQQQEA ATTQKAEKEVTR
EF-4	KEAAAQQQES ATVQKAEKEVTR
EF-5	KEAAAQQQEA AVVQKAEKEVTR
EF-6	KEAAAQQQEA AGGQKAEKEVTR
EF-7	KEAAAQQQES ATTQLAEKEVTR
EF-8	KEAAAQQQES ATTQKAELEVTR
EF-9	KEAAAQQQES ATTQLAELEVTR
EF-10	KEAAAQQQES ATTQKAQLEVTR
EF-11	KEAAAQQQES ATTQKAELQVTR
EF-12	KEAAAQQQES ATTQKAQKQVTR
EF-13	KEAAAQQQES ATTQKAQLQVTR
EF-14[b]	KEAAAQQQESA*TTQKAEKEVTR
EF-15	KEAATSLHGY SVTGPTGSNLTR
wt	KEAAAQQQES ATTQKAEKEVTR

[a] wt, wild-type bovine rhodopsin amino acid sequence (1–3).

[b] In mutant EF-14, the dipeptide Thr-Ser was inserted between Ala-241 and Thr-242.

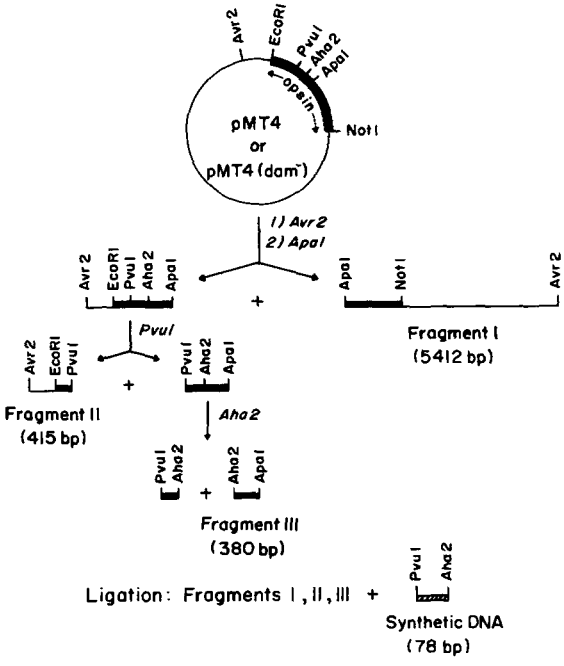

FIG. 2. **Outline of cloning strategy employed for construction of loop deletion mutants CD-Δ1 and CD-Δ2 and loop replacement mutant CD-7.** The amino acid sequences of the mutants are shown in Tables I (CD-Δ1 and CD-Δ2) and II (CD-7). Synthetic DNA duplexes containing the desired codon alterations were used to replace a *Pvu*I-*Aha*II restriction fragment in the synthetic rhodopsin gene. These restriction sites were not unique within the plasmid. Therefore, multiple restriction digests and restriction fragment purifications were required as described under "Experimental Procedures." In summary, each mutant was constructed in a four-component ligation consisting of the following: 1) a 5412-base pair *Apa*I-*Avr*II fragment, 2) a 415-base pair *Avr*II-*Pvu*I fragment, 3) a 380-base pair *Aha*II-*Apa*I fragment, and 4) a 78-base pair synthetic duplex containing the desired codon alterations. *bp*, base pairs.

pairs), a *Hin*fI-*Apa*I fragment (196 base pairs) containing the mutation, and an *Apa*I-*Eco*RI fragment (5330 base pairs). The ligation mixture was used to transform $CaCl_2$-treated *E. coli* strain DH1. Plasmid DNA was prepared from ampicillin-resistant colonies.

DNA Sequencing of Mutants—Each mutation was confirmed by dideoxy sequencing of plasmid DNA (19, 20) using [^{35}S]dATPαS and Sequenase DNA polymerase (U. S. Biochemical Corp.).

Expression of Rhodopsin Mutants in COS-1 Cells—The procedure for the transient transfection of COS-1 cells has been reported (14, 15). COS-1 cells were plated at a density of ~5 × 10^6 cells/10-cm culture plate and transfected within 14–18 h with 8 μg of CsCl-purified plasmid DNA/plate. Cells were harvested 72 h after transfection.

Binding of 11-cis-Retinal Chromophore and Purification of Rhodopsin Mutants—Freshly harvested COS cells (six plates in 6 ml of phosphate-buffered saline) were incubated with 35 μl of 11-*cis*-retinal in ethanol (1 mM) at 4 °C in the dark. A second aliquot of retinal solution (35 μl) was added after 45 min and incubated for an additional 45 min. The cells were collected by centrifugation, and the supernatant fraction was discarded. The cell pellet was resuspended in 10 ml of solubilization buffer (50 mM Tris-HCl, pH 6.8, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% dodecyl maltoside, 0.1 mM phenylmethylsulfonyl fluoride). After 30 min at 4 °C with gentle mixing, the insoluble material was pelleted by centrifugation at 100,000 × *g* for 30 min at 4 °C. The supernatant fraction, containing the solubilized rhodopsin, was incubated for 3 h at 4 °C with 150 μl of 1D4-Sepharose. The resin was collected by centrifugation, the supernatant fraction was removed, and the resin was resuspended in 5 ml of wash buffer (50 mM Tris-HCl, pH 6.8, 100 mM NaCl, 1 mM $CaCl_2$, 0.1% dodecyl maltoside) and washed for 5 min at 4 °C with gentle mixing. The wash procedure was repeated a total of five times. Elution of the rhodopsin from the immunoaffinity resin was carried out in 1 h in the presence of carboxyl-terminal peptide 1′–18′ (180 μg of peptide/ml of buffer) as previously described (14). The fraction containing eluted rhodopsin was centrifuged at 100,000 × *g* for 30 min.

Rhodopsin in Digitonin—Transfected COS-1 cells (12 dishes, 10-mm plates) were harvested, regenerated with 11-*cis*-retinal, solubilized, and incubated with the resin as described above. The resin was split into two fractions. One was treated as described above in dodecyl maltoside. The other fraction was washed and eluted in buffer containing 0.1% digitonin instead of dodecyl maltoside.

Characterization of Rhodopsin Mutants—Purified rhodopsin mutants were characterized in three ways: (*a*) UV-visible spectroscopy (rhodopsin concentrations were based on the absorbance difference at 500 nm before and after illumination assuming a molar absorption coefficient of ε = 42,700 M^{-1} cm^{-1}), (*b*) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualization of the protein bands by silver staining, and (*c*) transducin activation assay as described below.

Transducin Activation Assay—The rhodopsin mutants were assayed for their ability to stimulate GTPase activity of transducin in a light-dependent manner. The assay mixture (100 μl) contained 2.5 nM rhodopsin, 2.5 μM purified transducin, 20 μM [γ-^{32}P]GTP, 0.01% dodecyl maltoside, 10 mM Tris maleate, pH 7.2, 100 mM NaCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol. All the components except GTP were mixed in the dark, and the solution was equilibrated at 25 °C. Illumination was performed with a 150-watt fiber optic light source and a 495-nm cutoff filter in tandem with an IR filter. After continuous illumination for 1 min, the reaction was started by the addition of GTP. Aliquots (20 μl) were removed at 2, 4, 6, and 8 min and added to 200 μl of molybdic acid solution (6.25 g of MoO_3 dissolved in 35 ml of concentrated H_2SO_4 and diluted to 500 ml with H_2O). 0.1 ml of a reducing solution (5.7 g of $Na_2S_2O_5$, 0.2 g of Na_2SO_3, and 0.1 g of 1-amino-2-naphthol-4-sulfonic acid dissolved in 100 ml of water) was added, and the solution was mixed. The mixture was extracted by vortexing with 700 μl of isoamyl alcohol. After phase separation by

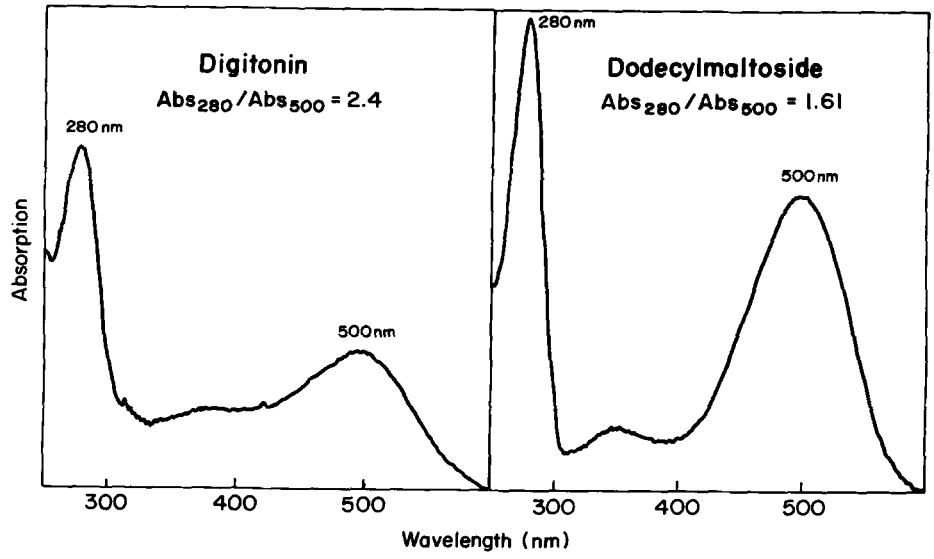

FIG. 3. **Comparison of UV-visible spectra of COS cell rhodopsin solubilized in digitonin or dodecyl maltoside.** *Left*, UV-visible absorption spectrum in digitonin. The procedure for the purification of rhodopsin and solubilization in digitonin has been described under "Experimental Procedures." Spectral ratios of <2.4 were not obtained using this procedure. *Right*, UV-visible absorption spectroscopy of rhodopsin in dodecyl maltoside. The procedure used is as described under "Experimental Procedures." The A_{280}/A_{500} spectral ratio is indicative of the purity of the preparation. Spectral ratios in the range of 1.6–1.8 were routinely obtained.

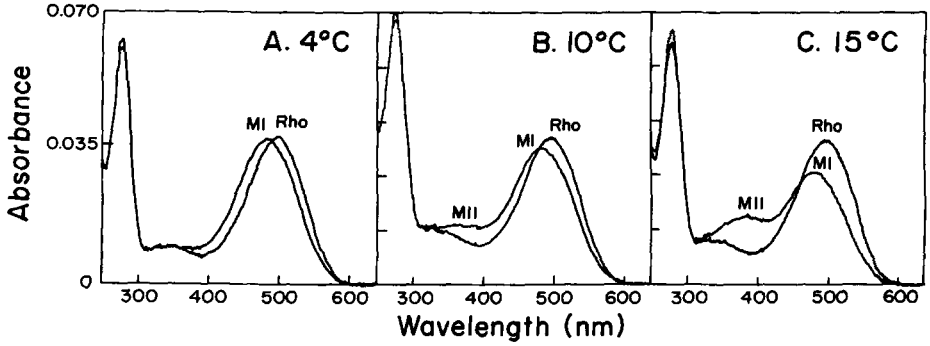

FIG. 4. **UV-visible spectroscopy of illuminated bovine rhodopsin in digitonin at different temperatures.** At each temperature, UV-visible spectra were taken before and immediately after a 3-s illumination with light >495 nm. At 4 °C, only MI (480 nm) was present. At 10 °C, MI and a small amount of MII (380 nm) was formed. At 15 °C, the amount of MII increased. In dodecyl maltoside detergent buffer, illumination of rhodopsin (*Rho*) at 4 °C or above resulted in complete conversion to MII (data not shown).

centrifugation, 0.6 ml of the organic layer was analyzed for P_i by scintillation counting.

RESULTS

Characterization of Rhodopsin Expressed in COS Cells: Influence of Detergents

UV-visible Spectral Characteristics—Rhodopsin prepared from COS cells using dodecyl maltoside showed an absorption ratio at 280 nm/500 nm of 1.6–1.7 (Fig. 3). Bovine rhodopsin, purified in parallel, gave the same spectral ratio. COS cell rhodopsin purified using digitonin gave A_{280}/A_{500} ratios of 2.4–4 for different preparations (Fig. 3).

Photoactivation and Stability of Intermediates—Illumination of rhodopsin in dodecyl maltoside with light >495 nm for 10 s quantitatively converted all of the pigment to MII (380 nm). No temperature effect was seen for this conversion between 4 and 25 °C. Illumination of rhodopsin in digitonin with a 495-nm cutoff filter gave a mixture of MI (480 nm) and MII species (Fig. 4), whose composition was temperature-dependent.

GTPase Activity in Transducin—The linear range for rhodopsin activation of the GTPase activity in transducin was determined by assaying rhodopsin at concentrations ranging from 200 pM to 5 nM (Fig. 5). The precision of this assay was estimated to be +10%. COS cell rhodopsin was assayed in parallel with each rhodopsin mutant as an internal standard. To determine the pH optimum for the rhodopsin-transducin GTPase assay, samples were prepared in Tris maleate at pH 5.45–8.2. The maximal activity was determined by plotting the P_i release against the pH of the reaction mixture (Fig. 6). Maximal activity was observed at pH 7.2.

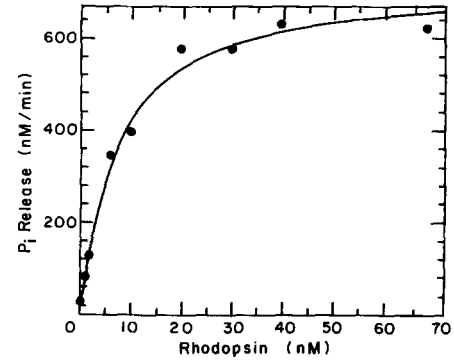

FIG. 5. **GTPase activity plotted as function of rhodopsin concentration to determine linear range of transducin GTPase assay in dodecyl maltoside detergent buffer.** The assay was carried out as described under "Experimental Procedures." The rate of P_i release was determined from the time course for each rhodopsin concentration shown (●), and the rates of P_i release were plotted against the corresponding rhodopsin concentrations. In the linear range of the assay (0.2–5 nM rhodopsin), the rate of P_i release was ~40 pmol of P_i/pmol of rhodopsin/min.

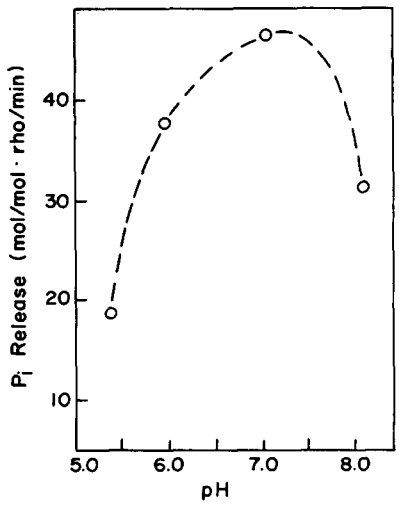

FIG. 6. **Determination of pH optimum for GTPase assay in dodecyl maltoside detergent buffer.** Rhodopsin (*rho*) purified from COS cells was assayed at a concentration of 2.5 nM in Tris maleate at pH 5.5–8.2. The rate of P_i release was plotted against the pH of the assay mixture. Differences in activity could be due to pH effects on rhodopsin or transducin or both.

TABLE IV

Functional assays of deletion mutations in loops CD and EF by transducin activation (GTPase) assay

The amino acid sequences of the loop regions in wild-type rhodopsin and rhodopsin mutants are shown in Table I. GTPase assays with purified mutants and transducin were carried out as described under "Experimental Procedures." The extinction coefficients of rhodopsin and the mutant pigments were assumed to be 42,700 M^{-1} cm^{-1} (29). Activities were not adjusted for any decay of metarhodopsin II that might have occurred during the 8-min course of the assay. Results are presented as mean ± S.D. averaged from two to four separate experiments as indicated by the numbers in parentheses. Purified rhodopsin mutants and COS cell rhodopsin were assayed in parallel. The mutant activities were normalized to the COS cell rhodopsin activities.

Mutant	GTPase activity (normalized)
Loop CD	
wt[a]	1.00
CD-Δ1	0.00 ± 0.0 (2)
CD-Δ2	ND[b]
Loop EF	
wt	1.00
EF-Δ1	0.00 ± 0.0 (2)
EF-Δ2	0.56 ± 0.04 (3)
EF-Δ3	0.03 ± 0.03 (3)
EF-Δ4	0.00 ± 0.0 (4)
EF-Δ5	0.00 ± 0.0 (2)

[a] wt, wild-type bovine rhodopsin amino acid sequence (1–3).
[b] ND, not determined. Mutant CD-Δ2 failed to bind 11-*cis*-retinal.

Deletions and Sequence Replacements in Loops CD and EF

Two questions were addressed in considering the mutations that were to be introduced. First, does the interaction between rhodopsin and transducin require participation of peptide sequences in one or both cytoplasmic loops of rhodopsin? Second, are there electrostatic or hydrogen bond interactions between specific amino acids in rhodopsin and transducin?

Loop CD—The deletions introduced in loop CD are shown in Table I. Mutant CD-Δ1, with a deletion of 8 amino acids (amino acids 143–150), bound 11-*cis*-retinal and formed the characteristic λ_{max} at 500 nm. However, it showed no activation of transducin (Table IV). Mutant CD-Δ2, with a 14-amino acid deletion, was expressed at normal levels in COS cells, but it failed to bind 11-*cis*-retinal. To remove possible constraint in the packing of helices, a 13-amino acid segment in loop CD was replaced by an amino acid sequence derived from the first intradiscal loop (mutant CD-7) (Table II). This mutant bound 11-*cis*-retinal and displayed a normal UV-visible spectrum, but showed no transducin activation (Table V).

Loop EF—Mutant EF-Δ1, with 6 amino acids deleted close to the beginning of helix F (amino acids 244–249), failed to show transducin activation. Deletion EF-Δ2, closer to the end of helix E, activated transducin at 54% of the wild-type level, whereas EF-Δ3, with a deletion 4 amino acids larger than that in EF-Δ2, showed only slight (3% of the wild type) transducin activity (Table IV). Mutants EF-Δ4 and EF-Δ5, which contained 13- and 19-amino acid deletions (Table I), respectively, both failed to stimulate the transducin GTPase activity, although they formed a normal chromophore with 11-*cis*-retinal.

Peptide Sequence Replacement in Loop EF—In mutant EF-15 (Table III), amino acid sequence 235–250 was replaced with an amino acid sequence from loop BC (positions 97–112, except for the change Cys-110 → Ser). This mutant was designed to remove sequence-specific interaction with transducin as in the other deletion mutants, but without affecting packing of the helices. The mutation retained a Thr at position 243. Mutant EF-15 activated transducin at a very low level (7%) (Table VI).

Loop CD—To investigate the role of the conserved charged pair Glu-134/Arg-135, three mutants with single amino acid substitutions and two mutants with double substitutions were constructed and characterized (Table II). Light and dark spectra of the purified retinal-regenerated mutants are shown in Fig. 7. Mutant E134Q (CD-2) showed 1.45 times higher activity than wild-type rhodopsin in the GTPase assay. Mutant E134D (CD-1) stimulated GTPase activity to 56% of the wild type. Mutant R135Q (CD-4) showed 8% of the wild-type GTPase activity. Both double mutants E134A/R135A (CD-5) and E134R/R135E (CD-6) failed to activate transducin.

Loop EF—Point mutants were introduced in the Ser and Thr residues in loop EF (Table III). Mutant S240A (EF-3) showed 60% GTPase activity compared to the wild type. Mutant T243V (EF-4) showed 40% activity in the GTPase assay. Mutant EF-6, with the 3 Ser and Thr residues in loop EF replaced (S240A, T242G, and T243G), showed only 8% of the wild-type GTPase activity. Mutant EF-5, which also had the 3 Ser and Thr residues replaced but with Val substituting for Thr (S240A, T242V, and T243V), showed higher GTPase activity (46%) than mutant EF-6.

To investigate the role of the charged amino acid residues,

TABLE V

GTPase activity of amino acid replacements in loop CD

The amino acid sequences of the loop region in wild-type rhodopsin and rhodopsin mutants are shown in Table II. Results are presented as mean ± S.D. averaged from two to three separate experiments as indicated by the numbers in parentheses. All the mutant activity values were normalized to COS cell rhodopsin controls assayed in parallel.

Mutant	GTPase activity (normalized)
wt[a]	1.00
CD-1	0.56 ± 0.02 (2)
CD-2	1.45 ± 0.27 (2)
CD-3	0.08 ± 0.0 (3)
CD-4	0.00 ± 0.01 (2)
CD-5	0.01 ± 0.02 (2)
CD-6	0.99 ± 0.11 (2)
CD-7	0.0 ± 0.0 (2)

[a] wt, wild-type bovine rhodopsin amino acid sequence (1–3).

TABLE VI

GTPase activity of amino acid replacements in loop EF

The amino acid sequences of loop EF in wild-type rhodopsin and rhodopsin mutants are shown in Table III. Results are presented as mean ± S.D. averaged from two to three separate experiments as indicated by the numbers in parentheses. All the rhodopsin mutant activity values were normalized to COS cell rhodopsin controls assayed in parallel.

Mutant	GTPase activity (normalized)
wt[a]	1.00
EF-1	0.87 ± 0.18 (3)
EF-2	0.97 ± 0.24 (2)
EF-3	0.60 ± 0.18 (2)
EF-4	0.40 ± 0.21 (2)
EF-5	0.34 ± 0.30 (3)
EF-6	0.10 ± 0.08 (2)
EF-7	0.98 ± 0.04 (2)
EF-8	0.72 ± 0.09 (2)
EF-9	0.61 ± 0.20 (2)
EF-10	1.10 ± 0.10 (2)
EF-11	1.01 ± 0.16 (2)
EF-12	1.01 ± 0.05 (2)
EF-13	0.99 ± 0.26 (2)
EF-14	0.56 ± 0.04 (2)
EF-15	0.07 ± 0.08 (2)

[a] wt, wild-type bovine rhodopsin amino acid sequence (1–3).

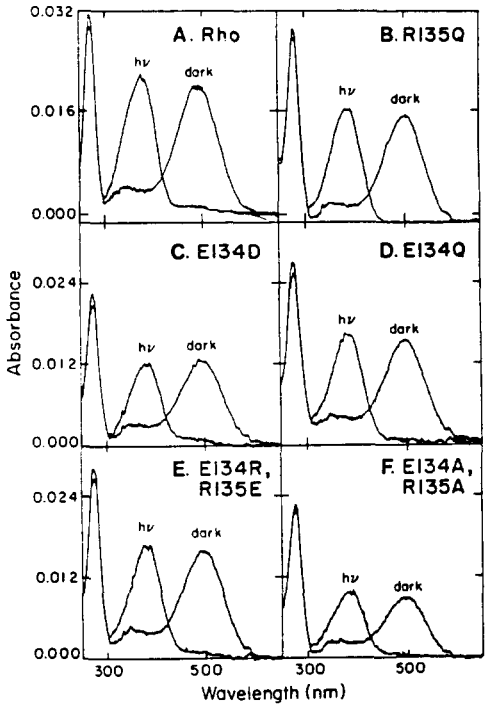

FIG. 7. **UV-visible absorption spectra of COS cell rhodopsin and five loop CD rhodopsin mutants prepared in dodecyl maltoside detergent buffer.** COS cell rhodopsin (*Rho*) displayed a spectrum with an A_{280}/A_{500} ratio of 1.6. All five pigment mutants had alterations in the charged pair Glu-134/Arg-135. Each of the mutants showed a normal λ_{max} value (500 nm), indicating that there was no influence of these amino acids on the spectral properties (18). Upon illumination, each pigment was completely converted to the MII form (380 nm). The capacity of these mutants to activate transducin is shown in Table V.

a set of nine mutants was prepared as shown in Table III. The mutations aimed at replacing the charged amino acids by neutral isosteric amino acids. Thus, mutant EF-1 contained two replacements, K231T and E232Q. This mutant showed nearly wild-type activity in the GTPase assay (87%) (Table VI). Mutant EF-2 (E239Q) (Table III) was previously reported to have a normal UV-visible spectrum and to display wild-type transducin activation in digitonin (15). This mutant was re-examined after purification in parallel in digitonin and dodecyl maltoside. In both detergents, mutant EF-2 showed a normal spectrum and transducin activation. The triple mutant EF-13 (E247Q/K248L/E249Q) also showed wild-type phenotype in both detergents. Mutant EF-8 (K248L) was previously reported to be inactive in the GTPase assay in digitonin. By using [γ-^{32}P]GTP of higher specific activity than that used in earlier experiments (15) and filtered light rather than white light, a residual activity of ~15% was detected in digitonin (Fig. 8). When assayed in dodecyl maltoside, mutant EF-8 displayed 72% of the wild-type activity.

Double and triple mutants were constructed to further investigate the role of the charged amino acids Glu-247, Lys-248, and Glu-249 (Table III), which are conserved in most visual pigments. Mutants EF-10 (E247Q/K248L), EF-11 (K248L/E249Q), EF-12 (E247Q/E249Q), and EF-13 (E247Q/K248L/E249Q) (Table III) all activated transducin normally (Table VI). However, EF9 (K245L/K248L) showed reduced activity of 61% (Table VI).

Loop EF Insertion Mutant—The middle region of loop EF has the potential for an α-helical secondary structure. An insertion mutation, EF-14 (Table III), was made that introduced 2 additional amino acids (Ser-Thr) after Ala-241 so as to extend the putative α-helix and to change its potential amphipathic character. The resulting mutant, EF-14, displayed ~55% GTPase activity.

DISCUSSION

By using site-specific mutagenesis, we have investigated the structural requirements for the interaction between transducin and the cytoplasmic loops CD and EF of rhodopsin. Of the two classes of mutations studied, one comprised deletions of varying lengths in the loop segments. These mutations were designed to identify particular peptide sequences that were important for the rhodopsin-transducin interaction. The second group of mutations consisted of substitutions of polar or charged amino acids by neutral or hydrophobic residues. These replacements were designed to evaluate the contributions of electrostatic interactions or hydrogen bonding in the rhodopsin-transducin association. Transducin activation by the rhodopsin mutants was measured throughout by the GTPase assay.

Retinal Binding and Chromophore Formation by Mutant

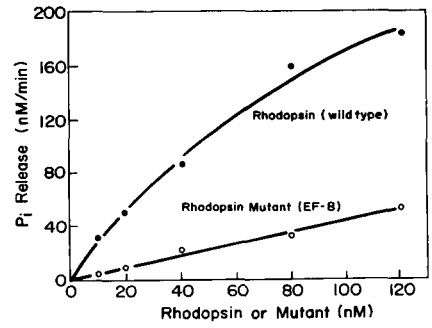

FIG. 8. **Determination of light-dependent GTPase activity *versus* rhodopsin concentration for mutant EF-8 and COS cell rhodopsin prepared in digitonin.** Assays were carried out with pigment concentrations ranging from 10 to 120 nM. The rate of P_i release was determined from the time course for each pigment concentration shown. The rate of P_i release was plotted against the corresponding pigment concentration. Rhodopsin (●) was significantly more active than mutant EF-8 (○) at all pigment concentrations assayed.

Opsins—Bovine rod opsin as expressed in COS-1 cells binds 11-*cis*-retinal and forms the UV-visible spectrum characteristic of native bovine rhodopsin. Binding of the retinal and formation of the characteristic chromophore provide a sensitive assay for the formation of correctly folded rhodopsin. Retinal binding occurred in all but one of the mutants studied, including those with large deletions of 15 and 19 amino acids in loop EF, even though in the 19-amino acid deletion, the extent of regeneration of the chromophore was low (Fig. 9). In the secondary structure model (Fig. 1), loop EF contains 21 amino acids. The finding that deletion of 19 amino acids allows chromophore regeneration suggests that either helixes E and F are very close to each other in the tertiary structure or that the membrane boundaries shown in Fig. 1 for helixes E and F are incorrect.

A deletion of 8 amino acids in the cytoplasmic loop CD formed an opsin with normal retinal binding properties. However, a deletion of 14 amino acids in the same loop caused inability to bind retinal. Replacement of the deleted sequence by an unrelated amino acid sequence restored retinal binding and formation of the correct chromophore, indicating that there was no sequence specificity in the loop CD region for the formation of the retinal-binding pocket. Thus, in general, the deletion mutations in the cytoplasmic loops do not impair opsin folding and chromophore formation. This made possible the study of the cytoplasmic mutations now reported. In contrast, the mutations in the intradiscal domain generally affect retinal binding (21).

Solubilization in Digitonin and Dodecyl Maltoside—Integral membrane proteins differ greatly in their behavior toward different detergents. Digitonin has been commonly used in the past for solubilization of rhodopsin (22, 23). In this work, the use of digitonin for the purification and characterization of rhodopsin and the mutants caused misinterpretations. As an example, mutant K248L, when assayed in digitonin for light-dependent transducin activation, was inactive (15). Reassay of this mutant in dodecyl maltoside showed 72% of the wild-type activity. GTPase activity assays in dodecyl maltoside, in general, gave 10 times higher activity than those in digitonin. Several possibilities could account for the large differences in activity between the two detergents. Since rhodopsin seems to be stable in both detergents, one possibility could be the use of white light in the earlier experiments (14, 15) with digitonin-solubilized samples. It has been reported (24) that constant illumination of rhodopsin with white light can cause the formation of photoisomers, thereby reducing the amount of MII in the pool of photoproducts. In fact, mutant K248L, which is inactive in digitonin under constant white light illumination, was partially active under light passed through a long-pass filter (Fig. 8). It is also possible that digitonin inhibits the formation of MII. As seen in Fig. 4, rhodopsin in digitonin formed a mixture of MI and MII. The ratio of MI to MII intermediates of rhodopsin is influenced dramatically by temperature. Increasing temperature favors the formation of MII. Under the same conditions, in dodecyl maltoside, rhodopsin is converted instantaneously to MII upon illumination. Finally, there is the possibility that digitonin inhibits rhodopsin-transducin interaction through an effect on rhodopsin or transducin or both. In summary, in our work, dodecyl maltoside has uniformly been superior to digitonin for rhodopsin purification and functional studies as judged by A_{280}/A_{500} ratios in absorption spectra, and by GTPase assays.

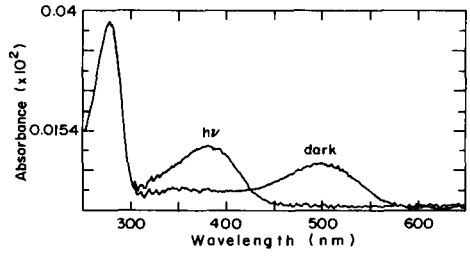

FIG. 9. **UV-visible absorption spectra of mutant EF-Δ5 (Δ234–252) (Table I).** This deletion mutant had 19 amino acids removed from loop EF. According to the secondary structure model (Fig. 1), only a 3-amino acid long linker would remain to connect helices E and F. The mutant bound 11-*cis*-retinal to give a λ_{max} of 500 nm and an A_{280}/A_{500} spectral ratio of 5.5. This low 11-*cis*-retinal regeneration could be due to structural constraints caused by the large deletion. Upon illumination, the pigment was converted to the characteristic MII form (380 nm) indistinguishable from that of wild-type rhodopsin.

Deletions in Loops CD and EF—Previously, König *et al.* (11) showed that synthetic peptides corresponding in sequence to the cytoplasmic rhodopsin loops competed for transducin binding to photolyzed rhodopsin. The results indicated that the cytoplasmic loops CD and EF were involved in interaction with transducin. The involvement of loop EF was also supported by an earlier study by Kühn and Hargrave (9), where limited proteolysis of loop EF caused the loss of light-induced transducin binding. Our earlier (12, 15) and present results support the involvement of loops CD and EF in transducin binding and activation. Thus, an 8-amino acid deletion mutant in loop CD (Δ143–150) failed to activate transducin, indicating that loop CD is essential for transducin activation. Furthermore, when 13 amino acids in loop CD were replaced by an unrelated sequence (CD-7) (Table II), the mutant bound 11-*cis*-retinal to form the normal chromophore, but it failed to activate transducin. This result further demonstrates the requirement of a specific sequence in loop CD for interaction with transducin.

The requirement of loop EF was also clearly shown by our results. All of the loop EF deletion mutations now described affected the ability of these mutants to activate transducin *in vitro*. A 4-amino acid deletion in the amino-terminal part of the loop (Δ236–239) caused a 50% reduction of transducin activation. Furthermore, a 6-amino acid deletion in the carboxyl-terminal portion of loop EF (Δ244–249) caused a complete inability to activate transducin. Similarly, all the other deletion mutations involving the carboxyl terminus of loop EF abolished transducin activation (Table IV).

Single and Multiple Amino Acid Substitutions in Loops CD and EF—A relatively large number of mutants with amino acid substitutions in loop EF were prepared to probe the role of polar and charged amino acids. There is a cluster of 2 threonine residues and 1 serine residue in the center of this loop. These residues are involved in light-induced phosphorylation (25). The role of these residues was investigated by carrying out a series of single and multiple amino acid substitutions. The GTPase activity results obtained with these mutants showed that Ser-240, Thr-243, and Lys-248 were important for transducin activation (Tables III and VI). Mutations that neutralized negatively charged amino acids (Table III) had no influence on spectral properties and no significant effect on transducin activation (Tables III and VI). The above results show a direct involvement of loop EF in transducin activation and are in agreement with peptide competition studies (11) in which a peptide corresponding to loop EF could compete with rhodopsin for transducin binding and with a previous study of rhodopsin loop mutants (15).

Other members of the seven-helical receptor family have also been shown to have an active involvement of loop EF in

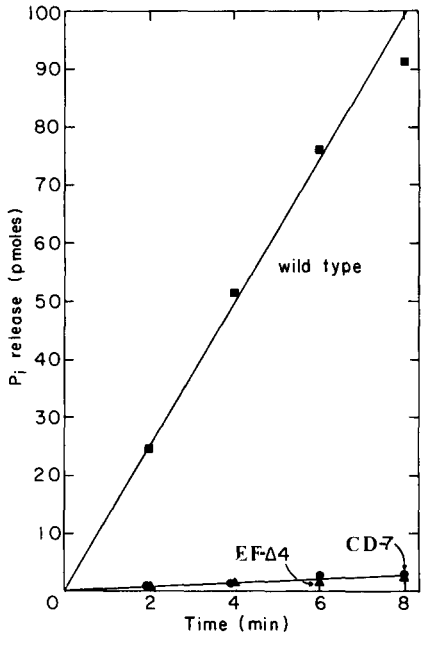

FIG. 10. **Mutants CD-7 and EF-Δ4 failed to activate transducin even at 4-fold increased concentration.** Under standard assay conditions, both mutants CD-7 and EF-Δ4 failed to activate transducin (Tables IV and V). This experiment tested whether activity could be recovered by increasing the concentration of the pigment in the assay mixture. When assayed at a pigment concentration of 10 nM, no detectable transducin activation was observed for either of the mutants. The P_i release observed under these conditions is identical to the intrinsic GTPase activity of transducin observed in the absence of pigment. The activity of wild-type rhodopsin is 12.5 pmol of P_i released per pmol of rhodopsin/min at this concentration (10 nM). This pigment concentration for wild-type rhodopsin is beyond the linear range of the assay (0.2–5 nM). A pigment concentration of 20 nM is saturating (Fig. 5). Even at a pigment concentration of 30 nM, no transducin activation was observed for either of the mutants.

G protein coupling. Kobilka *et al.* (26) suggested that with chimeric α_2- and β_2-adrenergic receptors, the specificity for coupling to the G protein is within loop EF. Kubo *et al.* (27) concluded that in the muscarinic acetylcholine receptor, the selective coupling of receptor subtypes I and II with different effector systems is due to the loop EF region in these receptors. Again, Wong *et al.* (28) indicated with chimeric muscarinic cholinergic/β-adrenergic receptors that the third cytoplasmic loop determines G protein specificity of the ligand-activated receptor.

The charged pair Glu-134/Arg-135 located at the cytoplasmic border of helix C is found in nearly every G protein-coupled receptor characterized to date. We investigated the role of this charged pair with a series of five mutants (Table II). All five mutants displayed normal UV-visible spectra. The amino acid substitutions had a drastic influence on rhodopsin-transducin interaction (Table V). Arg-135 is clearly very important for transducin interaction since substitution R135Q decreased transducin activation by more than an order of magnitude. Charge reversal and exchange of both residues with Ala caused complete inactivation of the resulting pigments in the transducin assay. In the case of the charge reversal mutant, we could not detect stimulation of the transducin GTPase activity even when the pigment concentration was increased 4- and 12-fold over the standard assay concentration (Fig. 10). We found previously that the charged pair plays an essential role in MII-transducin interactions that lead to binding of transducin (12).

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