

## A Single Amino Acid Substitution in Rhodopsin (Lysine 248→Leucine) Prevents Activation of Transducin\*

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Roland R. Franke‡, Thomas P. Sakmar§, Daniel D. Oprian¶, and H. Gobind Khorana

From the Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

In structure-function studies on bovine rhodopsin by *in vitro* site-specific mutagenesis, we have prepared three mutants in the cytoplasmic loop between the putative transmembrane helices E and F. In each mutant, charged amino acid residues were replaced by neutral residues: mutant 1, Glu<sup>239</sup>→Gln; mutant 2, Lys<sup>248</sup>→Leu; and mutant 3, Glu<sup>247</sup>→Gln, Lys<sup>248</sup>→Leu, and Glu<sup>249</sup>→Gln. The mutant rhodopsin genes were expressed in monkey kidney (COS-1) cells. After the addition of 11-*cis*-retinal to the cells, the rhodopsin mutants were purified by immunoaffinity adsorption. Each mutant gave a wild-type rhodopsin visible absorption spectrum. The mutants were assayed for their ability to stimulate the GTPase activity of transducin in a light-dependent manner. While mutants 1 and 3 showed wild-type activity, mutant 2 (Lys<sup>248</sup>→Leu) was inactive.

In visual transduction, the photopigment rhodopsin absorbs light and undergoes a series of structural changes. The rhodopsin photointermediate *meta*-rhodopsin II (1) activates transducin, a guanine nucleotide-binding protein (G<sub>αβγ</sub>).<sup>1</sup> The α-subunit of transducin (G<sub>α</sub>) exchanges bound GDP for GTP (2), dissociates from the complex of photolyzed rhodopsin and G<sub>βγ</sub>, and activates a rod outer segment (ROS) cyclic GMP phosphodiesterase. Modulation of the cyclic GMP concentration is believed to regulate the plasma membrane cation conductance and cause hyperpolarization of the rod cell. Clearly, the study of the light-induced structural changes in rhodopsin and the mechanism of the consequent activation of transducin are topics of great interest. G proteins have also been discovered in a variety of signal-transducing systems. There are structural and functional similarities among the G

proteins (3) and among the receptors that activate them. Therefore, the study of the visual system should also serve as a model for related transmembrane signaling systems. For structure-function studies of rhodopsin, we have carried out the synthesis of a gene for bovine rhodopsin (4, 5) which facilitates mutagenesis by restriction fragment replacement. The synthetic gene was expressed in COS-1 cells and the protein product, after *in vivo* reconstitution with 11-*cis*-retinal, was purified, and characterized. The purified COS-1 cell rhodopsin stimulated the GTPase activity of bovine transducin with a specific activity identical to that of rhodopsin purified from bovine retinas (6).

Using the above expression system and *in vitro* site-specific mutagenesis of the synthetic rhodopsin gene, we are now probing the sites of interaction between photolyzed rhodopsin and transducin. In this communication, we report on the effects of amino acid substitutions in the cytoplasmic loop linking putative transmembrane helices E and F in rhodopsin (7) (Fig. 1). Existing data from proteolysis experiments of rhodopsin (8) and from deletion mutagenesis of the hamster β-adrenergic receptor (9) suggested that this region was involved in G protein activation. Three mutant genes were constructed in which the following charged amino acids were replaced by neutral ones (Fig. 1): mutant 1, Glu<sup>239</sup>→Gln; mutant 2, Lys<sup>248</sup>→Leu; and mutant 3, a triple mutant, Glu<sup>247</sup>→Gln, Lys<sup>248</sup>→Leu, and Glu<sup>249</sup>→Gln. All of the purified mutant proteins displayed wild-type rhodopsin absorption spectra. When tested for the ability to activate transducin, mutants 1 and 3 showed wild-type responses while mutant 2 (Lys<sup>248</sup>→Leu) failed to activate transducin.

### EXPERIMENTAL PROCEDURES

**Materials**—The expression vector, pMT-2, a β-lactamase derivative of p91023 (10, 11), was generously provided by Dr. R. J. Kaufman, Genetics Institute, Inc., Cambridge, MA.

Sources of COS-1 cells, bovine retinas, 11-*cis*-retinal, detergents, concanavalin A-Sepharose 4B, and protein A, and the preparation of buffers and media have been described (6). Na<sup>125</sup>I (16.4 mCi/mg iodine), [γ-<sup>32</sup>P]GTP (30 Ci/mmol), and [<sup>35</sup>S]dATPαS (650 Ci/mmol) were purchased from Amersham Corp.

**Antibodies and Immunoaffinity Resin**—Rabbit polyclonal anti-rhodopsin IgG antibody was the generous gift of Dr. P. A. Hargrave, University of Florida, Gainesville, FL. The preparation and characterization of the anti-rhodopsin monoclonal antibody, 1D4 (12), and the coupling of the antibody to Sepharose 2B have been described (6). Peptide I, used to elute rhodopsin from the immunoaffinity resin, has also been described (6).

**Purification of Transducin and Rhodopsin from Bovine Retinas**—Rod outer segments were prepared from frozen bovine retinas according to the procedure of Hong and Hubbell (13) as modified by Fung *et al.* (14). Rhodopsin was purified on concanavalin A-Sepharose 4B (15) in 1% digitonin. Transducin was isolated from rod outer segments in room light according to Fung *et al.* (14). Protein was quantitated according to Lowry *et al.* (16) as modified by Bensadoun and Weinstein (17) with bovine serum albumin as the standard.

**Preparation of Oligonucleotides**—Oligonucleotides were synthesized on an Applied Biosystems Model 380A automated DNA synthesizer. The purification and characterization of the oligonucleotides were performed essentially according to Ferretti *et al.* (4).

**Cloning of the Synthetic Rhodopsin Gene into Expression Vector pMT-2**—The vector pMT-2 contained a *Pst*I-*Eco*RI cloning site downstream from the adenovirus major late promoter. Since the synthetic rhodopsin gene contained an internal *Pst*I site and a 5'-terminal *Eco*RI site, the pMT-2 cloning site was reconstructed to form an *Eco*RI-*Not*I cloning site. *Not*I was selected because it was

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¶ Present address: Graduate Dept. of Biochemistry, Brandeis University, Waltham, MA 02254.

<sup>1</sup> The abbreviations used are: G protein, guanine nucleotide-binding protein; ROS, rod outer segment; [<sup>35</sup>S]dATPαS, [<sup>35</sup>S]deoxyadenosine 5'-(α-thio)triphosphate.

not present in pMT-2 or in the synthetic rhodopsin gene. A 30-base pair synthetic duplex (*Pst*I'-*Eco*RI-spacer-*Not*I-*Eco*RI') was ligated into pMT-2 linearized with *Pst*I and *Eco*RI. The original *Pst*I and *Eco*RI sites were destroyed and an *Eco*RI-*Not*I cloning site was created. The cloning of the synthetic rhodopsin gene with the consensus sequence CCACC (18), immediately upstream of the initiation codon into the expression vector p91023(B), was previously described (6). A restriction fragment containing the 5'-1032 nucleotides of the 1048-nucleotide synthetic rhodopsin gene was isolated from this vector construction after *Eco*RI and *Bst*XI digestion. To allow cloning into the *Eco*RI-*Not*I site of the modified pMT-2 expression vector, the 3'-end of the gene after the *Bst*XI site was reconstructed to include a *Not*I site after the termination codon. A three-component ligation mixture contained: 1) the vector linearized by *Eco*RI and *Not*I digestion, 2) the *Eco*RI-*Bst*XI rhodopsin gene restriction fragment, and 3) the *Bst*XI-*Not*I synthetic duplex. The ligation mixture was used to transform CaCl<sub>2</sub>-treated *Escherichia coli* strain DH1. Plasmid DNA was prepared from an ampicillin-resistant colony.

**Construction of Rhodopsin Mutants**—Mutations were introduced into the synthetic rhodopsin gene by restriction fragment replacement (19). Fifty-seven base pair DNA duplexes (nucleotides 699-755 in the gene), corresponding to the restriction fragment *Pst*I-*Mlu*I, were synthesized with the desired codon alterations. The *Pst*I and *Mlu*I sites in the rhodopsin gene were also unique in the expression vector described above. A two-component ligation consisting of the expression vector/gene construction linearized with *Pst*I and *Mlu*I and the mutagenic duplex was carried out for each mutant. The plasmid DNAs were prepared from ampicillin-resistant colonies.

**DNA Sequencing of Mutants**—The nucleotide sequence of all cloned synthetic duplexes described was confirmed by the chain terminator method for DNA sequencing using [<sup>35</sup>S]dATPαS on CsCl-purified plasmid DNA (20, 21). For mutant 2, the entire gene was sequenced to confirm that the only nucleotide change present was that introduced by mutagenesis.

**Expression of Rhodopsin Mutants in COS-1 Cells**—Wild-type and the mutant rhodopsins were all prepared as follows. COS-1 cells were transfected with CsCl-purified plasmid DNA by treatment with DEAE-dextran (22) as reported previously (6). After incubation for 72 h, 11-*cis*-retinal (40 μM) was added to the cells. The cells were solubilized and rhodopsin was purified according to the immunoaffinity procedure of Oprian *et al.* (6).

**Characterization of Rhodopsin Mutants**—The wild-type rhodopsin and mutants were characterized by absorption spectroscopy, immunoblotting (23) using rabbit polyclonal anti-rhodopsin IgG, and polyacrylamide gel electrophoresis performed according to Laemmli (24) with visualization of protein bands by silver staining (25).

**Functional Assays**—The purified rhodopsin mutants were assayed for enhancement of light-dependent GTPase activity of rod outer segment transducin (26). The assays were performed as previously described (6). Reaction rates were corrected for the first-order decay of light-activated rhodopsin (0.053 min<sup>-1</sup>) which was measured independently under identical assay conditions (data not shown).

**Other Methods**—[γ-<sup>32</sup>P]Phosphate was measured by the method of Neufeld and Levy (27). Protein A was iodinated with Na<sup>125</sup>I using chloramine T (28).

## RESULTS

The three rhodopsin gene mutants shown in Fig. 1 were expressed in COS-1 cells. The levels of expression were comparable to that of the wild-type COS-1 cell rhodopsin as judged by immunoblotting (see Fig. 2A). The mutant rhodopsins were reconstituted in the COS-1 cells with exogenously added 11-*cis*-retinal and purified to apparent homogeneity by an immunoaffinity procedure previously reported (6). Silver staining (Fig. 2B) and immunoblotting (Fig. 2A) of polyacrylamide gels showed single bands for each of the purified rhodopsin mutants. A degree of smearing was noted above the bands and varied between different preparations as previously observed (6). The electrophoretic mobility of the mutants was identical to that of the wild-type rhodopsin produced by the COS-1 cells but it was consistently less than that of ROS rhodopsin. The difference in mobility between the ROS and COS-1 cell rhodopsins could be due to differences in glycosylation in the tissues involved or some other post-transla-

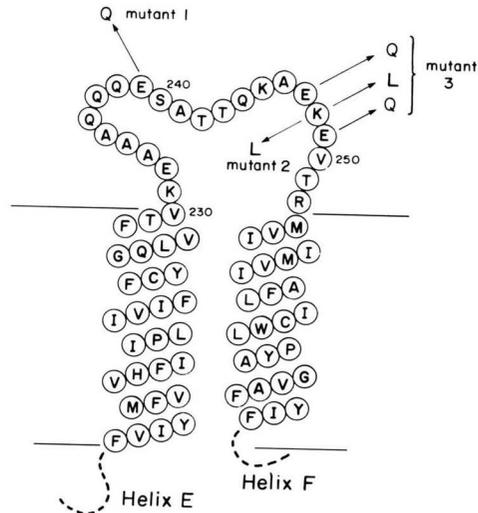


FIG. 1. A schematic representation of rhodopsin mutants. The amino acid sequence of bovine rhodopsin transmembrane helices E and F and the cytoplasmic loop linking these helices is shown. Three mutants were constructed as described under "Experimental Procedures": mutant 1, Glu<sup>239</sup>→Gln; mutant 2, Lys<sup>248</sup>→Leu; and mutant 3, Glu<sup>247</sup>→Gln, Lys<sup>248</sup>→Leu, and Glu<sup>249</sup>→Gln.

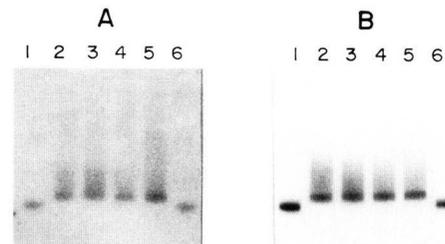
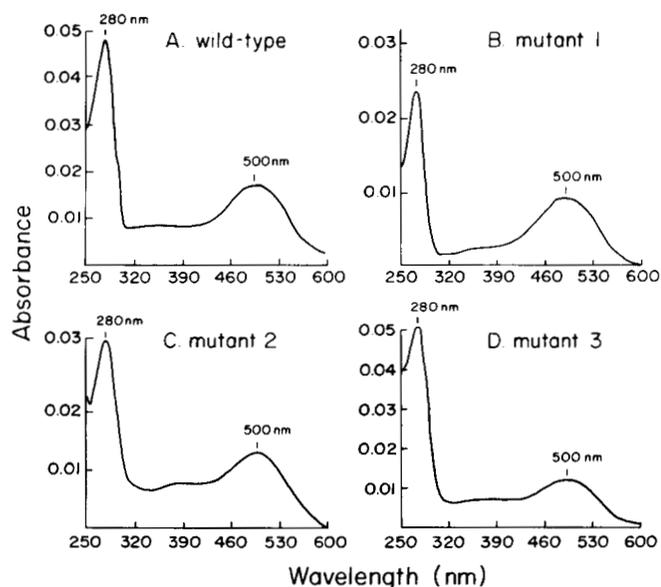


FIG. 2. Characterization of rhodopsin mutants. A, immunoblot analysis of rhodopsin mutants expressed in COS-1 cells. Lane 1, rhodopsin purified from ROS on concanavalin A (80 ng). Lane 2, purified rhodopsin mutant 1. Lane 3, purified rhodopsin mutant 2. Lane 4, purified rhodopsin mutant 3. Lane 5, purified COS-1 cell rhodopsin. Lane 6, rhodopsin standard as in lane 1. B, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified COS-1 cell rhodopsins visualized by silver staining. Lane 1, rhodopsin purified from ROS on concanavalin A (80 ng). Lane 2, purified rhodopsin mutant 1. Lane 3, purified rhodopsin mutant 2. Lane 4, purified rhodopsin mutant 3. Lane 5, purified COS-1 cell rhodopsin. Lane 6, rhodopsin standard as in lane 1.

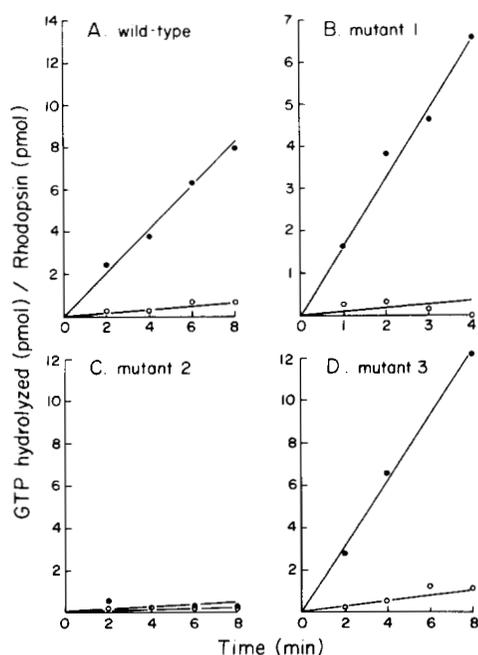
tional modification (6). This requires further investigation.

The absorption spectra of the purified wild-type and mutant rhodopsins are shown in Fig. 3. The visible absorption spectrum of each mutant ( $\lambda_{\text{max}} = 500 \text{ nm}$ ) was essentially identical to that of the wild-type. The ratio of absorbance ( $A_{280}/A_{500}$ ) varied: wild-type COS-1 cell rhodopsin showed a ratio of 2.8, while mutants 1, 2, and 3 purified from COS-1 cells showed ratios of 2.5, 2.4, and 4.3, respectively. Variability was also observed between different preparations of the same mutant. Since the purified proteins appeared to be homogeneous in every case, the differences in absorbance ratios were presumed to be due to varying amounts of rhodopsin apoprotein reconstituted with retinal (6). The important goal of obtaining fully reconstituted rhodopsin is the object of ongoing work.

The ability of the rhodopsin mutants to stimulate the GTPase activity of transducin was assayed as shown in Fig. 4. The assay performed measured GTP hydrolysis by bovine transducin after light-dependent activation by rhodopsin. Previously, COS-1 cell rhodopsin and ROS rhodopsin were shown to activate transducin with identical specific activities (6). In the data shown in Fig. 4, mutant 2 failed to stimulate



**FIG. 3. Absorption spectra of COS-1 cell rhodopsin and mutants.** The wild-type and mutant rhodopsins were purified from COS-1 cells as described under "Experimental Procedures." The  $\lambda$  maximum of each mutant was identical to that of wild-type COS-1 cell rhodopsin and ROS rhodopsin (500 nm). A, rhodopsin purified from COS-1 cells (wild-type); B, mutant 1; C, mutant 2; D, mutant 3.



**FIG. 4. Rhodopsin mutant 2 (Lys<sup>248</sup>→Leu) did not enhance the GTPase activity of transducin.** A time course of GTP hydrolysis by transducin is shown for COS-1 rhodopsin and for each mutant. A, rhodopsin purified from COS-1 cells (wild-type); B, mutant 1; C, mutant 2; D, mutant 3. GTP hydrolysis was measured as described under "Experimental Procedures." Activity is expressed as picomole of GTP hydrolyzed per picomole of rhodopsin as a function of time. Reactions were performed in the light (●) and in the dark (○). Mutant 2 did not activate transducin. The specific activities of the other 2 mutants (1.6 min<sup>-1</sup>) were similar to that of wild-type COS-1 cell rhodopsin (1.0 min<sup>-1</sup>). COS-1 cell rhodopsin and ROS rhodopsin were previously shown to have indistinguishable specific activities (6).

the GTPase activity of transducin under the conditions of the assay. Mutants 1 and 3 were able to activate transducin normally. The specific activities of these mutants appear slightly higher than wild-type COS-1 cell rhodopsin. This degree of variation in specific activity was observed between different preparations of the same mutant and is considered to be within the range of experimental error of the assay.

#### DISCUSSION

To elucidate the nature of the interactions between rhodopsin and transducin, we wish to introduce selected amino acid substitutions in the two proteins by the techniques of site-specific mutagenesis. In this initial study, we have reported on three rhodopsin mutants with amino acid substitutions in the cytoplasmic loop connecting the transmembrane helices E and F (Fig. 1). A proteolysis study had previously identified this cytoplasmic loop in rhodopsin as a potential site of interaction with transducin (8). An analogous region in the topographically similar hamster  $\beta$ -adrenergic receptor had also been suggested to interact with the stimulatory guanyl nucleotide-binding protein (9). The length and amino acid sequence of this loop is not well conserved in the family of homologous receptors that couple to G proteins. However, a cluster of charged amino acids near transmembrane helix F is a common feature, and Lys<sup>248</sup> is highly conserved in the G protein-coupled receptors sequenced to date (29) except for the human blue pigment which contains an Arg at this position (30). To test whether altering these charged amino acids in this cytoplasmic loop would affect transducin activation, we constructed three rhodopsin mutants (Fig. 1) and expressed them in COS-1 cells. The levels of expression for the three mutants were comparable and the purified mutant proteins bound retinal and displayed wild-type visible absorption spectra (Fig. 3). This result showed that the amino acid substitutions did not affect the correct folding of the protein as judged by the binding of 11-*cis*-retinal.

The rhodopsin mutants were assayed for the ability to stimulate the GTPase activity of transducin. Mutant 2, containing a single amino acid substitution, Lys<sup>248</sup>→Leu, did not activate transducin while the other two mutants activated transducin at wild-type levels. The dramatic result obtained by the substitution of a single charged amino acid suggests the involvement of an electrostatic interaction in transducin activation. However, a definitive conclusion regarding such an interaction cannot be put forward at this stage. The net charge at neutral pH of the wild-type amino acid residue 247–249 tripeptide (-Glu-Lys-Glu-) is -1. In mutant 2 (-Glu-Leu-Glu-) the net charge was -2 and in mutant 3 (-Gln-Leu-Gln-) the net charge was 0. In deducing the relevant overall charge of this domain, Glu<sup>239</sup> and Lys<sup>245</sup> may also be important. The functional analysis of additional mutants is underway. Nevertheless, the results presented here strongly support the involvement of the cytoplasmic loop linking helices E and F in transducin activation. This loop may interact directly with transducin or it may interact indirectly through another region of rhodopsin such as the carboxyl-terminal tail.

The assay employed to characterize the rhodopsin mutants measured light-dependent GTP hydrolysis. Activity in this assay requires 1) that a rhodopsin mutant undergo photoexcitation to form the photointermediate *meta*-rhodopsin II, 2) that the latter binds transducin and induces it to exchange GDP for GTP, and 3) that the resulting T<sub>α</sub>·GTP complex be able to hydrolyze GTP. The present assay cannot identify at which of these steps a block may occur. Alternative assays will be needed to determine if the inactive mutant retains the ability to undergo a normal photocycle and to bind transducin.

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