Mutagenesis Studies of Rhodopsin Phototransduction

*T.P. Sakmar, R.R. Franke, and H.G. Khorana**

The Rockefeller University, NY 10021, USA and
*Departments of Biology and Chemistry,
Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Abstract

Site-directed mutagenesis was employed in structure-function studies of bovine rhodopsin. Charged amino acids in the third transmembrane helix (Glu^{113}, Glu^{122}, Glu^{134}, and Arg^{135}) were systematically replaced. We conclude that Glu^{113} serves as the counterion to the retinylidene Schiff base. The second and third cytoplasmic loops linking transmembrane helices were studied by analysis of amino acid deletion and replacement mutations. Certain alterations of these loops produced rhodopsin mutants that bound but failed to activate transducin.

Introduction

Secondary structure models (Figure 1) indicate the presence of seven transmembrane domains in rhodopsin. This structural feature is shared among the members of the family of receptors that couple to guanine nucleotide-binding regulatory (G) proteins. The third transmembrane domain of bovine rhodopsin might contain up to four charged amino acids: Glu^{113}, Glu^{122}, Glu^{134}, and Arg^{135}. We investigated the possible involvement of these charged amino acids in the opsin shift and in transducin activation by site-directed mutagenesis. The role of the second and third cytoplasmic loops in transducin binding and activation was also evaluated. Mutant opsin genes were prepared by restriction fragment

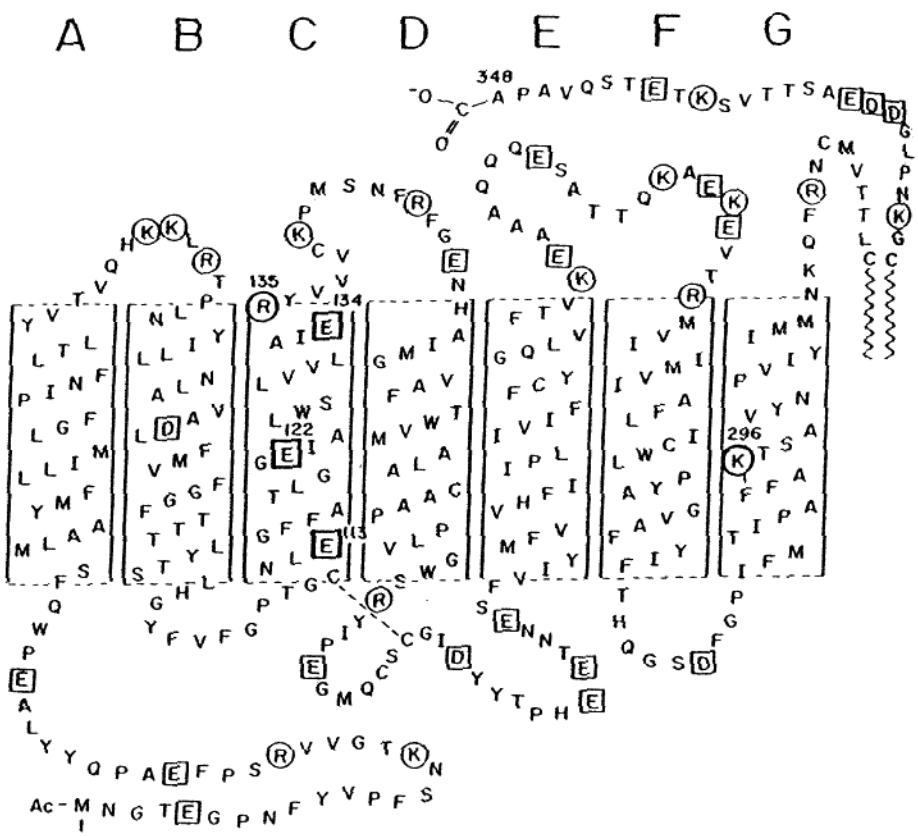

Figure 1: Schematic representation of bovine rhodopsin demonstrating the seven transmembrane helices (A-G). Potentially charged amino acids are highlighted and key positions in transmembrane helix C are numbered.

replacement in a synthetic opsin gene (Oprian et al. 1987). The genes were expressed in monkey kidney cells (COS-1) in tissue culture. After regeneration with retinal chromophore, the cells were solubilized in dodecyl maltoside detergent buffer and mutant pigments were purified by an immunoaffinity adsorption procedure (Oprian et al. 1987).

Results and Discussion

Glutamic acid 113 serves as the retinylidene Schiff base counterion. A series of mutants was prepared in which Glu^{113} was replaced. Replacement of Glu^{113} by Lys (E113K) formed a protein that did not bind 11-*cis* -retinal. It displayed a multiple band pattern on a polyacrylamide gel similar to that seen with cysteine mutants (Karnik et al. 1988) and intradiscal mutants that failed to fold properly in the membrane bilayer (Doi et al. 1990). This result was consistent with Glu^{113} being localized within the bilayer region of the protein where an uncompensated positive charge would prevent proper folding and 11-*cis* - retinal binding.

Mutant E113D in which Glu^{113} was replaced by aspartic acid bound 11-*cis* -retinal to yield a red-shifted chromophore absorbing at 505 nm (Figure 2). Upon illumination, the 505 nm peak was converted to 480 nm consistent with a metarhodopsin I-like (MI) (all-*trans*-retinal) species. This 480 nm species decayed slowly in the dark to a 380 nm form. The MI-like (protonated Schiff base) to metarhodopsin II-like (MII) (unprotonated Schiff base) transition was slowed down by orders of magnitude in E113D with respect to rhodopsin. In rhodopsin, conversion from MI to MII involves deprotonation of the Schiff base imine. In E113D, the corresponding deprotonation of the Schiff base may be hampered, resulting in a stabilized 480 nm MI-like intermediate. Mutant E113D was able to activate transducin at nearly normal levels (Sakmar et al. 1989).

Mutant E113Q in which Glu^{113} was replaced by glutamine bound 11-*cis* -retinal to yield a pigment that absorbed predominantly in the near ultraviolet range at 380 nm with a small second component at about 490 nm at neutral pH (Figure 2). The proportion of these two absorbance maxima depended on pH. Acidification of E113Q converted the 380 nm peak to 490 nm. This conversion was reversible and a single isosbestic point was observed. The two forms of the E113Q pigment appeared to exist in a pH-dependent equilibrium mixture of unprotonated (380 nm) and protonated (490 nm) Schiff base species.

Illumination of E113Q at neutral pH caused the disappearance of the 490 nm peak with a concomitant increase in the 380 nm peak (Figure 2). The light-activated E113Q pigment activated transducin slightly more efficiently than did rhodopsin (Figure 4). One explan-

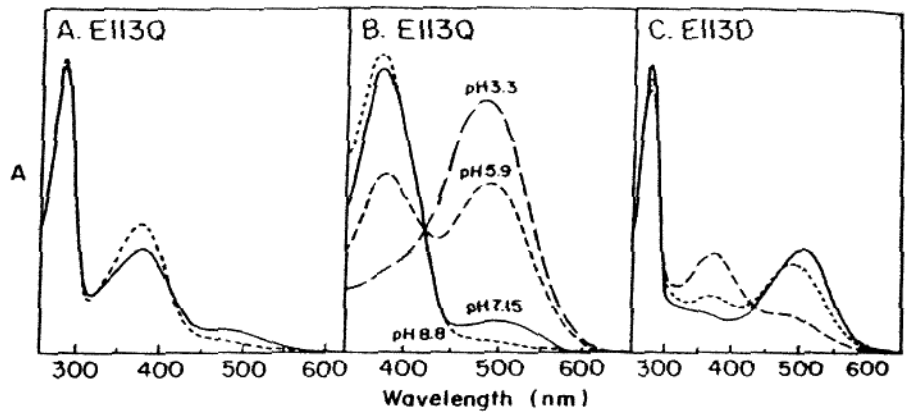

Figure 2: UV-visible spectroscopy of the Glu^{113} mutant pigments. A. Mutant E113Q regenerated with 11-*cis* -retinal showed a dark spectrum with a λ_{max} of 380 nm with a small second peak at 490 nm (——). Illumination with light of greater than 495 nm wavelength produced a loss of the 490 nm peak with an increase in the 380 nm peak. (- - -). B. The mutant pigment E113Q spectrum was pH dependent. The 380 nm and 490 nm peaks were interconvertible in the dark by pH titration. The interconversion by titration was reversible and a consistent isosbestic point was noted. C. Mutant E113D regenerated with 11-*cis*- retinal displayed a red-shifted λ_{max} of 505 nm (——). Upon brief illumination, a shift to 480 nm consistent with the stable formation of MI-like species was observed (- - -). This species decayed in the dark to a 380 nm peak (- - -) (Sakmar et al. 1989).

ation for this activity increase was that the light-activated E113Q pigment had a longer half-life time than rhodopsin (150 min. versus 18 min.). The slight increase in transducin activation of E113Q over rhodospin may be due to the slower decay of the MII-like species. In dodecyl maltoside solubilized samples, the decay of MII corresponded to hydrolysis of the retinylidene Schiff base linkage. The marked increase in stability of photoactivated mutant pigment E113Q suggested that Schiff base hydrolysis may be slowed down. Therefore, Glu^{113} may be

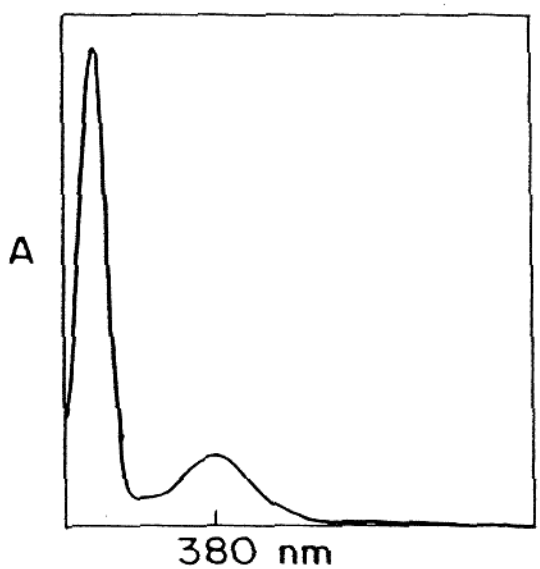

Figure 3: UV-visible spectrum of mutant E113Q regenerated with all-*trans* - retinal. A Schiff base linkage was formed between the chromophore and opsin (Sakmar et al. 1989).

involved in a catalytic hydrolysis of the Schiff base linkage in MII. Catalysis of the Schiff base hydrolysis in rhodopsin by a carboxyl group has been proposed (Cooper et al. 1987).

Mutant E113Q bound all-*trans* -retinal and formed a Schiff base linkage to give a pigment that absorbed maximally at about 380 nm with no peak in the visible spectrum (Figure 3). The E113Q pigment regenerated with all-*trans* -retinal was able to stimulate the GTPase activity of transducin in the dark (Figure 4). Dark activity levels were about 50% of wild-type MII rhodopsin levels. Opsin and E113D apoproteins incubated with all-*trans* -retinal under identical conditions did not activate transducin in the dark. The dark activity of this pigment decayed at a rate similar to that of the photoactivated E113Q 11-*cis* -retinal regenerated pigment (half-time of about 150 min.). This pigment appeared to be locked into a MII-like conformation. Furthermore, it could bypass the usual intermediates in the rhodopsin photoacti-

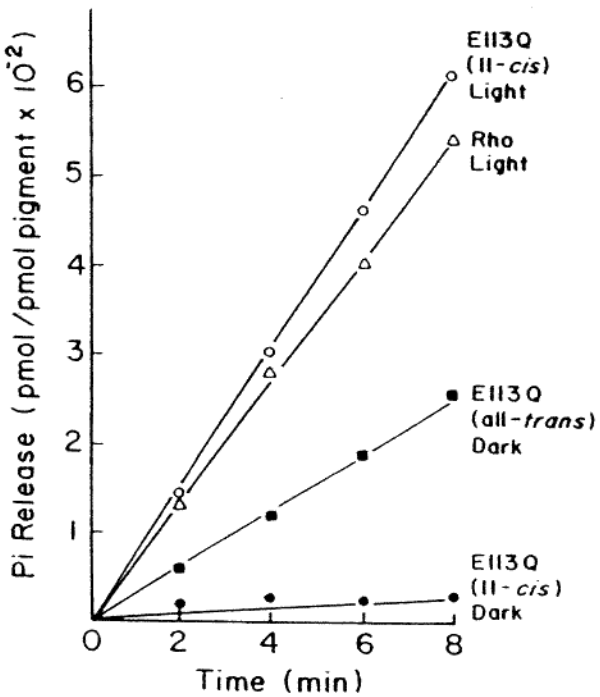

Figure 4: Stimulation of transducin GTPase activity by mutant pigments (Sakmar et al. 1989).

vation pathway. Thus, in this mutant, a binding pocket occupied by all-*trans*- retinal linked to the protein by an unprotonated Schiff base was sufficient for transducin activation (Sakmar et al. 1989).

More recent work on additional Glu^{113} replacements has shown that the amino acid at position 113 affects the apparent pKa of the Schiff base imine. Furthermore, the λ_{max} value of the visible form of some Glu^{113} mutant pigments depends upon the concentration and type of solute anion. Thus, it appears that a solute anion can substitute for the Glu^{113} carboxlyate group to compensate for the Schiff base positive charge in the acid form of certain mutant pigments (Sakmar et al. in press).

The results of the Glu^{113} mutagenesis studies are consistent with Glu^{113} serving the role of counterion to the retinylidene Schiff base imine. Glu^{113} is thus unprotonated and charged in rhodopsin, and it is this negative charge that compensates for the positive charge of

the Schiff base. Without a carboxylate group at position 113, the Schiff base will not become protonated under physiological pH and ionic strength conditions. The exact environment in the Schiff base region is not known from these experiments. However, a proximity of Glu^{113} to the Schiff base is supported by the effects on the imine pKa and the stability of the Schiff base linkage after illumination as described above.

Evidence that glutamic acid 122 is uncharged in rhodopsin. Glu^{122} was replaced by lysine (E122K), glutamine (E122Q), or aspartic acid (E122D). Mutant opsin E122K did not bind 11-*cis* -retinal as seen with E113K. It is likely that introduction of a positive charge into the transmembrane helix prevented proper folding of the mutant protein such that it would not bind retinal. Replacement of Glu^{122} by glutamine or aspartic acid produced blue-shifted pigments absorbing at 480 nm and 475 nm respectively (Figure 5). Thus, a portion of the opsin shift may result from an interaction between the retinal chromophore and Glu^{122}. Since the replacement of Glu^{122} by an aspartic acid, retaining a carboxyl group, and by glutamine resulted in mutants with similar λ_{max} values, it is possible that Glu^{122} is present in the protonated and uncharged state in rhodopsin (Sakmar et al. 1989) .

The role of the conserved charged pair glutamic acid 134, arginine 135. The charged pair Glu^{134}, Arg^{135} in rhodopsin is conserved in all G protein-coupled receptors as either Glu, Arg or Asp, Arg (Figure 1). These amino acids are likely to form the cytoplasmic border of helix C in rhodopsin and related receptors. Three single mutations (E134D, E134Q, and R135Q) and two double mutations (E134A, R135A and E134R, R135E) were prepared (Sakmar et al. 1989). Each of the five mutants bound 11-*cis* -retinal to give a normal spectrum with λ_{max} of 500 nm (Figure 5). Thus, these amino acids are not involved in the chromophore-opsin interactions controlling the opsin shift. The ability of these mutant pigments to activate transducin in a light-dependent manner was evaluated. Substitution of Arg^{135}, either singly or in combination with Glu^{134}, severely reduced the ability of the resulting mutant to activate transducin. Charge reversal, or neutralization of both charges by substitution with alanines, completely abolished transducin activation. Neutralization of Glu^{134}, resulting in an uncompensated positive charge at Arg^{135}, led to an enhancement of

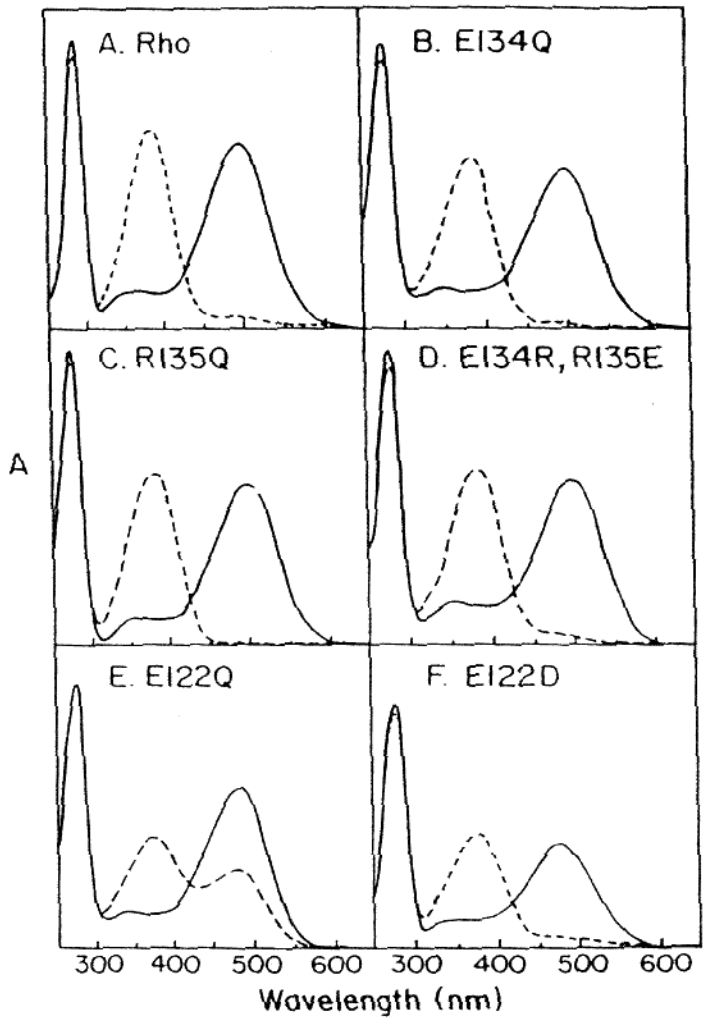

Figure 5: UV-visible spectroscopy of mutant pigments in the dark (——) and after 10 sec. illumination with light of greater than 495 nm wavelength (- - -). Each of the mutants was regenerated with 11-*cis* -retinal. A. COS cell rhodopsin showed the characteristic λ_{max} of 500 nm. B. Mutant E134Q displayed a λ_{max} of 500 nm. C. Mutant R135Q displayed a λ_{max} of 500 nm. D. Mutant E134R, R135E displayed a λ_{max} of 500 nm. E. Mutant E122Q displayed a λ_{max} of 480 nm. Illumination produced less 380 nm species than was the case for rhodopsin. F. Mutant E122D displayed a λ_{max} of 475 nm.

transducin activating ability. Replacement of Glu^{134} by aspartic acid resulted in a moderate reduction in activity. These results were consistent with the Glu^{134}, Arg^{135} charged pair playing a role in transducin binding or activation.

The charge reversal mutant (E134R, R135E) was evaluated further by a flash photolysis method (Franke et al. 1990). This mutant failed to bind transducin indicating that a direct interaction exists between the charged pair and transducin, or that this charged pair may be involved in the cytoplasmic domain that binds transducin.

Rhodopsin cytoplasmic loop mutants that bind but fail to activate transducin. A number of mutants were constructed involving replacements, deletions, and insertions of amino acids in the cytoplasmic loops linking transmembrane helices C and D (loop CD) and E and F (loop EF) (Franke et al. 1988; Franke et al. in preparation). Deletions of as many as 19 amino acids from loop EF did not affect 11-*cis* -retinal binding. However, a deletion of 14 amino acids from loop CD prevented 11-cis -retinal binding. A loop CD mutant was prepared in which 13 amino acids were replaced by an unrelated sequence derived from amino-terminal tail of rhodopsin. Several mutants with alterations in loop CD and loop EF were identified that failed to activate transducin (Franke et al. in preparation). Two of these mutants, a replacement of 13 amino acids in loop CD, and a deletion of 13 amino acids from loop EF were studied by flash photolysis. These mutants formed MII normally and bound transducin normally, but failed to release transducin in the presence of GTP (Franke et al. 1990). Therefore, it appears that at least the second and third cytoplasmic loops of rhodopsin are required to activate bound transducin. When these sites were altered, an inactive receptor-G protein complex was formed.

REFERENCES

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

Cooper, A., Dixon, S.F., Nutley, M.A., & Robb, J.L. (1987) *J. Am. Chem. Soc.* **109**, 7254-7263

Karnik, S.S., Sakmar, T.P., Chen, H.-B., & Khorana, H.G. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8459-8463

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

Franke, R.R., Sakmar, T.P., Oprian, D.D., & Khorana, H.G. (1988) *J. Biol. Chem.* **362**, 2119-2122

Franke, R.R., König, B., Sakmar, T. P., Khorana, H.G., & Hofmann, K.P. (1990) *Science* **250**, 123-125

Franke, R.R., Sakmar, T.P., Graham, R.M., & Khorana, H.G. in preparation

Sakmar, T.P., Franke, R.R., & Khorana, H.G. (1989) *Proc. Natl. Acad. Sci. USA.* **86**, 8309-8313

Sakmar, T.P., Franke, R.R., & Khorana, H.G. in press