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Relocating the active-site lysine in rhodopsin: 2. Evolutionary intermediates

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Abstract

The visual pigment rhodopsin is a G protein-coupled receptor that covalently binds its retinal chromophore via a Schiff base linkage to an active-site Lys residue in the seventh transmembrane helix. While this residue is strictly conserved among all type II retinylidene proteins, we found previously that the active-site Lys in bovine rhodopsin (Lys296) can be moved to three other locations (G90K, T94K, S186K) while retaining the ability to form a pigment with retinal and to activate transducin in a light-dependent manner [Devine *et al.* (2013) *Proc. Natl. Acad. Sci. USA* 110, 13351–13355]. Since the active-site Lys is not functionally constrained to be in helix seven, it is possible that it could relocate within the protein, most likely via an evolutionary intermediate with two active-site Lys. Therefore, in this study we characterized potential evolutionary intermediates with two Lys in the active site. Four mutant rhodopsins were prepared in which the original Lys296 was left untouched and a second Lys residue was substituted for G90K, T94K, S186K, or F293K. All four constructs covalently bind 11-*cis*-retinal, form a pigment, and activate transducin in a light-dependent manner. These results demonstrate that rhodopsin can tolerate a second Lys in the retinal binding pocket and suggest that an evolutionary intermediate with two Lys could allow migration of the Schiff base Lys to a position other than the observed, highly conserved location in the seventh TM helix. From sequence based searches, we identified two groups of natural opsins, insect UV cones and neuropsins, that contain Lys residues at two positions in their active sites and also have intriguing spectral similarities to the mutant rhodopsins studied here.

Graphical Abstract

The visual pigment rhodopsin from vertebrate rod photoreceptor cells is a prototypical type II retinylidene protein as well as a member of the large class of G protein-coupled receptors

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NOTES

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SUPPORTING INFORMATION AVAILABLE

Dark-state acid trap of the chromophore in the double-Lys mutant pigments.

(GPCRs)^{1, 2}. It is an integral membrane protein composed of a polypeptide chain (opsin) with 7 transmembrane helices (TM) and an 11-*cis*-retinal chromophore (Figure 1). The chromophore is attached to the protein covalently by means of a protonated Schiff base linkage to the ϵ -amino group of a Lys residue in TM7 (K296 in bovine rhodopsin), a residue that is strictly conserved among all type II retinylidene proteins. The positive charge on the Schiff base nitrogen, which is critical for the visible absorption maximum of the pigment ($\lambda_{\text{max}} = 500 \text{ nm}$), is stabilized by a glutamate counterion at position 113 in TM3³. Absorption of a photon causes isomerization of the chromophore to the all-*trans*-form and consequent deprotonation of the Schiff base nitrogen resulting in a shift of the absorption maximum to 380 nm. An associated conformational change in the protein produces the intermediate metarhodopsin II (MII), responsible for activation of the G protein transducin³. Deactivation of rhodopsin leads to the release of all-*trans*-retinal from opsin, which is held in an inactive state by a salt bridge between the Lys296 and Glu113⁴. A new 11-*cis*-retinal molecule must be bound before the protein can be activated again⁵.

In a previous study looking at the evolutionary connection among retinylidene proteins, we asked if the invariant Lys296 could be moved to a different location in the protein⁶. We tested five different locations within the active site of rhodopsin (Figure 1): two positions in TM2 (90 and 94), one in TM3 (117), one on the two-stranded β -sheet connecting TM4 and TM5 (186), and one in a different location in TM7 (293). Surprisingly, four of the five mutants (90, 94, 186, and 293) combine with 11-*cis*-retinal to form pigments with near wild-type spectral properties, and three of these four (90, 94, and 186) activate transducin in a light-dependent manner with specific activities approaching that of wild-type rhodopsin⁶. These results demonstrate that the conserved location of the active site Lys in TM7 is not required for rhodopsin's photosensitive function, contradicting a key prediction of functional constraint for convergent evolution for the retinylidene proteins.

If not from a functional constraint, why then is the location of the Lys invariant among type II retinylidene proteins? Perhaps this is a consequence of an initial location of the Lys in TM7 combined with the absence of viable evolutionary pathways to move the Lys to a different location. Two mutations are required to move the Lys: one at 296, removing the Lys, and a second, at another position, introducing a Lys that is capable of supporting the formation of a Schiff base linkage with the retinal chromophore. It is highly unlikely that these two mutations would occur simultaneously, and evolution would need to progress one mutation at a time. Therefore, we consider the two alternative pathways involving an intermediate with a single amino acid change (i.e., with either no Lys in the retinal binding pocket or with two; Figure 2). If Lys296 mutated first, the resulting protein would be incapable of binding the retinal chromophore, it would be unresponsive to light, and it would likely be constitutively active, leading to a dominant negative phenotype inconsistent with this protein being a viable evolutionary intermediate in relocation of the Lys⁴. This leaves only the pathway involving an intermediate with two Lys in the active site: the second Lys is introduced before loss of the Lys in TM7. These mutants have not previously been described.

In this study, we prepared four mutant rhodopsins containing two Lys residues in the active site of the protein, the original Lys296 that was left untouched and a second Lys residue

substituted for G90K, T94K, S186K, or F293K. All four mutant proteins form a pigment with 11-*cis*-retinal and all four activate transducin in a light-dependent manner, demonstrating that rhodopsin can tolerate a second Lys in the retinal binding pocket. These results suggest that an evolutionary intermediate with two Lys in the retinal binding pocket could lead to the movement of the Schiff base Lys to a different position in the protein, leaving open the question of why the active site Lys location is invariant. The evolutionary implications of these data are discussed in the context of criteria used to identify type II retinylidene proteins in the genomic database.

EXPERIMENTAL PROCEDURES

Materials

All reagents, including retinal, transducin, guanine nucleotides, and the 1D4-antibody were purchased or prepared as described previously⁶.

Mutagenesis

All mutations were made in the thermally stable N2C/D282C mutant of bovine rhodopsin, which was considered to be “wild-type” (WT) in this study. The N2C/D282C mutant contains an engineered disulfide bond between two introduced cysteine residues, N2C and D282C⁷. The engineered disulfide confers enhanced thermal stability to the opsin form of the protein^{7, 8}. Crystal structures of the N2C/D282C mutant, of both the dark pigment⁹ and active MII form^{10, 11}, show the protein to be identical to that of native rhodopsin^{12–15}, except for the missing oligosaccharyl chain at position 2 and the presence of electron density corresponding to a disulfide bond connecting the two side chain sulfur atoms at positions 2 and 282. In addition, functional studies performed to date show that this mutant behaves the same as WT in all ways except with respect to stability of the opsin form in detergent solution^{7, 8}. Mutations were introduced into the WT cDNA by either cassette or quick-change (Stratagene) mutagenesis. Oligonucleotides used for mutagenesis were purchased from Integrated DNA Technologies (Coralville, Iowa).

Expression and Purification of the Proteins

All rhodopsin mutants were expressed transiently in HEK293S-GnT1⁻ cells using calcium phosphate precipitation for transfection¹⁶. Cells were harvested 72 hours after transfection. Proteins were purified and then reconstituted with 11-*cis*-retinal (while bound to the immunoaffinity matrix) essentially as previously described⁷ except that mutants were eluted from the 1D4-Sepharose matrix following a 1 hour incubation with 0.02% (w/v) DDM in 5 mM HEPES buffer, pH 7.5, 0.1 mM MgCl₂, 3 mM NaN₃ containing 80 μM 1D4-peptide at room temperature. The 1D4-peptide used in this work had a sequence corresponding to either the carboxy-terminal eight or nine amino acids of rhodopsin.

Absorption Spectroscopy

UV-visible absorption spectra were recorded on a Varian Cary 50 Bio UV/Visible spectrophotometer. All spectra were recorded with samples at 25 °C and a path length of 1.0 cm. Pigments were bleached by exposure to light from a 300 W tungsten bulb filtered through a 475 nm cut-on filter for 30 seconds.

Transducin Activation Assays

A filter-binding assay, described previously^{7, 17, 18}, was used to monitor the ability of mutant pigments or opsins ($\epsilon_{280} = 65000 \text{ M}^{-1} \text{ cm}^{-1}$ used for all mutants) to catalyze the exchange of GDP for [³⁵S]-GTP γ S in transducin. All assays contained 5 nM pigment (or opsin), 1 μ M transducin, 3 μ M GTP γ S + [³⁵S]-GTP γ S (5 Ci/mmol), in 10 mM Tris buffer, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, and 0.01% (w/v) DDM.

RESULTS

Four mutants were constructed in which a single Lys residue was added to the active site of bovine rhodopsin. In each case, the added Lys was introduced in a position known from previous work to be able to substitute for Lys296 in formation of a Schiff base with the 11-*cis*-retinal chromophore. The four mutants were: G90K, T94K, S186K, and F293K (Figure 1). Each was expressed in transiently transfected HEK293S-GnT1⁻ cells, reconstituted with 11-*cis*-retinal, and purified to homogeneity in detergent solution by immunoaffinity chromatography. Absorption spectra for the four pigments, as well as the parental N2C/D282C “WT”, are presented in Figure 3. Each mutant is expressed in good yield relative to the WT protein, and each is clearly able to combine with retinal to form a pigment with long-wavelength absorbance ($\lambda_{\text{max}} \approx 500 \text{ nm}$) reminiscent of that of the WT protein. However, it is also evident from the figure that each pigment displays a significant peak in the spectrum with maximum at 380 nm, characteristic of either an unprotonated Schiff base or free retinal (Figure 3 and Table 1). The fraction of pigment with $\lambda_{\text{max}} = 380 \text{ nm}$ clearly varies with mutation and increases in the order F293K < T94K < S186K < G90K.

Acid-trapping of the chromophore was used to determine whether the 380 nm peak corresponds to free retinal or a covalently bound, unprotonated Schiff base. In these experiments, the pigment solution is rapidly brought to pH 3.5 and 0.5% (w/v) SDS to denature the protein and trap any covalently bound chromophore as a protonated Schiff base, characterized in the denatured pigment by an absorption maximum at 440 nm, independent of the protein to which it is bound. Under these conditions, the absorption spectrum of free retinal remains unchanged, characterized by its λ_{max} at 380 nm. While these experiments were performed with pigments not previously exposed to light (Figure S1), we present here (Figure 4) the results for the pigments after exposure to light. In each case, the protein purified in the dark (black spectrum) was exposed to light resulting in significant conversion of the long-wavelength peak ($\lambda_{\text{max}} \approx 500 \text{ nm}$) to another species with $\lambda_{\text{max}} = 380 \text{ nm}$ (blue spectrum). The solution was then acidified (as described above), trapping any covalently bound retinal as a protonated Schiff base (red spectrum). It is clear from the loss of absorbance at 380 nm upon acid-trapping that the light-generated 380 nm species has a covalently bound retinal, and likely represents an active MII intermediate (*vide infra*). It is also clear from the fact that the absorbance of the acid-trapped pigment at 380 nm (red spectrum) is below that of the dark state pigment (black spectrum) that the 380 nm peak in the dark state corresponds to a covalently bound retinal with an unprotonated Schiff base. An identical conclusion is reached from acid-trapping data for the dark-state pigments (Figure S1). Thus, the chromophore in each of the mutant pigments is composed of a mixture of protonated and unprotonated Schiff bases of retinal.

In some rhodopsin mutants, the ratio of unprotonated to protonated Schiff base can be influenced by the external solution. For example, the E113Q mutant removes the counterion and causes the Schiff base to be unprotonated at pH 7.5 and low salt (Figure 5)^{19, 20}. Adding NaCl (225 mM final concentration) provides a chloride ion to the binding pocket that can act as a counterion. As a consequence, the absorbance at 380 nm decreases and the absorbance at 500 nm increases, as some of the Schiff base becomes protonated (Figure 5)^{20, 21}. This effect is accentuated by dropping the pH to 6 with 100 mM sodium phosphate buffer (final concentration)^{19–21}. Under the same conditions, spectra of the double-Lys mutants, like WT, do not change (Figure 5). The lack of change shows that the binding pocket is not influenced by these changes to the solvent, and the protonation state of the Schiff base is controlled by the protein internally.

An active MII-like intermediate and an inactive state with an unprotonated Schiff base cannot be distinguished spectrally. Both display absorption maxima at 380 nm, and both trap with acid to give a 440 nm peak. For this reason, the pigments ability to activate transducin was tested to determine if the 380 nm peaks are from an active or inactive state. As seen in Figure 6, none of the mutants activate transducin in the dark. Therefore, the 380 nm peaks seen in the dark spectra are from an inactive state containing an unprotonated Schiff base.

Upon exposure to light, all four mutants activate transducin (Figure 6). The activity of F293K approaches WT, whereas the extent of reaction for T94K and S186K is about half that of WT. G90K shows a weaker response with only a third of the activity seen with WT. Unlike the Lys relocation mutants⁶, the differences in extent of transducin activation are not explained by differences in stability of the various MII-like intermediates. All the mutants shift to a MII-like intermediate, with a 380 nm absorbance maximum, when exposed to light from a 300 W tungsten bulb filtered through a 475 nm cut-on filter for 30 seconds (Figure 4). However, none of the mutants display early breakdown of MII, as all yield a 440 nm pigment when trapped with acid after exposure to light (Figure 4). The activities correlate roughly with the amount of long-wavelength peak observed for each mutant and likely reflect the fraction of each pigment activated by light from the 475 nm cut-on filter. We have also shown that the G90K mutant can be activated by UV light (375 $\pm$ 10 nm), but this result is difficult to interpret as WT rhodopsin is also activated by the same light source (not shown)²².

The second Lys adds another polar amino acid to the retinal-binding pocket, which raised concern that the mutant opsins might display constitutive activity as a consequence disrupting the inactivating salt-bridge. However, none of the mutants were found to constitutively activate transducin (Figure 7), suggesting that the second Lys does not interfere with the salt bridge.

DISCUSSION

We have shown previously that the invariant active-site Lys residue in type II opsins is not functionally constrained to a location in the seventh transmembrane segment. This is also the case for the active-site Lys in the type I microbial opsins²³. Why then has the active-site Lys remained fixed during evolution and not migrated to a different site in the protein?

Relocating the Lys to a different location requires a minimum of two mutations, one to remove the original active-site Lys and another to introduce a new active-site Lys somewhere else. It is unlikely that both mutations would occur simultaneously. Alternatively, a stepwise evolutionary pathway would involve either an intermediate with no active-site Lys or an intermediate with Lys residues at both locations. K296G and other mutants that remove Lys296 are incapable of forming pigments with the retinal chromophore, suggesting that evolutionary intermediates with no active site Lys would not be viable^{24, 25}. Hence, the most promising evolutionary pathway for moving the active site Lys involves intermediates with two Lys in the active site.

We characterized four mutant rhodopsins containing a second Lys residue in the active site (positions corresponding to Gly90, Thr94, Ser186, and Phe293 in wild-type rhodopsin). All four mutants are functional: they form a pigment with 11-cis-retinal, and they activate transducin in a light-dependent manner. None of the mutants are constitutively active, which indicates that the second Lys does not disrupt the active-site salt bridge in the opsin. Intriguingly, all of the mutants display absorption spectra with both a long-wavelength peak typical of wild-type rhodopsin and a peak with an absorption maximum at about 380 nm. The short-wavelength peak is typical of an unprotonated Schiff base, but it is not influenced by the salt concentration or the pH of the external solution. The ratio of unprotonated to protonated Schiff base varies with mutation, with G90K displaying the highest amount.

While each of the mutants is composed of two distinct spectral species, we currently do not know if the two species represent protonated and unprotonated forms of a single pigment (i.e., a protein with a single site of attachment to the retinal chromophore), or if the two species represent two different populations of pigments with distinct sites of attachment to retinal. Identification of which Lys are involved is the subject of current efforts.

How often do we expect a double-Lys mutant to arise in nature? We can estimate the frequency with a rough back-of-the-envelope calculation. If we assume 10^{-8} mutations per site per year occur in any given individual, and a one in twenty chance that the mutation inserts a Lys, there is about a 10^{-10} chance of a random mutation adding a second active-site Lys in an individual per year. With a conservative effective population size of 10^7 for metazoans, random mutation will generate a type II opsin with two active-site Lys in a species on the order of once every 10^3 years. If these intermediates are beneficial, they could become fixed in a genome relatively quickly.

Given that type II opsins have existed for over a billion years, we examined an alignment of available type II opsin protein sequences²⁶ to see if any contain two active-site Lys. We identified two subgroups of opsins that contain Lys residues at two positions in the putative active site: insect UV cones and neuropsins (Figure 8). Insect UV cones have been well studied, and it is known that the second-site Lys (corresponding to G90 in bovine rhodopsin) is responsible for the UV sensitivity of the pigment²⁷. Neuropsins are relatively recently identified proteins that have Lys in positions equivalent to Phe91 and Lys296 of bovine rhodopsin. While neuropsin function has not yet been determined, they are known to be UV sensitive as well^{28, 29}.

The unique spectral properties of G90K bovine rhodopsin are intriguingly similar to the insect UV cone opsins. The 380 nm absorption maximum observed with G90K is consistent with the spectral sensitivity of fruit fly vision²⁷. Perhaps insect UV cone opsins and neuropsins have opened up potential evolutionary pathways for relocating the active-site Lys.

Modern type II opsins containing two binding site Lys exist in nature, so why has the active-site Lys not moved to a new location? A single point mutation could eliminate the native binding-site Lys, which should occur relatively frequently. Perhaps a nascent relocated Lys would be functionally suboptimal, at least initially, and hence be selected against with respect to the original opsin with a Lys in the conventional active site position. For instance, both neuropsins and insect UV cone opsins are UV sensitive. Loss of the native Lys should restore long-wavelength sensitivity, which may have no fitness advantage, especially relative to existing opsins with highly optimized long-wavelength sensitivity. Another possibility is that proteins with relocated active-site Lys do exist but have not yet been identified. The only sequence feature definitively separating type II opsins from other class A GPCRs is the Lys on TM7. Without this sequence feature, identification of a type II opsin requires biochemical characterization to show binding of a retinal chromophore. To date no such protein has been identified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

GPCR	G protein-coupled receptor
TM	transmembrane helix
MII	metarhodopsin II
WT	wild-type

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Figure 1.

Rhodopsin structure. A) Top down cartoon representation of bovine rhodopsin (PDB: 1U19) with the viewpoint from the cytoplasmic surface. Helices are rainbow colored from N-terminus (blue) to C-terminus (red). The 11-*cis*-retinal (purple), active-site Lys296, Glu113 counterion, and the five positions (Gly90, Thr94, Ala117, Ser186, and Phe293) at which Lys was tested to replace Lys296⁶ are shown as sticks. B) Simplified helix bundle view of rhodopsin structure showing helices as circles numbered from 1 (N-) to 7 (C-terminus) showing the retinal chromophore (purple line) covalently bound to Lys296 (black line) on TM7.

Figure 2.

Scheme for relocation of the binding site Lys in type II opsins. Two mutations are needed to relocate the binding site Lys: one to remove Lys296 and another to insert a Lys at the new position. Mutants without an active site Lys are not functional, indicating a functional intermediate with two Lys in the retinal binding pocket is required. A subsequent mutation could then remove Lys296, forming a type II opsin with relocated binding site Lys.

Figure 3. Normalized UV-visible absorption spectra of the double-Lys mutant pigments. Spectra are for dark-adapted pigments, formed with 11-*cis*-retinal, in 0.02% (w/v) DDM, pH 7.5, at room temperature. UV-visible spectra were normalized for absorbance at 280 nm.

Figure 4.

Formation of MII intermediates by the double-Lys mutant pigments. *Black*, dark-adapted pigment from Figure 3; *blue*, after 30 s exposure to light from a 300 W tungsten bulb filtered through a 475 nm cut-on filter; *red*, protein acid-trapped four min after light exposure with 50 mM sodium phosphate buffer, pH 3.5, containing 0.5% (w/v) SDS (final concentrations).

Figure 5.

Salt and pH sensitivity of the double-Lys mutant pigments. *Black*, spectra for dark-adapted pigments, formed with 11-*cis*-retinal, in 0.02% (w/v) DDM, pH 7.5, at room temperature; *blue*, after addition of 225 mM NaCl (final concentration) to provide Cl⁻ as a potential counterion; *green*, after the pH was dropped to 6.0 with 100 mM sodium phosphate buffer (final concentration).

Figure 6.

Light-dependent activation of transducin by the double-Lys mutant pigments. Transducin activation was monitored by binding of [35 S]-GTP γ S as described in Experimental Procedures. Each reaction contained 5 nM pigment, 1 μ M transducin, and 3 μ M GTP γ S, in 10 mM Tris buffer, pH 7.5, containing 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, and 0.01% (w/v) DDM. Reactions were initiated in the dark and exposed to light from a 300 W tungsten bulb filtered through a 475 nm cut-on filter at 5.5 min. *Blue circles*, WT; *maroon diamonds*, F293K; *black inverted triangles*, T94K; *orange triangles*, S186K; *green squares*, G90K. *Grey shading*, time points taken in the dark. Error bars represent the standard deviation (n=4). Initial rates for the light-dependent reaction are (pmol/min $\pm$ SEM): N2C/D282C, 3.49 $\pm$ 0.32; F293K, 2.15 $\pm$ 0.18; T94K, 1.49 $\pm$ 0.14; S186K, 1.33 $\pm$ 0.10; and G90K, 0.83 $\pm$ 0.09.

Figure 7.

Assay for constitutive activation of transducin by the double-Lys mutants. Transducin activation was monitored by binding of [³⁵S]-GTPγS as described in Experimental Procedures. Each reaction contained 5 nM pigment, 1 μM transducin, and 3 μM GTPγS, in 10 mM Tris buffer, pH 7.5, containing 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, and 0.01% (w/v) DDM. *Light blue open squares*, K296G; *blue circles*, WT; *green squares*, G90K; *maroon diamonds*, F293K; *orange triangles*, S186K; and *black inverted triangles*, T94K. Error bars represent the standard deviation (n=4).

Figure 8.

Partial sequence alignments identifying type II opsins with two Lys in the retinal binding site. Full sequence alignment includes 489 type II opsins²⁶. Two groups were identified with 2 Lys residues in the retinal binding site: A) insect UV cone opsins and B) neuropsins. Selected sequences are shown aligned with bovine rhodopsin. Numbering is for bovine rhodopsin.

Table 1

UV-Vis absorption maxima of double-Lys mutants in the dark

Mutant	Short Wavelength λ_{max} (nm)	Long Wavelength λ_{max} (nm)
G90K	384	480
T94K	375	506
S186K	373	494
F293K	372	504