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Contribution of Glutamic Acid in the Conserved E/DRY Triad to the Functional Properties of Rhodopsin

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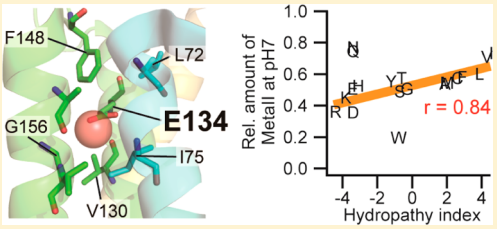
ABSTRACT: Rhodopsin is a G protein-coupled receptor specialized for photoreception and contains a light-absorbing chromophore retinal that binds to the lysine residue of opsin through a protonated Schiff base linkage. Light converts rhodopsin to an equilibrium mixture of the active state metarhodopsin II (MII) and its precursor, metarhodopsin I (MI), which have deprotonated and protonated Schiff base chromophores, respectively. This equilibrium was thought to depend on the pK_a of not the Schiff base chromophore but glutamic acid E134 in the highly conserved E/DRY triad in helix III. We performed mutational analyses of E134 and nearby residues to examine whether the equilibrium is really dependent on the pK_a of E134 and to obtain clues about the contribution of E134 to the G protein activation characteristics of rhodopsin. All the single mutants at position 134 except for E134D lost the characteristic pH-dependent equilibrium, indicating that the carboxyl group of E134 is responsible for the equilibrium. Interestingly, mutation at position 134 caused little change in the MI or MII spectra or G protein activation efficiency of MII, while it caused a shift of the MI−MII equilibrium. The mutants containing hydrophobic or amide-containing residues at position 134 formed an equilibrium in favor of MII, resulting in an increase in light-induced G protein activation efficiency. On the other hand, the wild type exhibited an opsin activity lower than those of the mutants, which exhibited reasonable light-dependent activities. These results strongly suggest that the evolutionary significance of E134 is not an increase in G protein activity but rather suppression of the opsin activity.



The G protein-coupled receptors (GPCRs) form an active state by receiving various kinds of extracellular stimuli and trigger an intracellular signal transduction cascade through activation of their cognate G protein subtypes. They are membrane proteins with seven transmembrane α-helices connected by three extracellular and three cytoplasmic loops. Rhodopsin is one GPCR specialized for photoreception and contains an intrinsic ligand, 11-*cis*-retinal, in its protein moiety, opsin.[1] 11-*cis*-Retinal is bound to a lysine residue of opsin through a protonated Schiff base linkage, which results in the visible light sensitivity of opsins. On the other hand, 11-*cis*-retinal acts as a pharmacologically inverse agonist that suppresses the G protein activity of opsin. Light causes isomerization of the 11-*cis*-retinal to a highly twisted all-*trans* form in the restricted chromophore binding site.[2] In vertebrate rhodopsins, the highly twisted all-*trans* chromophore then induces stepwise changes of the protein that finally lead to the formation of the intermediate, known as metarhodopsin II (MII), responsible for G protein activation.[3,4] MII is in a direct equilibrium with its precursor, metarhodopsin I (MI).[5]

The chromophore of MII is a deprotonated all-*trans*-retinylidene Schiff base, whereas that of MI is its protonated form.[6] Previous experiments indicated that the MI−MII equilibrium is pH-dependent but does not follow the pK_a of the Schiff base chromophore, in contrast to the case of invertebrate rhodopsins such as squid and Go rhodopsins.[7,8] That is, deprotonation of the Schiff base chromophore in MII is favored under acidic conditions, whereas that in the active state (acid metarhodopsin) of invertebrate rhodopsin is favored under alkaline conditions. Recently, it was suggested that a glutamic acid in the E/DRY triad highly conserved in the family 1 GPCRs could be a titratable functional group that accounts for the unique pH profile of the MI−MII equilibrium in vertebrate rhodopsins;[9−13] although at least two deprotonated Schiff base products promptly follow MI, the term MII here refers to the equilibrated mixture of those protonation forms. Therefore, in this study, we first confirmed that E134 in the E/DRY triad of bovine rhodopsin is responsible for the pH-dependent shift of the MI−MII equilibrium. Next we investigated the functional significance of the glutamic acid at this position by measuring the G protein activation efficiencies of mutants having different amino acid residues at this position. The results clearly showed that the characteristic pH profile was observed in only the WT and the E134D mutant, indicating that the presence of a carboxyl-bearing amino acid residue at this position is indispensable for the characteristic pH profile. On the other hand, the measurement of G protein activation efficiencies showed that several mutants exhibit light-induced G protein activation efficiencies higher than that of the WT. These results indicated that the presence of a carboxyl-bearing amino acid at this position has not been indispensable for the optimization of the light-dependent G protein activation efficiency during the course of molecular evolution. In contrast, we found that the WT exhibited an opsin activity lower than

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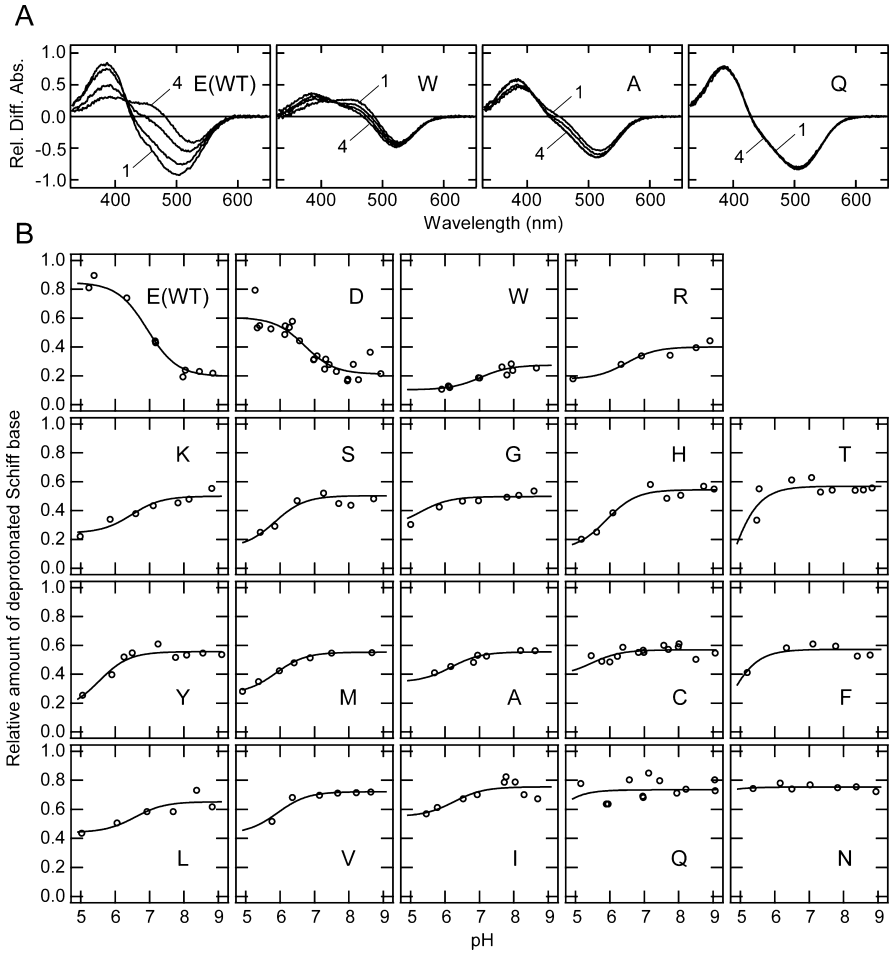

Figure 1. pH-dependent equilibrium between MI and MII in E134 mutants. (A) Set of difference spectra calculated from the spectra before and after light irradiation in WT, E134W, E134A, and E134Q bovine rhodopsin. All the spectra were recorded at 0 °C. For the WT, difference spectra were recorded at pH 5.2, 6.3, 7.2, and 8.8 (curves 1−4, respectively). For E134W, difference spectra were recorded at pH 5.9, 7.0, 8.0, and 9.2 (curves 1−4, respectively). For E134A, difference spectra were recorded at pH 5.7, 6.8, 8.2, and 8.6 (curves 1−4, respectively). For E134Q, difference spectra were recorded at pH 5.2, 6.6, 7.5, and 9.1 (curves 1−4, respectively). (B) Relative amounts of an intermediate having a deprotonated Schiff base, MII, as a function of pH for E134 mutant rhodopsins. The amounts of MII produced by irradiation of E134 mutants with >550 nm light for 30 s at various pHs are plotted as a function of the pH of the sample. The amount of MII was estimated by simulating each difference spectrum with the absorption spectra of MI and MII. Solid lines are the best-fit curves calculated according to the Henderson−Hasselbalch equation.

those of any of the mutants exhibiting reasonable light-dependent activities. These results strongly suggested that the evolutionary conservation of E134 is significant not because of an increase in the light-dependent activity but because of the suppression of the opsin activity. We discuss the significant contribution of E134 to the activation process in bovine rhodopsin.

MATERIALS AND METHODS

Sample Preparation. The cDNAs encoding site-directed bovine rhodopsin mutants were prepared by using the QuikChange mutagenesis method (Stratagene). Wild-type and mutant cDNAs were introduced into mammalian expression vector pcDNA3.1 (Invitrogen). The wild type and all the mutants were expressed as previously reported[14] in the HEK293S cell line. All procedures were conducted on ice unless otherwise noted. For spectroscopic measurements, the purified rhodopsin and mutant rhodopsins were prepared as previously reported with minor modifications.[14] The opsin-expressing cells were collected and incubated with 11-*cis*-retinal to generate photosensitive pigments that were then extracted with buffer E [0.75% CHAPS, 1 mg/mL PC, 140 mM NaCl, 3 mM $MgCl_2$, and 50 mM HEPES (pH 7.5)] from the cell membranes and purified using a column containing anti-bovine rhodopsin monoclonal antibody Rho1D4. For measurement of the transducin activation efficiencies of the mutants, opsin-containing membranes were prepared by the sucrose floatation method.[14] In short, the opsin-expressing cells were suspended in 50% sucrose in buffer A [140 mM NaCl, 3 mM $MgCl_2$, and 50 mM HEPES (pH 7.0)]. After centrifugation, the membranes that floated were harvested and diluted by adding 2 volumes of buffer A. This diluted suspension was centrifuged to precipitate the membranes. The supernatant was discarded, and the pellet was suspended again in buffer A. This sample was separated into two aliquots: one for the estimation of the expression level and the other for the transducin activation assay. The expression level was estimated as follows. 11-*cis*-Retinal (20 μM) was added to the membrane suspension to regenerate rhodopsin. After overnight incubation at 4 °C, the sample was washed once with buffer A. Rhodopsin was extracted from the membrane with buffer A containing 1% CHAPS. After centrifugation, the supernatant was irradiated with yellow

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light (>500 nm) in the presence of 50 mM hydroxylamine. The expression yield of rhodopsin was calculated on the basis of the change in absorbance at 500 nm.

Spectrophotometry. Absorption spectra were recorded using a Shimadzu UV2400 spectrophotometer and an optical cell (width, 2 mm; light path, 1 cm).[14] To control the sample temperature, an optical cell holder was connected to a Neslab RTE-7 temperature controller. Accordingly, the sample temperature was kept at 0 ± 0.1 °C. The sample was irradiated with light from a 1 kW tungsten halogen lamp (Rigaku Seiki) that had been passed through a glass cutoff filter (VO57, Toshiba).

Transducin Activation Assay. The transducin activation efficiencies of the opsin-containing membranes were estimated by the GTPγS binding assay as previously described,[14] and the GDP−GTPγS exchange rate was compared among wild-type and mutant rhodopsins. Basal activity was measured at 20 °C, and light-induced activity was measured at 0 °C after incubation with 11-*cis*-retinal and irradiation with >500 nm light for 30 s at this temperature. The assay mixture contained 1 μM [^{35}S]GTPγS, 140 mM NaCl, 8 mM $MgCl_2$, 50 mM HEPES (pH 7.0), and 0.6 μM bovine transducin. Additionally, 2.5 μM GDP was added for the measurement of light-induced activity. Control measurement of basal activity was conducted by using the membrane preparation of mock-transfected cells.

RESULTS AND DISCUSSION

pH Dependency of the Equilibrium between MI and MII of E134 Mutants. To demonstrate that E134 is the titratable group responsible for the pH profile of the MI−MII equilibrium, we expressed a series of mutants whose E134 was replaced with other amino acids in HEK293S cells. Although the E134P mutant showed a level of expression remarkably lower than that of the WT (~10%), all the other mutants showed reasonable expression levels and could be purified by affinity column chromatography after solubilization with CHAPS detergent. Therefore, we characterized the molecular properties of the mutants except for the E134P mutant. Because of the turbid nature of the membrane fractions containing opsins, we were unable to precisely measure the spectra of the dark state and the MI and MII intermediates of WT and mutant rhodopsins by using the membrane fractions as samples over the whole pH range.

Figure 1A shows the light-dependent spectral changes of the WT and three mutants at various pHs. These mutants exhibited three kinds of stereotypic results with regard to the amounts of MII at equilibrium. The absorption spectra of the mutants before irradiation were identical in shape to that of the WT (500 nm). Additionally, the spectra of MI and MII in the WT were very close to those of the corresponding intermediates in all the mutants. As previously reported, MI and MII in the WT were stabilized at alkaline and acidic pH, respectively.[5] However, mutation of E134 [except for that resulting in replacement with aspartic acid (D)] altered the pH-dependent profile of the MI−MII equilibrium (Figure 1A). In E134Q, MII was the major component even at alkaline pH, which is consistent with previous reports.[11,13] On the other hand, irradiation of E134A resulted in the formation of roughly equal amounts of MI and MII, and MI was predominantly formed at equilibrium from E134W. These three mutants exhibited a pH-dependent spectral shift significantly smaller than that of the WT. By decomposing the difference spectra of all the E134 mutants into a linear combination of the pure MI and MII spectra,[15] we obtained the relative amount of MII at equilibrium at a particular pH. We then plotted it against pH (Figure 1B).

Among the mutants, E134D exhibited a pH profile of the equilibrium similar to that of the WT. However, all the other mutants showed a loss of pH dependency or a gain of inverse pH dependency. These results indicated that the carboxyl-bearing amino acid at position 134 is an essential component for the unique pH dependency of the MI−MII equilibrium in rhodopsin. MI/MII ratios of several E134 mutants, such as E134W, E134A, and E134Q, were also estimated by using membrane fractions containing 50% sucrose, and the ratios were not so different from those measured in CHAPS/PC detergent (data not shown).

Transducin Activation Efficiencies of E134 Mutant Rhodopsins. We next investigated the light-induced G protein activation efficiencies of the mutants. Probably because CHAPS could affect the receptor−G protein interaction and the integrity of the G protein,[16,17] we could not measure transducin activation efficiency by using the samples identical to those used in the spectroscopic measurement. Therefore, we used membrane preparations to measure the transducin activation efficiency. The efficiencies of the mutants were changed 0.2−2.2-fold relative to that of the WT (Figure 2A). Interestingly, there was the close relationship between the amount of MII in

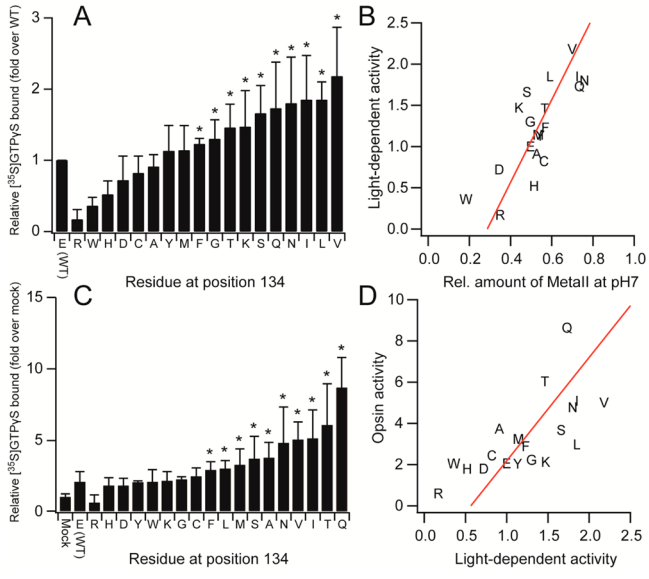

Figure 2. Transducin activation efficiency of E134 mutant rhodopsins in membrane preparations. (A) Light-dependent activities of E134 mutants. Solid bars represent means ± the SD obtained from at least three independent experiments. The activities are indicated as the values relative to that of the wild type. In E134F, -G, -T, -K, -S, -Q, -N, -I, -L, and -V, the light-dependent activities are significantly higher than that of the wild type (*p < 0.05). (B) Correlation between the light-dependent G protein activation efficiency and the relative amount of MII at pH 7 in E134 mutants. The solid line represents the linear regression (n = 19; r = 0.77). (C) Basal activities of ligand unbound states of E134 mutants. Solid bars represent means ± the SD obtained from at least three independent experiments. The activities are indicated as the values relative to that of mock. In E134F, -L, -M, -S, -A, -N, -V, -I, -T, and -Q, the basal activities are significantly larger than that of the wild type (*p < 0.05). (D) Correlation between the light-dependent and basal G protein activation efficiencies of E134 mutants. The solid line represents the linear regression (n = 19; r = 0.68).

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the equilibrium and the G protein activation efficiency (Figure 2B). This fact indicated that the light-dependent activity of a mutant is largely determined by the amount of MII, rather than by the difference in activation efficiency of MII among mutants. However, the fact that the linear regression line does not go through the origin suggests that there were some effects of the detergent used for the spectroscopic measurements and/or that some deviations of the G protein activation efficiencies of MII were caused by the mutations. The small effect of the mutation on the G protein activation efficiency is consistent with the structural evidence that light absorption causes flipping of E134 from the G protein interaction site where it forms an ionic lock with R135 in the dark state.

Figure 2C shows the opsin activities of the WT and mutants in the absence of the chromophore. The opsin activities of the mutants changed 0.3−4.2-fold relative to that of the WT. Although there was some difference in mutational effect between opsin activity and light-dependent rhodopsin activity, a strong correlation was observed between them (Figure 2D). This is consistent with the fact that the structure of light-induced active state MII is almost identical with that of the opsin active state.

It should be noted that the WT exhibited an opsin activity lower than those of any of the mutants exhibiting reasonable light-dependent activities. E134R showed no detectable increase in opsin activity compared to that of mock-transfected cells (Figure 2C). It also exhibited little light-dependent activity (Figure 2A), indicating that this mutant has little ability to activate G protein. Because a higher opsin activity would generate more noise in the signal transduction of rod cells, rhodopsin should become optimized to suppress opsin activity during the course of molecular evolution. These results strongly suggested that the evolutionary significance of E134 is not the increase in G protein activity but rather the suppression of the opsin activity.

Relationship between the Amount of MII and the Physicochemical Properties of the Amino Acids at Position 134 and Nearby Amino Acids. To investigate the relationship between the physicochemical properties of the amino acid residue introduced at position 134 and the amount of MII present in the equilibrium, we analyzed a total of 544 descriptors of the physicochemical properties of natural amino acids registered in AAindex.[18] As a result, we found a close relationship between the amount of MII at equilibrium and the hydropathy index of the amino acid residue at position 134 (Figure 3). Those of the E134Q, E134N, and E134W mutants deviate from the relationship, probably because some physicochemical property other than hydrophobicity is responsible for the shift in the equilibrium in these mutants. E134Q and E134N contain a hydrophilic amide group in their side chains, which could mimic the protonated carboxyl group of E134 formed in wild-type MII. Therefore, they form a pH-independent equilibrium possessing a large amount of MII. In contrast, the E134 site in MII would not accommodate the large side chain introduced in the E134W mutant, resulting in the formation of predominantly MI. Via the exclusion of three mutants, E134W, -N, and -Q, as outliers, a relationship between the amount of MII and the hydrophobicity index emerges with correlation coefficients of >0.8. Therefore, we further investigated the relationship between the amount of MII and hydrophobic amino acid residues at position 134.

The change in the stability of an intermediate caused by the mutation of an amino acid should originate from the change in

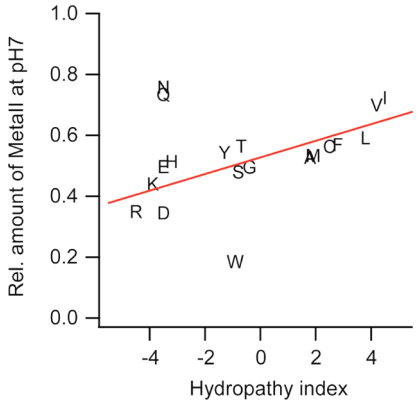

Figure 3. Correlation between the relative amount of MII at pH 7 in E134 mutants and the hydropathy index of the introduced amino acid. The solid line represents the linear regression (n = 16; r = 0.84). E134N, -Q, and -W were discarded as outliers.

the interactions of the mutated amino acid with nearby amino acids, and it should be possible to infer these changes from the three-dimensional (3D) structures of the intermediates. The atomic structure of MII has already been published,[19] but that of MI is still unavailable. However, spectroscopic studies[20] in combination with a low-resolution crystal structure of MI[21] demonstrated that conformational changes in the protein moiety are still restricted to the area near the chromophore. Thus, it is reasonable to speculate that conformational changes near the cytoplasmic region of helix III, including those in the ERY triad, do not occur in MI. Therefore, we speculate here that the structure near the ERY triad in MI is similar to that in the dark state rhodopsin.

There is a general consensus that the carboxyl group of E134 forms a salt bridge with the guanidyl group of R135 to stabilize a resting state conformation around the ERY triad in rhodopsin.[11,22,23] Therefore, replacement of E134 with a hydrophobic amino acid results in breakage of the salt bridge and destabilizes the region. If MI has a structure in this region similar to that of rhodopsin, the replacement would destabilize MI, resulting in a shift of the MI−MII equilibrium in favor of MII. In contrast, the side chain of E134 in MII is located near the side chains of L72, I75, V130, F148, and G156 in addition to a water molecule,[19] suggesting that E134 is located in a rather hydrophobic environment. It should be noted that these amino acids, except for F148, do not interact with E134 in rhodopsin. To test this suggestion about the environment, we investigated the shift in the equilibrium caused by replacements of these residues with alanine to reduce (L72, I75, and V130) or increase (G156) the hydrophobicity.

L72A and I75A caused an increase in the amount of MII at equilibrium, whereas no shift in the amount at equilibrium was observed for V130A (Figure 4A). In contrast, the amount of MII at equilibrium decreased in G156A. These results strongly suggested that the amount of MII tends to increase when the hydrophobicity or bulkiness of residues near E134 decreases.

The results described above are quite interesting because E134 is surrounded by amino acids that destabilize this region even in the WT. A close look at the 3D structure of MII suggested that the side chains of these residues destabilize E134 by interacting with the carboxyl group of E134.[19] Therefore, we performed a similar mutational analysis using the E134I mutant instead of the WT. We found that I75A, and also V130A, caused a decrease in the amount of MII due to a decrease in the

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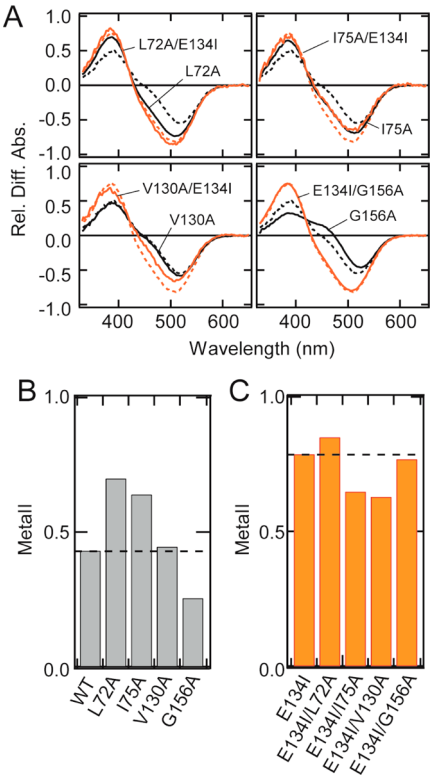

Figure 4. Effect of replacements of the residues surrounding the amino acid at position 134 in the MII structure on the MI−MII equilibrium. (A) Set of difference spectra calculated from the spectra before and after light irradiation of bovine rhodopsin mutants. Black and orange dashed curves show the difference spectra of the WT and E134I, respectively. All the spectra were recorded at pH 7.2 and 0 °C. (B and C) Effects of replacements of the residues surrounding E134 and I134, respectively, in the MII structure on the amount of MII. The amounts of MII were estimated on the basis of the difference spectra. Dashed lines indicate the amount of MII of the WT (B) or E134I (C).

level of hydrophobic interaction between the side chain of the introduced isoleucine residue and those of nearby amino acid residues, and that the effect of G156A on the amount of MII was canceled by E134I due to removal of the destabilization of E134 by A156 (Figure 4B,C). These results clearly show that E134 was really destabilized by the surrounding amino acid residues.

Another important finding is that mutation of E134 as well as mutation of residues near E134 caused the increase in the amount of MII at equilibrium. As described above, the increase in the amount of MII caused by mutation of E134 increases light-induced G protein activation efficiency. Taken together, these findings led us to speculate that vertebrate rhodopsin has the potential to improve the efficiency of G protein activation by increasing the preference of the receptor conformation for the active state.

Difference in the Process of Formation of the Active State between Rhodopsin and Other GPCRs. Substitutions of the glutamic/aspartic acid in the E/DRY triad have been reported to enhance the basal activity of several family 1 GPCRs.[24−28] In particular, in the α1B-adrenergic receptor, it has been shown that the introduction of threonine, glutamine, or a hydrophobic residue such as isoleucine results in a high constitutive activity.[28] This is quite similar to our observation for bovine rhodopsin. Thus, these GPCRs could share a common molecular mechanism for regulating the interconversion between the resting and active states, probably the mechanism predicted from the structural comparison between the dark state rhodopsin and the active state MII.[19,29,30] On the other hand, all the mutations examined elevated the basal activity of the α1B-adrenergic receptor,[28] which is in contrast with the result that some mutations did not significantly elevate the basal activity of bovine rhodopsin. This moderate elevation by the substitution of the residue at position 134 in rhodopsin implies that rhodopsin has a molecular mechanism that suppresses spontaneous receptor activation in addition to the "ionic lock" between helix III and helix VI involving E134.

Irradiation of invertebrate rhodopsins also leads to the formation of the equilibrium among the active intermediate, acid metarhodpsin, and alkaline metarhodopsin.[7,31] Acid or alkaline metarhodopsin has a protonated or deprotonated Schiff base, respectively, and acidic conditions favor acid metarhodpsin.[32] That is, the Schiff base of the active state in invertebrate rhodopsins would be directly accessible from the aqueous phase. The difference in pH dependency of the formation of the active state between vertebrate and invertebrate rhodopsins showed that vertebrate rhodopsins have acquired a special molecular architecture during the evolutionary process after having diverged from other opsin groups.[8] However, the carboxylic residue in the E/DRY triad is also conserved among almost all the opsin groups. Therefore, other amino acid residues would contribute to this special molecular architecture responsible for the pH dependency of the formation of the active state of vertebrate rhodopsins. Future studies of various cone pigments and nonvisual opsins phylogenetically close to vertebrate rhodopsins will inform us about the key residues that modulate the molecular mechanism of MII formation.[8,14,33,34]

In conclusion, the glutamic acid at position 134 plays the roles of stabilizing the inactive conformation in bovine rhodopsin through forming a salt bridge with the guanidyl group of R135 and partially stabilizing the active conformation through interaction with a nearby water molecule after protonation of the carboxyl group. Some of the mutations at this position increased the G protein activation efficiency compared with that of the WT. In contrast, WT exhibited an opsin activity lower than those of any of the mutants exhibiting reasonable light-dependent activities. These results strongly suggested that the evolutionary conservation of E134 can be attributed not to an increase in G protein activity but rather to the suppression of the opsin activity.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine from egg yolk; WT, wild type; MI, metarhodopsin I; MII, metarhodopsin II; SD, standard deviation.

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