The role of the retinylidene Schiff base counterion in rhodopsin in determining wavelength absorbance and Schiff base pKₐ

(transmembrane receptor/visual pigment-retinal chromophore/site-directed mutagenesis/synthetic gene)

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ABSTRACT Glu-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. Purified mutant rhodopsin pigments were prepared in which Glu-113 was replaced individually by Gln (E113Q), Asp (E113D), Asn (E113N), or Ala (E113A). E113Q, E113N, and E113A existed as pH-dependent equilibrium mixtures of unprotonated and protonated Schiff base (PSB) forms. The Schiff base pKₐ values determined by spectrophotometric titration were 6.00 (E113Q), 6.71 (E113N), and 5.70 (E113A). Thus, mutation of Glu-113 markedly reduced the Schiff base pKₐ. The addition of NaCl promoted the formation of a PSB in E113Q and E113A. An exogenously supplied solute anion replaced Glu-113 to compensate for the positive charge of the PSB in these mutants. The λₘₐₓ values of the PSB forms of the mutants in NaCl were 496 nm (E113Q), 506 nm (E113A), 510 nm (E113D), and 520 nm (E113N). To evaluate the effect of different types of solute anions on λₘₐₓ values, mutants were prepared in sodium salts of halides, perchlorate, and a series of carboxylic acids of various sizes and acidity. The λₘₐₓ values of E113Q and E113A depended on the solute anion present and ranged from 488 nm to 522 nm for E113Q and from 486 nm to 528 nm for E113A. The solute anion affected the λₘₐₓ values of E113N and E113D to lesser degrees. The reactivities of the mutants to hydroxylamine were also studied. Whereas rhodopsin was stable to hydroxylamine in the dark, E113N reacted slowly and E113Q reacted rapidly under these conditions, indicating structural differences in the Schiff base environments. The λₘₐₓ values and solute anion dependencies of the Glu-113 mutants indicate that interactions between Schiff base and its counterion play a significant role in determining the λₘₐₓ of rhodopsin.

Rhodopsin belongs to the family of receptors that activates guanine nucleotide-binding regulatory proteins in signal transduction. The primary structure of rhodopsin has been determined (1–3), and models propose the existence of seven transmembrane segments, a common motif in this membrane receptor family (4). In rhodopsin, these transmembrane segments form a binding pocket for the 11-cis-retinal chromophore that is linked to the opsins through a protonated Schiff base (PSB) at Lys-296. Rhodopsin has a λₘₐₓ at 500 nm; human cone pigments, which also contain 11-cis-retinal, display λₘₐₓ values ranging from 440 nm (blue) to 530 nm (green) to 560 nm (red) (5). A central problem has been to understand how the interactions between opsin proteins and retinal chromophores determine their spectral properties.

Recently, we investigated the role of charged amino acids in transmembrane helix C on the structure and function of bovine rhodopsin by site-directed mutagenesis and concluded that Glu-113 served as the counterion of the retinylidene Schiff base (6). Zhukovsky and Oprian (7) and Nathans (8) came to the same conclusion. We further studied a set of four mutants, each of which contains a single amino acid substitution at position 113, (E113D, E113Q, E113N, and E113A). Mutant pigments E113Q, E113N, and E113A existed as pH-dependent equilibrium mixtures of an unprotonated Schiff base (SB) form absorbing in the near-UV range and a PSB form absorbing in the visible range. Spectrophotometric titrations of the mutants showed that the amino acid at position 113 affected the pKₐ of the Schiff base imine.

The λₘₐₓ values of the PSB forms of the mutant pigments were determined in sodium salts of halides, perchlorate, and a collection of carboxylic acids of various sizes and acidity. The λₘₐₓ values of E113Q and E113A depended on the solute anion present. Exogenously supplied solute anions could substitute for the Glu-113 anion in mutants E113Q and E113A to allow Schiff base protonation. These results help to define the role of Glu-113 in promoting Schiff base protonation and regulating its pKₐ. The interaction between Schiff base and its counterion may also play a significant role in determining the λₘₐₓ of visual rhodopsin.

METHODS

Preparation of Rhodopsin Mutants. The mutant opsin genes were prepared by restriction fragment replacement in a synthetic opsin gene (9, 10). Mutations were confirmed by dideoxynucleotide sequencing of plasmid DNA. Mutant genes were expressed in monkey kidney cells (COS-1) (11). After addition of 11-cis-retinal in the dark, the cells were solubilized in 1% dodecyl maltoside buffer. Pigments were purified by immunoaffinity adsorption (10, 11). All experiments were performed in darkness or under dim red light.

UV–Visible Spectroscopy. Spectroscopic measurements were performed on a Perkin–Elmer λ-7 UV–visible spectrophotometer on purified dodecyl maltoside detergent-solubilized samples at 20°C in a cell with a 1-cm path length.

Measurement of pKₐ Values. Mutant pigments were prepared as above except that washes of the pigments immobilized on the immunoaffinity support were carried out in 0.1% dodecyl maltoside in 100 mM NaCl. pH readings were taken in the cuvette with a pH electrode. Samples were gradually titrated with dilute HCl or NaOH, and pH readings and spectra were recorded.

The Effect of NaCl on Spectral Properties of Rhodopsin Mutants. UV–visible spectroscopy was carried out on mutant pigments in 0.1% dodecyl maltoside in water and in the presence of increasing NaCl concentrations.

The Effect of Solute Anion on λₘₐₓ Values of Rhodopsin Mutants. Solutions of sodium salts of various anions and carboxylic acids were prepared at a 10× (500 mM) concent-

Abbreviations: SB, unprotonated Schiff base; PSB, protonated Schiff base; rhodopsin mutants are designated by the wild-type amino acid residue (single-letter code) and its position number followed by the substituted amino acid residue.

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tration: fluoride, chloride, bromide, iodide, formate, acetate, perchlorate, tartrate, citrate, benzoate, chloroacetate, dichloroacetate, and trichloroacetate. For benzoate, the 10× solution was 200 mM. The salt solutions were titrated with their respective acids so as to give pH 5.0 upon dilution to 1×. Mutant pigments were prepared in 0.1% dodecyl maltoside in water, and then the individual 10× salt solutions were added to give a final 1× (50 mM or 20 mM) concentration.

Reaction of Mutant Rhodopsin Pigments with Hydroxylamine. COS cell rhodopsin, E113Q, and E113N were prepared in 0.1% dodecyl maltoside/100 mM NaCl, pH 5. A solution of hydroxylamine hydrochloride adjusted to pH 5 with NaOH was added to a final concentration of 10 mM to start the reaction. The reaction was carried out in the dark at 20°C. Spectroscopy was repeated periodically to monitor the loss of pigment and formation of retinal oxime. The pH was measured again at the end of the reaction.

RESULTS

UV–Visible Spectroscopy of Glu-113 Mutants. The mutant pigment E113Q existed as a pH-dependent equilibrium mixture of SB (λ\text{max} = 385 nm) and PSB (λ\text{max} = 496 nm) species. Mutant pigment E113D displayed a λ\text{max} = 510 and did not show a blue-shifted UV form. Mutant pigment E113A regenerated with 11-cis-retinal absorbed maximally in the near-UV range at 385 nm with a small second component in the visible range (Fig. 1). Acidification of the pigment with HCl converted the 385-nm peak to 517 nm. A single isosbestic point was observed for this conversion, and the two forms of the pigment existed in a pH-dependent equilibrium as was found with mutant E113Q. Mutant pigment E113A regenerated with 11-cis-retinal absorbed maximally in the near-UV range at 386 nm with a small second component in the visible range (Fig. 1). Acidification of the pigment with HCl converted the 386-nm peak to 506 nm. A single isosbestic point was observed for this conversion, and the two forms of the pigment existed in a pH-dependent equilibrium. Under extreme acid conditions (pH 2), where the protein cannot interact with retinal, a 440-nm peak characteristic of a PSB was formed for each of the pigments (data not shown).

Schiff Base pK_a Determination. The pK_a of the transition between SB and PSB forms in mutant E113Q was estimated to be about 6 (6, 7). Spectrophotometric titrations of the transition between visible (PSB) and near-UV (SB) forms as a function of pH were carried out (Fig. 2). Analysis of the titration curves yielded the following pK_a values (mean ± SD): 6.0 ± 0.11 (E113Q), 6.71 ± 0.04 (E113N), and 5.70 ± 0.09 (E113A). COS cell rhodopsin and E113D each displayed a visible λ\text{max} that was stable over a pH range from 5.2 to 8.3 (Fig. 2).

The Effect of NaCl on Spectral Properties. Pigment E113Q absorbed predominantly at 385 nm when prepared in deionized water, but it absorbed at 496 nm in 100 mM NaCl (Fig. 3). This indicated that the presence of NaCl facilitated the protonation of the Schiff base. The behavior of E113A was similar to that of E113Q. It absorbed predominantly at 386 nm when prepared in deionized water and was converted to a 506-nm species upon addition of NaCl (Fig. 3). In the case of E113N, the presence of NaCl had no significant effect on the λ\text{max} of the acidic form of the pigment. The pigment absorbed at 520 nm when prepared in water or 100 mM NaCl (Fig. 3). There was no salt effect on the proportion of UV to visible form of this pigment. In E113D, there was no significant difference between the λ\text{max} of the pigment prepared in deionized water (513 nm) or 100 mM NaCl (510 nm) (Fig. 3).

The proportion of visible to UV forms increased as a function of NaCl concentration for mutants E113Q and E113A (Fig. 4). To control for the effect of any pH changes resulting from the addition of the NaCl or from pH electrode instability at low salt concentrations, the pH was measured as a function of NaCl concentration with the same electrode used to measure the pigment samples (Fig. 4A). No significant difference was noted in the pH value over the range of 1–500 mM NaCl.

Effect of Exogenously Supplied Anions on λ\text{max} Values. Mutant pigments were prepared in solutions of various sodium salts of halides, perchlorate, and carboxylic acids. The λ\text{max} values for the visible form of pigment E113Q varied significantly in different anion solutions (Table 1). The extreme values were 488 nm in formate and 522 nm in acetate. Likewise, the λ\text{max} values for mutant E113A varied significantly in the presence of different anions (Table 1). The extreme values were 486 nm in fluoride and 528 nm in benzoate. Varying the solute anion had only a relatively small effect on the λ\text{max} of pigment E113N. The λ\text{max} values for E113N ranged from 512 nm in bromide to 524 nm in both citrate and benzoate (Table 1). For E113D, the λ\text{max} values ranged narrowly from 501 nm for acetate to 510 nm for chloride and iodide.

The λ\text{max} values of mutants E113Q and E113A varied in different halide anions (Fig. 5). However, there was no straightforward relationship observed between λ\text{max} values and halide ionic radii. For example, even though the ionic

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**Fig. 1.** UV–visible spectroscopy of Glu-113 mutant pigments regenerated with 11-cis retinal. (A) Mutant E113N in the dark at pH 7.7 and pH 5. (B) Mutant E113A in the dark at pH 7 and pH 5.

**Fig. 2.** Determination of the pK_a values for the transition between near-UV and visible forms of mutant pigments. The amount of visible-absorbing pigment was determined as a function of pH. (A) Mutant pigment E113D. (B) Mutant pigment E113Q. (C) Mutant pigment E113N. (D) Mutant pigment E113A.
Glu-113 serves as the counterion to the protonated retinylidene Schiff base in bovine rhodopsin (6–8). Replacement of Glu-113 by Gln (E113Q) removed the ionizable group from that position. However, Gln still had the potential to interact with the chromophore through hydrogen bonding or through electrostatic effects as a weak permanent dipole. The replacements of Glu-113 by Asn (E113N) and by Ala (E113A) were carried out to evaluate these other potential interactions. Replacement of Glu-113 by Asn (E113N) or Ala (E113A) made the protonation state of the Schiff base pH-dependent as reported for E113Q (6). At basic pH these mutant pigments absorbed predominantly in the near-UV range. However, at acidic pH, the pigments absorbed in the visible range characteristic of PSB pigments. The $\lambda_{\text{max}}$ values of these pigments at acidic pH were 496 nm (E113Q), 520 nm (E113N), and 506 nm (E113A) (Table 1). The methyl group of Ala has no hydrogen-bonding potential and no permanent dipole. Therefore, it might be expected that the $\lambda_{\text{max}}$ of E113A would be blue-shifted with respect to the $\lambda_{\text{max}}$ values for E113Q or E113N, since a protonated retinylidene Schiff base with no protein interactions absorbs at 440 nm (12). The fact that the $\lambda_{\text{max}}$ of E113A fell between those of E113Q and E113N makes a simple interpretation difficult. Possibly, substitution at position 113 can not only change the specific interaction between position 113 and the chromophore but may also influence the interaction of the chromophore with other amino acids in the protein as well.

**DISCUSSION**

Glu-113 serves as the counterion to the protonated retinylidene Schiff base in bovine rhodopsin (6–8). Replacement of Glu-113 by Gln (E113Q) removed the ionizable group from that position. However, Gln still had the potential to interact

![Fig. 3](image-url) **Fig. 3.** Effect of NaCl on spectral properties of the mutant pigments. A dark spectrum for each pigment in 0.1% dodecyl maltoside in water is compared to that in 100 mM NaCl. (A) COS cell rhodopsin (Rho) displayed identical spectra under both conditions. (B) Mutant pigment E113D. (C) Mutant pigment E113Q. (D) Mutant pigment E113N. (E) Mutant pigment E113A.

Radii increase from chloride to bromide to iodide, the $\lambda_{\text{max}}$ value for each mutant in the presence of bromide was less than that in the presence of chloride (Table 1).

**Hydroxylamine Reactivity of the Glu-113 Mutants.** Rhodopsin is insensitive to hydroxylamine in the dark but reacts rapidly upon illumination. Mutant E113Q was sensitive to hydroxylamine bleaching in the dark (6, 7). Reaction rates of hydroxylamine with E113Q and E113N were compared (Fig. 6). The reaction was carried out in the dark at acidic pH so that only the visible-absorbing PSB species of each mutant was present. Under these conditions, rhodopsin did not react, E113N reacted very slowly ($k = 0.0008 \text{ min}^{-1}$), and E113Q reacted about 15 times more rapidly ($k = 0.012 \text{ min}^{-1}$).

![Fig. 4](image-url) **Fig. 4.** UV–visible spectroscopy of mutant pigments E113Q and E113A in the presence of various NaCl concentrations. (A) pH was measured as a function of NaCl concentration in deionized water with the same pH electrode used to perform subsequent experiments on the pigments. (B) E113Q spectra were recorded in the presence of various NaCl concentrations. The proportion of visible-absorbing form increased with increasing salt concentration. The $\lambda_{\text{max}}$ value in the absence of NaCl (515 nm) was red-shifted with respect to the $\lambda_{\text{max}}$ value in 100 mM NaCl (496 nm) (see Table 1). (C) E113A spectra were recorded in the presence of various NaCl concentrations as listed. The proportion of the visible-absorbing form increased with increasing salt concentration. The $\lambda_{\text{max}}$ value in the absence of NaCl (528 nm) was red-shifted with respect to the $\lambda_{\text{max}}$ value in 100 mM NaCl (506 nm) (see Table 1).
Table 1. Effect of solute anions on $\lambda_{\text{max}}$ values of rhodopsin mutants

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\*The final anion concentration was 50 mM (pH 5) except benzoate, which was 20 mM.
\*Values are mean values of independent pigment preparations (SE < 1.5 nm; $n = 2$–7). See Fig. 3 for examples of individual experiments.
\*For E113Q and E113A, only a very small visible component existed in deionized water (see Figs. 2 and 3).

Rhodopsin and mutant E113D did not show spectral changes throughout a pH range of about 5–9. The other three pigments existed as pH-dependent equilibrium mixtures of visible (PSB) forms and near-UV (SB) forms. Since retinylidene pigments with SB linkages absorb in the near-UV range (<440 nm) and those with PSB linkages absorb in the visible range (>440 nm), the transition between the two forms of the pigments should represent a change in the Schiff base protonation state. A pH titration of the pigments should reflect the pKₐ of the Schiff base imine. The pKₐ value determined for each of the mutant pigments was in the range for those of model retinylidene Schiff bases in detergent solutions (13). The fact that the values varied from 5.7 to 6.7, depending on the amino acid at position 113, suggests an interaction between Schiff base imine and Glu-113. Possible effects due to changes in protein conformation cannot be ruled out. Also, it is possible that side chains in some amino acids change ionization states during titration. However, it is likely that the changes measured in visible absorbance resulted only from the change in ionization state of the Schiff base imine because single isosteric points were noted for the titrations.

The question posed previously of what served as the Schiff base counterion in the acidic form of the E113Q pigment led us to the hypothesis that a solute anion such as chloride could substitute for the normal amino acid counterion and allow Schiff base protonation (6). Thus, if chloride is needed to form the PSB form of the pigment, then the absence of chloride should hamper its formation. In E113Q and E113A, the presence of salt had a marked effect on the formation of the pigment absorbing in the visible range (Fig. 3). Without salt, these pigments formed only the UV-absorbing (SB) forms. The proportions of the PSB forms of E113Q and E113A depended on the concentration of NaCl (Fig. 4).

The NaCl effect was not observed in rhodopsin or in mutant E113D in which Asp-113 can presumably act as the counterion in place of Glu. Interestingly, this effect was also not observed in E113N despite the fact that a pH-dependent equilibrium between SB and PSB forms of the pigment was noted. This raises the possibility that the Schiff base environment of E113N is different from that of E113Q or E113A. Perhaps another ionizable amino acid in the protein forms the Schiff base counterion in the acidic form of E113N. We concluded that Glu-122 was protonated (uncharged) in rhodopsin (6). It is possible that, in mutant E113N, Glu-122 may become deprotonated to fulfill the role of Schiff base counterion. It is also possible that a water molecule or other amino acid side chains can interact with the Schiff base to compensate for the positive charge. Furthermore, Asn-113 might undergo a deamidation reaction to form an $\alpha$-carboxylic acid (14).

The $\lambda_{\text{max}}$ values of protonated retinylidene model Schiff bases are sensitive to the anion present (15). Under certain conditions the $\lambda_{\text{max}}$ values of the chromophores become red-shifted, presumably as the radius of the anion and consequently the charge separation distance increases.
There is a red-shifted series of protein itself.

In general, anion could substitute for Glu-113, the \( \lambda_{\text{max}} \) values of mutants E113Q and E113A should be sensitive to the type of anion present. As postulated, the \( \lambda_{\text{max}} \) values for E113Q and E113A varied widely in different anion solutions (Table 1). In general, smaller anions (formate or fluoride) tended to give \( \lambda_{\text{max}} \) values of shorter wavelength, whereas larger anions or anions with multiple ionizable groups tended to be associated with \( \lambda_{\text{max}} \) values of longer wavelength. In rhodopsin or E113D, where a protein carboxylate can compensate for the positively charged PSB, the \( \lambda_{\text{max}} \) values did not vary significantly for different solute anions. The observation that the \( \lambda_{\text{max}} \) of mutant E113N was not particularly sensitive to the type of solvent anion also supports the possibility that the counterion of the PSB form of this pigment is a part of the protein itself.

Blatz et al. (15) reported that the \( \lambda_{\text{max}} \) value of a retinylidene PSB with chloride as the counterion was 437 nm in benzene. With different halide ions, the \( \lambda_{\text{max}} \) value was progressively red-shifted as the ionic radius increased in the series chloride, bromide, and iodide. However, this anion-induced \( \lambda_{\text{max}} \) dependency was not observed in polar solvents such as methanol or ethanol. In mutants where a solute anion is substituting for the Glu-113 carboxylate, would we expect the \( \lambda_{\text{max}} \) values to vary as for the model retinylidene compounds in organic solvents? In mutants E113Q and E113A, \( \lambda_{\text{max}} \) values varied significantly for different halide anions tested (Table 1). However, the pattern observed in nonpolar solvents (15) was not seen. The halide dependency of the mutant pigment \( \lambda_{\text{max}} \) was as follows: \( \text{Br}^- (493 \text{ nm}) < \text{Cl}^- (496 \text{ nm}) < I^- (504 \text{ nm}) < F^- (509 \text{ nm}) \) for E113Q and \( F^- (486 \text{ nm}) < \text{Br}^- (500 \text{ nm}) < \text{Cl}^- (506 \text{ nm}) < I^- (507 \text{ nm}) \) for E113A. In both mutants, if the \( \lambda_{\text{max}} \) changes were determined only by charge separation between PSB and counterion, then the order \( \text{Cl}^- < \text{Br}^- < I^- \) should be maintained (15). Fluoride has unique properties within the halide group because of its electron orbital occupancy. In fluoride, E113Q gave the most red-shifted \( \lambda_{\text{max}} \) (508 nm) and E113A gave the most blueshifted \( \lambda_{\text{max}} \) (486 nm) when compared with other halides. There are several possible explanations for the lack of halide dependency on \( \lambda_{\text{max}} \) based simply on ionic radius. (i) Aqueous solutions are polar solvents and do not prevent hydrogen bonding to the PSB proton which can influence \( \lambda_{\text{max}} \). (ii) The opsin shift in rhodopsin results from interactions with the polyene chain in addition to those involving the PSB (6). (iii) A mutation at 113 can affect factors other than the Schiff base interactions that determine \( \lambda_{\text{max}} \). (iv) A mutation at 113 can affect the ability of a solute anion to interact directly with the PSB. (v) Resonance structures with partial positive charges located on retinylidene C-15 or C-3 may become relatively stabilized in mutants. In a recent study, a pattern of halide anion dependence of \( \lambda_{\text{max}} \) shift was noted in rhodopsin mutant E113Q under one set of experimental conditions (8).

Hydroxylamine can be used as a probe of the Schiff base environment in rhodopsin mutants. Rhodopsin is stable to hydroxylamine bleaching in the dark. Mutants E113N and E113Q were treated with hydroxylamine under conditions where only the PSB forms of the pigments were present. E113Q reacted rapidly with hydroxylamine to form free retinal oxime and opsin, whereas E113N reacted slowly (Fig. 6). These differences in hydroxylamine reactivity among rhodopsin, E113Q, and E113N imply differences in their Schiff base environments that influence hydroxylamine accessibility.

In conclusion, Glu-113 influences the protonation state of the Schiff base and its \( K_{a} \). The negatively charged carboxylate allows Schiff base protonation by compensating for the PSB positive charge. In mutant pigments E113Q and E113A, an exogenously supplied solute anion could fulfill one role of Glu-113 to form the PSB counterion. The degree of \( \lambda_{\text{max}} \) shifts in these mutant pigments in the presence of different solute anions also indicates that the interaction between Schiff base and its counterion is a significant mechanism that determines the \( \lambda_{\text{max}} \) of rhodopsin and possibly other visual pigments.

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