# Determinants of Visual Pigment Absorbance: Identification of the Retinylidene Schiff's Base Counterion in Bovine Rhodopsin†

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ABSTRACT: The role of negatively charged residues in tuning the absorbance spectrum of bovine rhodopsin has been tested by mutating each aspartate and glutamate to asparagine and glutamine, respectively. Previous work demonstrated that aspartate[83], glutamate[122], and glutamate[134] can be replaced by neutral residues with little or no effect on the absorbance spectrum of the resulting pigment [Nathans, J. (1990) *Biochemistry 29*, 937–942]. With one exception, mutations at the remaining 19 aspartate and glutamate residues result in very nearly wild-type absorbance spectra. The exception is glutamate[113]: mutation to glutamine causes the pigment to absorb at 380 nm, reflecting deprotonation of the retinylidene Schiff's base. Upon addition of either chloride, bromide, or iodide, the absorbance rapidly shifts to 495, 498, or 504.5 nm, respectively, reflecting protonation of the Schiff's base. The progressive red shift observed upon addition of halides with larger atomic radii strongly suggests that halides are serving as the Schiff's base counterion. Halides have no effect on the absorbance spectrum of wild-type rhodopsin. I infer, therefore, that glutamate[113] is the retinylidene Schiff's base counterion in wild-type rhodopsin. Sakmar et al. [(1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8309–8313] and Zhukovsky and Oprian [(1989) *Science 246*, 928–930] have arrived at the same conclusion based upon a related series of experiments. These data support a model in which spectral tuning in bovine rhodopsin results from interactions between the polyene chain of 11-*cis*-retinal and uncharged amino acids in the binding pocket.

Visual pigments are the light-absorbing proteins in the retina that initiate phototransduction. Each consists of an integral membrane protein, opsin, covalently joined via a protonated Schiff's base to a chromophore, 11-*cis*-retinal (or, in some instances, 11-*cis*-3,4-dehydroretinal). All visual pigments have in common a broad bell-shaped absorbance spectrum, resembling in shape that of retinal free in solution. The pigment spectra differ from one another and from that of the free chromophore in their positions along the wavelength axis: points of maximal absorbance have been found throughout the visible range, from near-ultraviolet to far-red. Bovine rhodopsin, the object of the present study, absorbs maximally at 498 nm.

How does binding to the visual pigment modify the absorbance spectrum of 11-*cis*-retinal? Part of the modification derives from Schiff's base formation: when measured in methanol, free retinal has an absorbance maximum of 380 nm, whereas the chloride salt of a protonated *N*-butylamine Schiff's base of retinal absorbs maximally at 440 nm (Pitt et al., 1955). (The latter compound is the standard against which the visual pigments and other retinal derivatives are compared.) During the past 3 decades, a number of plausible models have been advanced to account for the spectral tuning of retinal. All rest upon the observation that photoexcitation of 11-*cis*-retinal induces a significant increase in $\pi$ electron delocalization, and a corresponding change in its dipole moment (Kropf & Hubbard, 1958; Mathies & Stryer, 1976). Any protein–chromophore interaction that increases delocalization will lead to a smaller energy difference between ground and excited states and therefore a red shift in the absorbance spectrum. Conversely, any interaction that decreases delocalization will lead to a larger energy difference between ground and excited states and therefore a blue shift in the absorbance spectrum (Hubbard & Sperling, 1973).

Most models propose that a negatively charged amino acid serves as the Schiff's base counterion. Changes in the distance or charge density of the putative counterion have been hypothesized to play a role in generating the differences in absorbance maxima that distinguish the visual pigments from one another, from their photointermediates, and from the reference protonated Schiff's base (Blatz et al., 1972; Baasov & Sheves, 1986; Fukada et al., 1990). Alternatively, the protonated Schiff's base in the visual pigment could be paired to a counterion from solution, for instance, chloride, or be stabilized by a surrounding shell of polar residues.

With respect to interactions along the polyene chain, one model proposes that in bovine rhodopsin a negatively charged amino acid selectively stabilizes the photoexcited state by contacting the polyene chain near carbon 13 (Honig et al., 1976, 1979). This point charge model is consistent with the red shifts seen in comparing a series of dehydroretinal analogues of bovine rhodopsin to the corresponding *N*-butylamine salts (Koutalos et al., 1989). Interactions along the polyene chain that do not involve charged residues have also been considered. Twisting of the chromophore about double or single bonds would produce, respectively, a red shift or a blue shift (Blatz & Liebman, 1973); polarizable groups near the chromophore would stabilize the photoexcited state by compensatory electronic movements and therefore produce a red shift; and polar groups would produce either a red shift or a blue shift depending upon their orientation with respect to the dipole moment of the chromophore (Irving et al., 1969, 1970; Hays et al., 1980).

In an earlier study, I examined the role of charged amino acids at six positions within the putative membrane-spanning segments in tuning the absorbance spectrum of bovine rhodopsin (Nathans, 1990). These six positions (83, 86, 122, 134,

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135, and 211) are the ones at which charged amino acids are found in one or more of the four human visual pigments (Nathans & Hogness, 1984; Nathans et al., 1986). These positions were chosen because experiments with bovine rhodopsin strongly suggest that 11-*cis*-retinal binds within the hydrophobic core of the visual pigment [see Nathans (1987) for a recent review.] Single and double amino acid substitutions were introduced by site-directed mutagenesis to produce at these six positions the distribution of charged or uncharged residues seen in each of the three human cone pigments as well as to change to neutral those charged residues that are common to all human pigments. Upon joining to 11-*cis*-retinal, all of the site-directed rhodopsin mutants were found to have absorbance maxima very close to that of wild-type bovine rhodopsin. This result rules out a significant electronic interaction between these residues and the chromophore.

Because current models of transmembrane topography and of the retinal binding pocket are still preliminary, the present study was undertaken to systematically test the role of each of the remaining 19 negatively charged amino acids in determining the absorbance spectrum of bovine rhodopsin. (Throughout this paper, the term "charged" will be used to refer to those amino acid residues that are most commonly ionized—aspartate, glutamate, lysine, arginine, and histidine. The possibility exists, however, that one or more of these residues may not be in its ionized state in rhodopsin.)

## Materials and Methods

*Plasmids and Cells.* The rhodopsin expression plasmid pCIS-cRho contains a bovine rhodopsin-coding region (Nathans & Hogness, 1983) under the control of a human cytomegalovirus immediate early enhancer and promoter, an SV40 poly(A) addition site, and an SV40 origin of DNA replication. (Gorman et al., 1990; Nathans et al., 1989). In a typical experiment, 100 μg of a pCIS-cRho mutant plasmid and 10 μg of pRSV-TAg (an SV40 T-antigen expression plasmid) were coprecipitated onto 20 10-cm diameter plates of 293S cells (a suspension-adapted variant of a human embryonic kidney cell line; ATCC CRL 1573) by the calcium phosphate method (Gorman, 1985).

*In Vitro Mutagenesis.* Oligonucleotide-directed mutagenesis was performed as described (Nathans, 1990). To confirm the predicted mutations and to rule out spurious changes, the entire rhodopsin-coding region of each mutant was sequenced on one strand by the dideoxy method.

*Membrane Preparation and Incubation with 11-cis-Retinal.* Solutions for membrane preparation were chilled on ice, and all centrifugation steps were performed at 4 °C. Sixty hours after transfection, the cells were collected by vigorously washing the plates with phosphate-buffered saline (PBS) containing 5 mM EDTA. The cells were centrifuged at 1000*g* for 10 min, washed once with 20 mL of PBS, and resuspended in 10 mL of 0.1 M sodium phosphate, pH 6.5, 1 mM EDTA, 250 mM sucrose, and 0.2 mM PMSF. The cell suspension was homogenized for 30 s in a Polytron homogenizer (Brinkmann) as a setting of 5.5, diluted to 25 mL with the same buffer, and then layered on top of a 10-mL 1.5 M sucrose cushion containing 0.1 M sodium phosphate, pH 6.5, and 1 mM EDTA. The homogenate was centrifuged in a swinging-bucket rotor (SW28) at 105000*g* for 30 min. Membranes were collected from the interface between the homogenization buffer and the sucrose cushion, diluted with 8 volumes of 0.1 M sodium phosphate, pH 6.5, and 1 mM EDTA, and centrifuged in a swinging-bucket rotor (SW28) at 105000*g* for 30 min. The resulting membrane pellet was solubilized in 0.3 mL of 0.1 M sodium phosphate, pH 6.5, 1 mM EDTA, and 5% digitonin (Kodak) at 23 °C.

All manipulations involving 11-*cis*-retinal were performed under dim red light. 11-*cis*-Retinal (stored dry in the dark under argon at −80 °C) was first dissolved in ethanol to a concentration of $4 \times 10^{-3}$ M and then diluted with 20 volumes of 0.1 M sodium phosphate, pH 6.5, 1 mM EDTA, and 5% digitonin; 0.1 mL of the aqueous 11-*cis*-retinal solution was added to the solubilized preparation and incubated at 23 °C for 2 h in the dark. The reaction was terminated by the addition of 0.05 mL of 0.6 M hydroxylamine (adjusted to pH 6.0 with sodium hydroxide) and the sample incubated for an additional 30 min at 23 °C (Hubbard et al., 1971).

For measuring the reaction rate with hydroxylamine and for monitoring the effect of halide addition, the protocol was modified as follows. Reconstitution with 11-*cis*-retinal was performed in the presence of 1 mM DTT at 23 °C overnight with membranes collected from the sucrose step gradient. To remove excess 11-*cis*-retinal, the membranes were diluted with 8 volumes of 0.1 M sodium phosphate, pH 6.0, containing 4% bovine serum albumin and centrifuged in a swinging-bucket rotor (SW28) at 105000*g* for 30 min. The resulting membrane pellet was solubilized in 0.3 mL of 0.1 M sodium phosphate, pH 6.0, and 5% digitonin at 23 °C.

*Absorbance Spectra.* Before measurement of the absorbance spectrum, samples were centrifuged at 11 000 rpm in an Eppendorf microfuge for 5 min. Spectra were obtained on the supernatant before and after 10 min of photobleaching with a 15-W incandescent bulb placed behind a glass shield and a 495-nm shortwave cutoff filter approximately 10 cm from the cuvette. Spectra were recorded in a water-jacketed cuvette holder at 20 °C with a Kontron Instruments Uvikon spectrophotometer. Kontron Instruments software was used to calculate the difference spectra and determine the absorbance maxima shown in Figure 2. Earlier work has shown that for absorbance maxima averaging 0.075 ODU, this method has a mean variation in duplicate experiments of 2.4 nm (Nathans, 1990). For the halide addition experiments shown in Table I and Figures 5 and 6, approximately 2-fold greater accuracy in estimating absorbance maxima was obtained by transferring the photobleaching difference spectra to a MacIntosh computer, calculating for each spectrum the best-fitting fifth-order polynomial, and determining the maximum of the fitted curve.

*Protein Blots.* Membrane samples containing expressed opsin were diluted with 3 volumes of SDS sample buffer, electrophoresed on an SDS/12% polyacrylamide gel, and electroblotted onto nitrocellulose (Burnette, 1981). Opsin was detected by using a mouse monoclonal antibody directed against residues 3–14 of bovine rhodopsin (B6-30; a kind of gift of Dr. Paul Hargrave) and an alkaline phosphatase conjugated goat anti-mouse second antibody.

## Results

*Aspartate and Glutamate Mutants.* To test the role of each negatively charged amino acid in determining the absorbance spectrum of bovine rhodopsin, 20 site-directed mutants were constructed. Each glutamate was replaced by glutamine and each aspartate by asparagine. A triple-substitution mutant was constructed at positions 330, 331, and 332; at all other positions, single-substitution mutants were constructed. Figure 1 shows the locations of these residues in the transmembrane model of Hargrave et al. (1983); similar models have been proposed by Ovchinnikov et al. (1982) and by Nathans and Hogness (1983). Mutants are referred to by the single-letter amino acid designation of the wild-type residue followed by the position number in the polypeptide chain followed by the

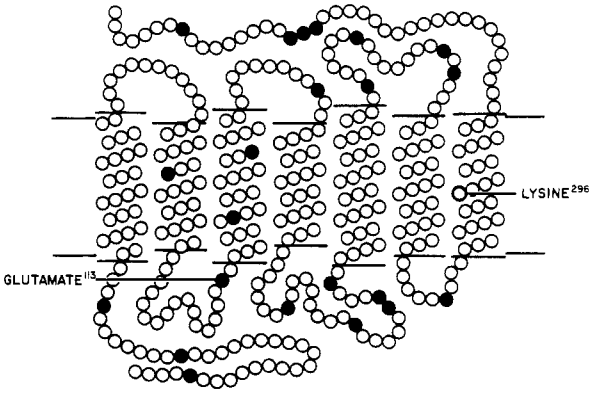


FIGURE 1: Aspartate and glutamate residues in bovine rhodopsin (filled circles). The transmembrane topography is based on the model of Hargrave et al. (1983). The amino terminus is at the lower left and resides on the extracellular face of the membrane.

single-letter amino acid designation of the introduced residue; e.g., E201Q refers to the substitution of glutamate$^{201}$ by glutamine.

Each mutant cDNA was inserted into an expression plasmid, mixed with a plasmid directing expression of SV40 large T-antigen, and transfected into a human embryonic kidney cell line, 293S. Sixty hours after transfection, approximately 2 $\mu$g of opsin accumulates per $10^7$ cells, as determined spectrophotometrically following reconstitution with 11-*cis*-retinal and by protein blotting.

In an initial series reconstitution experiments, total cell membranes were prepared in sodium phosphate and EDTA, solubilized in sodium phosphate, EDTA, and digitonin, and incubated in the dark with 11-*cis*-retinal. To convert excess retinal to retinal oxime, hydroxylamine was then added to 50 mM, and the samples were incubated for an additional 30 min at room temperature (Hubbard et al., 1971). Absorbance spectra were recorded before and after photobleaching and the difference spectra calculated (Figure 2). For completeness, difference spectra for D83N, E122Q, and E134Q are reproduced from Nathans (1990). Of the 20 mutants, 19 have photobleaching difference spectra closely resembling that of wild-type rhodopsin: 18 mutants have absorbance maxima within 8 nm of the wild type; E122Q is blue-shifted 17 nm. Under these conditions, E113Q differs markedly from the wild type: it does not show an absorbance peak in the visible region, and it exhibits no change in absorbance after exposure to light.

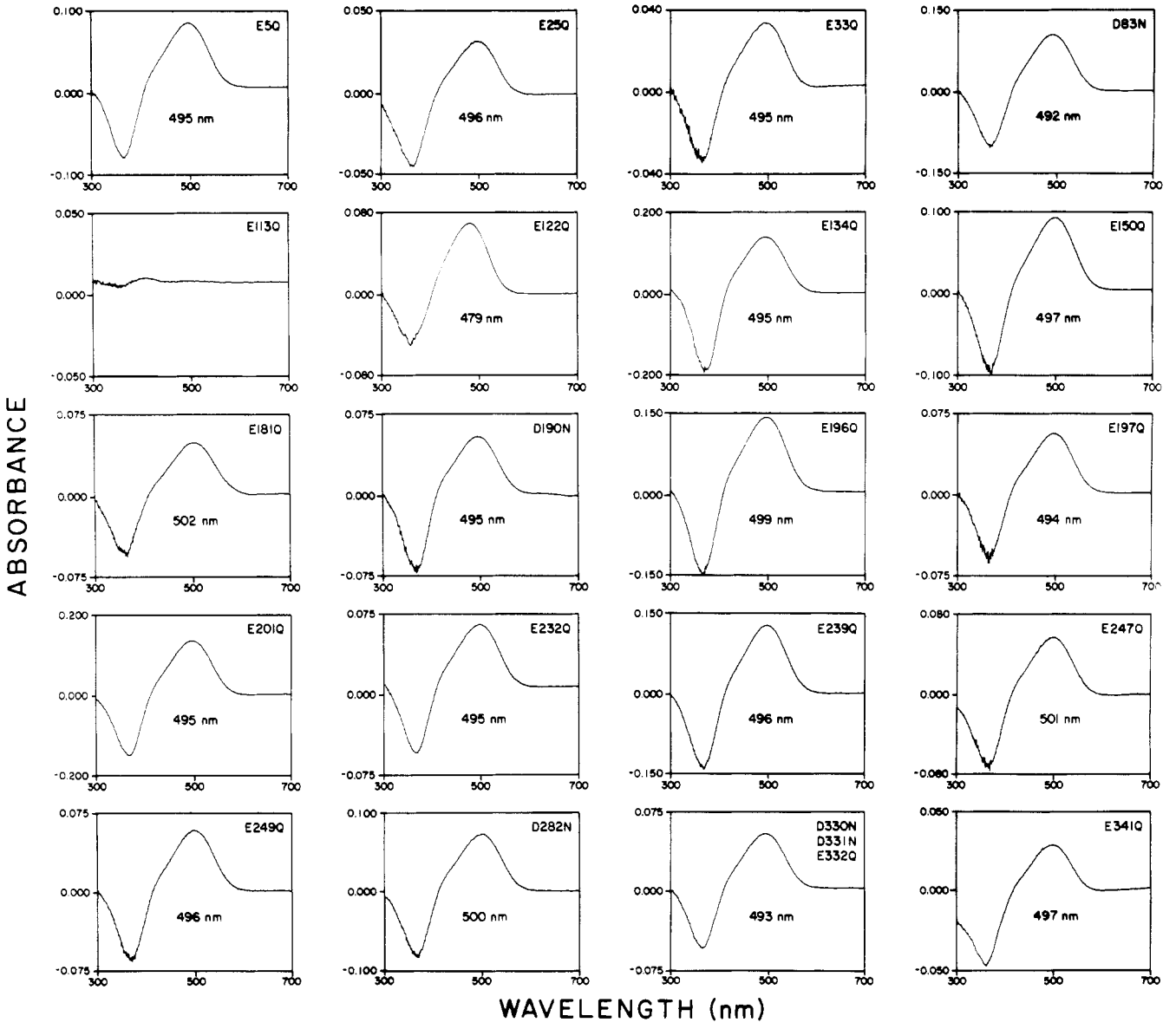


FIGURE 2: Photobleaching difference spectra of expressed mutant rhodopsins in 0.1 M sodium phosphate, pH 6.5, 1 mM EDTA, 5% digitonin, and 50 mM hydroxylamine. The absorbance maximum is indicated below each spectrum.

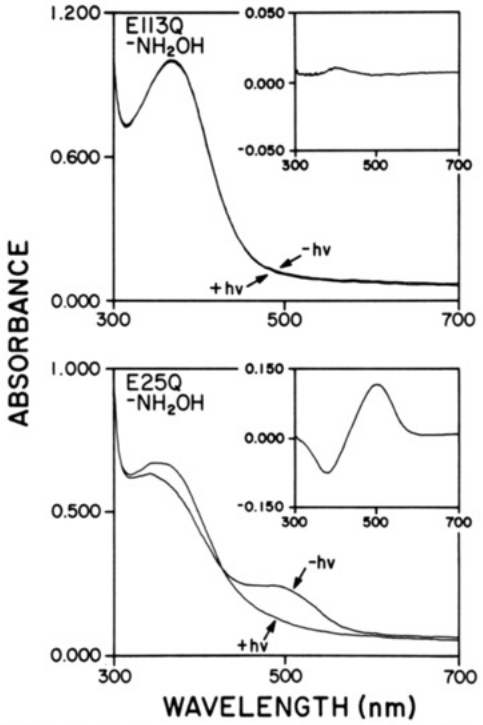


FIGURE 3: Spectra in the absence of hydroxylamine. Absorbance spectra before and after photobleaching (main panel) and photobleaching difference spectra (inset) of rhodopsin mutants E113Q and E25Q were measured in 0.1 M sodium phosphate, pH 6.5, 1 mM EDTA, and 5% digitonin.

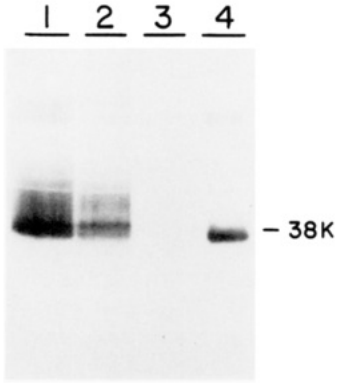


FIGURE 4: Protein blot of exposed rhodopsin mutants. Each lane of an SDS/12% polyacrylamide gel was loaded with the equivalent of 4% of the sample used for spectrophotometry and visualized with a monoclonal mouse anti-bovine rhodopsin primary antibody and alkaline phosphatase conjugated goat anti-mouse secondary antibody. Lanes: 1, E25Q; 2, E113Q; 3, mock-transfected control; 4, rod outer segments containing 300 ng of bovine rhodopsin.

To further characterize the anomalous behavior of E113Q, photobleaching difference spectra were obtained as described above except in the absence of hydroxylamine (Figure 3). Under these conditions, E113Q produces no absorbance change upon exposure to light. Figure 4 shows that E113Q is glycosylated to the same extent as E25Q and that it accumulates to a similar level.

*E113Q: Effects of Halides and Hydroxylamine.* The experiments described above suggest a model in which glutamate[113] provides a negative charge that is essential for normal visual pigment formation. This model assumes that the anions present in solution, phosphate and EDTA, are unable to substitute for the missing negative charge. To examine the possibility that a smaller anion could substitute for the charge normally present at position 113, chloride (sodium chloride, 0.1 M final concentration) was added to a sample of E113Q that had been incubated with 11-*cis*-retinal. An increase in absorbance in the visible region, maximal at 487 nm, and a concomitant decrease in the ultraviolet, maximal at 380 nm, were observed (Figure 5). The chloride-dependent absorbance change was complete before a spectrum could be recorded, a delay of approximately 15 s. The chloride-dependent peak in the visible region resembles that of a bovine rhodopsin in its absorbance maximum (487 nm vs 498 nm) and photolability (Figure 6). Photobleaching generates a product that is identical in its absorbance spectrum with free *all-trans*-retinal. The magnitude of the chloride-dependent absorbance change is greater at pH 6.0 than at pH 7.0. The experiments described below were therefore performed at pH 6.0.

Table I: Visual Pigment Absorbance Maxima Determined from Difference Spectra[a]

| halide added | wild type[b] +halide, ±*hν* | E113Q[b] +halide, ±*hν* | E113Q[c] ±halide, −*hν* |
|---|---|---|---|
| chloride | 498.0 ± 1.0 ($n$ = 3) | 495.0 ± 2.0 ($n$ = 5) | 487.5 ± 1.5 ($n$ = 3) |
| bromide | 499.0 ± 1.0 ($n$ = 3) | 498.5 ± 1.5 ($n$ = 5) | 490.0 ± 2.0 ($n$ = 3) |
| iodide | 498.5 ± 2.0 ($n$ = 3) | 504.5 ± 2.5 ($n$ = 5) | 497.0 ± 2.5 ($n$ = 3) |

[a] Absorbance maxima (in nanometers) are presented as the mean ± standard deviation; the number of independent experiments is shown in parentheses. [b] Derived from photobleaching difference spectra in the presence of 0.1 M of the indicated halide (Figure 6). [c] Derived from difference spectra obtained by subtracting the absorbance before halide addition from the one obtained after halide addition (Figure 5).

Chloride addition converts E113Q from a form that absorbs maximally at 380 nm to one that absorbs maximally at 487 nm. The 380-nm absorbance peak matches that of an unprotonated Schiff's base of retinal, as seen in metarhodopsin II and in Schiff's bases of retinal in solution, all of which absorb at wavelengths less than 400 nm. The 487-nm absorbance peak is presumed to derive from a protonated Schiff's base, as seen in wild-type rhodopsin and in protonated Schiff's bases of retinal in solution, which absorb at wavelengths greater than 400 nm. The simplest interpretation of this experiment is that glutamate[113] normally serves as the Schiff's base counterion. Mutation of glutamate[113] to glutamine favors deprotonation of the Schiff's base. Addition of chloride to E113Q leads to protonation of the Schiff's base because chloride serves as a surrogate counterion. Presumably, phosphate and EDTA, the two other solution anions present in the sample, are too bulky to enter the retinal binding site.

To test this interpretation, the absorbance spectra and the photobleaching difference spectra of E113Q were measured following addition of either chloride, bromide, or iodide to 0.1 M (Figures 5, 6, and 7). This experiment was inspired by the observation of Blatz et al. (1972) that the absorbance maxima of *N*-butylamine protonated Schiff's bases of retinal move to progressively longer wavelengths when paired with halide counterions of increasing atomic radius. For example, in benzene, the chloride salt absorbs maximally at 437 nm, the bromide salt at 448 nm, and the iodide salt at 457 nm. In tetrahydrofuran, the absorbance maxima are 430, 432, and 441 nm, respectively. The red shifts in absorbance reflect in increase in $\pi$ electron delocalization.

When added to E113Q, each halide induces a rapid conversion of the 380-nm species to one absorbing maximally near 495 nm. Table I lists the absorbance maxima derived from difference spectra following halide addition in the absence of light (Figure 5; three independent experiments) and following photobleaching in the presence of 0.1 M halide (Figures 6 and 7; five independent experiments). There is a reproducible 7–8-nm difference between the absorbance maxima measured from the two types of difference spectra. The cause of this discrepancy is not known. In the discussion of these pigments,

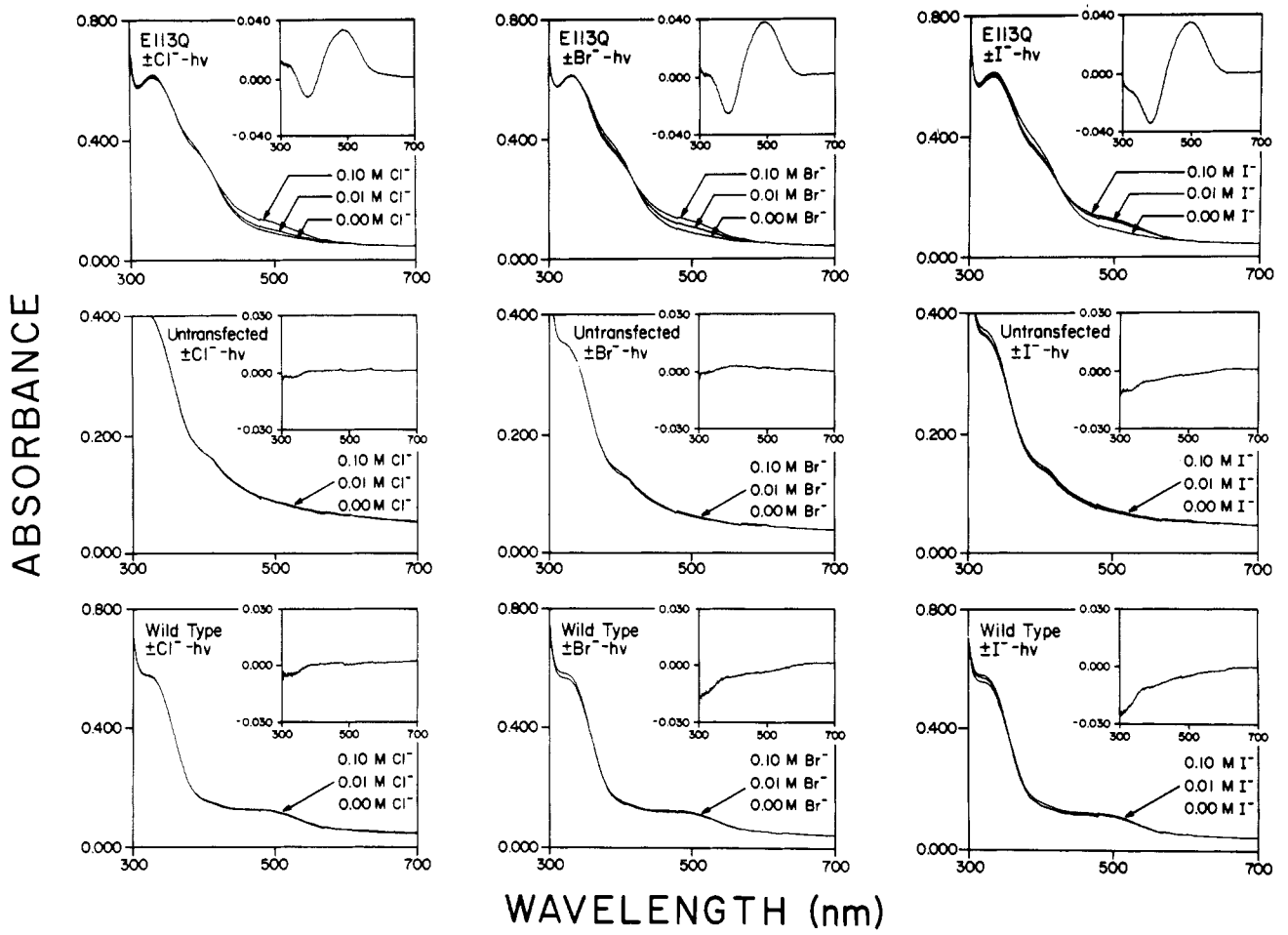


FIGURE 5: Absorbance spectra before and after addition of sodium chloride, bromide, or iodide to final concentrations of 10 and 100 mM (main panels). The insets show difference spectra in which the 0 mM halide absorbance spectrum has been subtracted from the 100 mM halide absorbance spectrum. The samples contained 0.1 M sodium phosphate, pH 6.0, and 5% digitonin. Top panels, E113Q; middle panels, untransfected cell membranes; bottom panels, wild-type rhodopsin expressed in 293S cells.

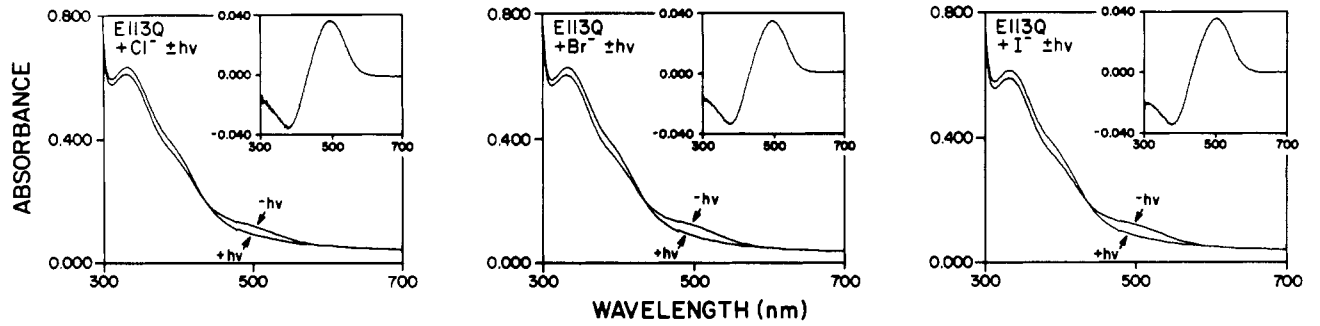


FIGURE 6: Absorbance spectra before and after photobleaching (main panel) and photobleaching difference spectra (inset) of rhodopsin mutant E113Q in the presence of 100 mM sodium chloride, bromide, or iodide. The samples were prepared as shown in Figure 5.

the photobleaching absorbance maxima will be considered the true maxima. Control samples prepared identically from untransfected cells or from cells expressing wild-type rhodopsin show no significant absorbance change upon halide addition. Table I lists the absorbance maxima derived from photobleaching difference spectra of wild-type rhodopsin in the presence of chloride, bromide, and iodide. These values are identical within experimental error.

The hypothesis that halides act as counterions in E113Q predicts that the Schiff's base in E113Q should be accessible to other small molecules in the aqueous environment. To test this prediction, the time course of reaction between hydroxylamine and the chromophore in E113Q was determined (Figure 8). At 20 °C in 0.1 M chloride, the half-life of E113Q in 50 mM hydroxylamine is 12 min, as measured by the disappearance of the absorbance peak at 487 nm. Under identical conditions, free 11-*cis*-retinal and wild-type rhodopsin have half-lives of 1 min and greater than 10 h, respectively.

## Discussion

Negatively charge amino acids in bovine rhodopsin have been hypothesized to play two distinct roles in modifying the absorbance spectrum of 11-*cis*-retinal. Most models propose that one negatively charged amino acid serves as the retinylidine Schiff's base counterion (Honig et al., 1976). The point charge model proposes that a second negative charge lies along the polyene chain and enhances electron delocalization (Kropf & Hubbard, 1958; Honig et al., 1976, 1979). On the basis of the absorbance shifts seen when bovine rhodopsin is reconstituted with a series of dehydroretinals, the point charge

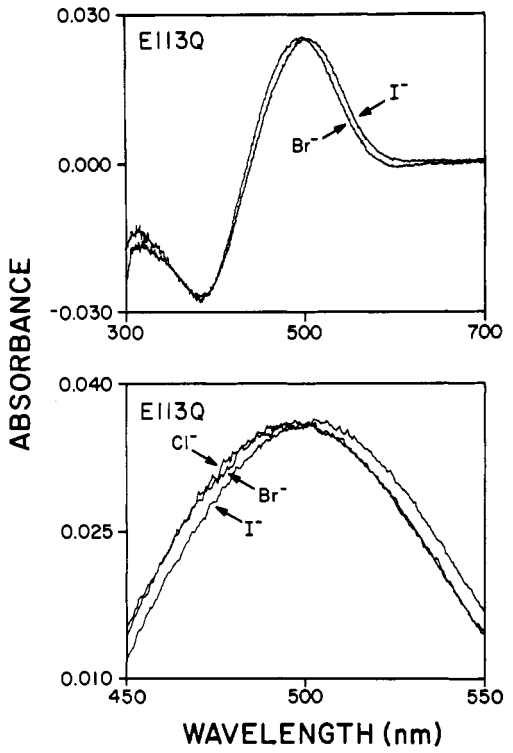


FIGURE 7: Superposition of photobleaching difference spectra of rhodopsin mutant E113Q in 100 mM sodium iodide or bromide (upper panel) and in 100 mM sodium iodide, bromide, or chloride (lower panel, enlarged). The samples were prepared as shown in Figures 5 and 6.

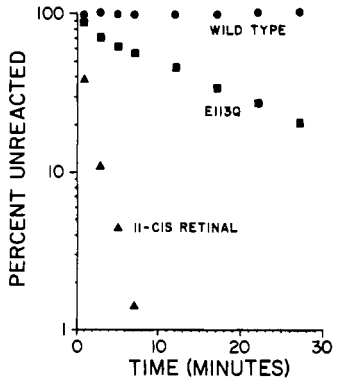


FIGURE 8: Time course of hydroxylamine reactivity with wild-type rhodopsin, rhodopsin mutant E113Q, and free 11-*cis*-retinal at 20 °C. All samples contained equal amounts of 293S membranes, 0.1 M sodium phosphate, pH 6.0, 5% digitonin, and 0.1 M sodium chloride. The reaction was initiated by addition of hydroxylamine to a final concentration of 50 mM.

is proposed to reside near C-13 of 11-*cis*-retinal (Koutalos et al., 1989). Substitution of the putative point charge by a neutral residue should lead to a blue shift in the absorbance spectrum, reflecting a decrease in $\pi$ electron delocalization. Substitution of the putative Schiff's base counterion by a neutral residue should lead either to a red shift, reflecting an increase in $\pi$ electron delocalization, and/or to a deprotonation of the Schiff's base. Unprotonated retinylidene Schiff's bases absorb at approximately 380 nm.

An earlier study of charged residues in the putative transmembrane segments demonstrated that aspartate[83], glutamate[122], and glutamate[134] do not make electronically significant contact with the chromophore (Nathans, 1990). The present study was undertaken to test the role of each of the remaining 19 negatively charged amino acids in determining the absorbance spectrum of bovine rhodopsin. In designing these experiments, only aspartate and glutamate were considered as negatively charged residues; the possibility exists, however, that one or more tyrosinates may be present in bovine rhodopsin or other visual pigments.

In the present study, each aspartate and glutamate residue not examined in the previous study was mutated to asparagine and glutamine, respectively. Of these, E113Q is the only mutant that differs markedly from the wild-type. Upon reconstitution with 11-*cis*-retinal and in the absence of small solution anions, E113Q forms a pigment absorbing at 380 nm. This pigment is presumed to contain an unprotonated retinylidene Schiff's base. In the presence of halides, the absorbance of E113Q shifts to approximately 495 nm, reflecting the protonation of the Schiff's base. In these experiments, the UV-absorbing form of E113Q has not been observed directly; its existence is inferred by the shift from UV to visible absorbance upon halide addition.

It appears that in E113Q the normal Schiff's base counterion is absent and that a halide from solution can serve as a surrogate Schiff's base counterion. This interpretation is supported by the observation of a progressive red shift upon addition of halides with progressively larger atomic radii (chloride vs bromide vs iodide). The direction and magnitude of the red shifts resemble those seen by Blatz et al. (1972) with protonated Schiff's bases of retinal in solution, in particular in tetrahydrofuran. Halides have no effect on the absorbance spectrum of wild-type rhodopsin. These characteristics of E113Q, together with the nearly wild-type behavior of all of the other glutamate and aspartate mutants, identify glutamate[113] as the retinylidene Schiff's base counterion in bovine rhodopsin. Alternative mechanisms for stabilizing the protonated Schiff's base in wild-type rhodopsin—i.e., pairing to a counterion from solution or interacting with favorably disposed dipoles—are unlikely to be relevant. Zhukovsky and Oprian (1989) and Sakmar et al. (1989) have arrived at the same conclusion based upon a related series of experiments. Glutamate is present at the homologous location in all of the other vertebrate pigments sequenced to date: chicken (Takao et al., 1988), mouse (Baehr et al., 1988), and human (Nathans & Hogness, 1984) rhodopsins, and the three human cone pigments (Nathans et al., 1986). It is interesting that aspartate[113] in the $\beta$-adrenergic receptor, which is located at a position similar to that of glutamate[113] in bovine rhodopsin, is proposed to ion-pair with the primary amine in adrenergic ligands (Strader et al., 1987, 1988).

The halide salts of E113Q can be compared to the corresponding halide salts of a protonated retinylidene Schiff's base in solution without the confounding effect of a change in counterion. This comparison suggests that the red shift induced in 11-*cis*-retinal upon forming the halide salts of E113Q is not caused by a decrease in counterion charge density or an increase in counterion distance relative to that of the reference protonated Schiff's base in methanol. Given the nearly identical red shifts induced in 11-*cis*-retinal upon combining with wild-type rhodopsin or with E113Q in the presence of halides, it appears that the tuning mechanisms operating in E113Q can account for the full extent of the wild-type rhodopsin red shift. This observation is consistent with dehydroretinal experiments in implicating an electrostatic perturbation along with polyene chain in spectral tuning (Koutalos et al., 1989).

The present set of experiments together with those described earlier (Nathans, 1990) eliminates all aspartate and glutamate residues as candidates for a point charge lying along the polyene chain. Instead, they are consistent with a more general

interpretation of the dehydroretinal data, namely, that spectral tuning could be due to either charged, polar, or polarizable residues in the neighborhood of the chromophore. Future experiments will be aimed at testing the latter two possibilities.

The identification of glutamate[113] as the Schiff's base counterion requires a revision in the working model of rhodopsin's transmembrane topography (Figure 1). To move glutamate[113] closer to the center of the membrane, the first extracellular loop should be shortened by approximately five residues, and the second intracellular loop should be correspondingly lengthened. This identification provides a constraint on the spatial proximity of two positions (113 and 296) that are widely separated on the linear sequence. A second constraint of this type has been defined by Karnik et al. (1988) between cysteine[110] and cysteine[187], which form a disulfide bond. These constraints provide two useful starting points for prediction of tertiary structure.

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