Light-Dependent Transducin Activation by an Ultraviolet-Absorbing Rhodopsin Mutant†

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ABSTRACT: The photoactivation pathway of an ultraviolet-absorbing rhodopsin mutant was studied. The mutant pigment, in which the retinylidene Schiff base counterion, Glu^{113}, was replaced by glutamine (E113Q), was known to exist in a pH-dependent equilibrium between spectral forms absorbing at about 380 and 490 nm. The 380-nm form contains an unprotonated Schiff base chromophore linkage, whereas the 490-nm form contains a protonated Schiff base chromophore linkage. The role of the Schiff base proton in photoactivation was investigated by measuring transducin activation as a function of photoactivation wavelength. The transducin activation action spectra of rhodopsin and of mutant E113Q were found to be very similar to their UV–visible absorption spectra. Thus, the 380-nm UV form of the mutant E113Q could be activated directly by UV light to catalyze nucleotide exchange by transducin. The quantum efficiency of photoactivation of the UV-absorbing form of E113Q was similar to that of its visible-absorbing form. These results show that the presence of a protonated Schiff base in the ground state is not necessarily required for efficient photoactivation of visual pigments. They support the hypothesis that the key role of the protonated Schiff base in visible-absorbing pigments is to stabilize the ground state and to allow absorbance at wavelengths above about 420 nm. The findings are also consistent with transducin activation studies of mutant apoproteins regenerated with *all-trans*-retinal, or of mutant apoproteins alone, suggesting that the active state of rhodopsin can be formed via a number of pathways. UV-absorbing mutants of rhodopsin can be used as a model system for the study of naturally occurring vertebrate UV photoreceptors.

Rhodopsin is the visual pigment of retinal rod cells (Dratz & Hargrave, 1983; Khorana, 1992; Liebman et al., 1987; Nathans, 1992). Light is absorbed by the 11-*cis*-retinal chromophore bound to opsin via a protonated Schiff base (Oseroff & Callender, 1974). Photoisomerization of the chromophore to the all-trans form evokes conformational changes in the protein to form the spectroscopically defined photointermediate metarhodopsin II (MII),[1] in which the Schiff base is deprotonated (Doukas et al., 1978). Schiff base deprotonation is required to form the activated form of the receptor (R*) (Longstaff et al., 1986). The cytoplasmic surface of R* interacts with the G protein transducin to catalyze guanine nucleotide exchange (i.e., transducin activation). In the unphotolyzed state of rhodopsin, the Schiff base proton is stabilized by electrostatic interaction with Glu^{113} of the opsin (Nathans, 1990; Sakmar et al., 1989; Zhukovsky & Oprian, 1989). This contributes to an unusually high acidity constant (pK_a) of the Schiff base in rhodopsin (Steinberg et al., 1993). Replacement of Glu^{113} by Gln (E113Q), and regeneration of the mutant opsin with 11-*cis*-retinal, leads to a pigment with a dramatically lowered Schiff base pK_a (Sakmar et al., 1989, 1991; Zhukovsky & Oprian, 1989). The mutant pigment exhibits an absorption maximum at 380 nm in addition to a second absorption maximum at about 490 nm. It was concluded that mutant E113Q exists in an equilibrium between a protonated and an unprotonated Schiff base in the dark, and titration revealed a pK_a of 6 for the transition between the 380- and 490-nm forms. Resonance Raman spectroscopy provided evidence that direct titration of the Schiff base proton caused the pH-induced changes in chromophore absorption (Lin et al., 1992). In particular, the resonance Raman study showed a covalent attachment of the chromophore to the mutant opsin. Therefore, the UV absorption of the mutant pigment under investigation is not caused by a noncovalently entrapped retinal chromophore.

The mutant pigment E113Q containing a protonated Schiff base was found to form a MII-like photoproduct with the typical 380-nm absorption upon illumination with light of wavelengths above 495 nm. This mutant MII-like photoproduct could activate transducin. Furthermore, the mutant pigment could also activate transducin in the dark if regenerated with *all-trans*-retinal (Sakmar et al., 1989). These results suggested that the minimal determinants of the R* state in rhodopsin are a neutralized counterion and an unprotonated Schiff base of *all-trans*-retinal in the binding site of opsin. Recent studies on constitutively active opsin mutants which activate transducin in the absence of a chromophore showed that breakage of the salt bridge between Lys^{296} (forming the Schiff base in rhodopsin) and its counterion Glu^{113} was crucial for R* formation even in the absence of a chromophore (Cohen et al., 1992; Robinson et al., 1992).

In rhodopsin, deprotonation of the Schiff base must be linked to the breakage of this intramolecular salt bridge. However, it is not clear that the net proton transfer from the Schiff base either to an acceptor group of the protein or to the aqueous phase is mechanistically required, or whether a different pathway to the same active molecular state is possible. The results with the *all-trans*-retinal-regenerated form of the mutant E113Q suggested that net proton transfer from the Schiff base to opsin may not be an essential trigger for R*

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[1] Abbreviations: G protein, guanine nucleotide-binding regulatory protein; MII, metarhodopsin II; R*, photoactivated rhodopsin.

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formation. However, under these conditions, R* forms independently of light, and the protonation state that a putative Schiff base proton acceptor might adopt during chromophore regeneration is not known. Likewise, constitutively active mutants possess an active conformation but presumably lack the switch mechanism for activation. Therefore, to assess the role of net deprotonation of the Schiff base using mutational modification of the Schiff base environment, it is essential to study a system that can still be triggered by light.

In this study, we measure the ability of mutant pigment E113Q regenerated with 11-*cis*-retinal to activate transducin as a function of the wavelength of photoactivation. We show that the 380-nm form of rhodopsin mutant E113Q can be activated by UV light to catalyze nucleotide exchange by transducin. The quantum efficiency of photoactivation of the UV-absorbing pigment by 380-nm light is comparable to that of the visible-absorbing form illuminated by 500-nm light. This is the first direct demonstration that a protonated Schiff base in the unphotolyzed state is not a prerequisite for light activation of a visual pigment. The UV-absorbing rhodopsin mutant described may be considered to be a model of some naturally occurring UV-photoreceptor pigments.

MATERIALS AND METHODS

Materials. Sources of materials have been previously reported (Franke et al., 1992; Sakmar et al., 1989, 1991). Nucleotides were purchased from Boehringer Mannheim.

Preparation of Recombinant Pigments and Bovine Transducin. Mutant E113Q was constructed in a synthetic gene (Ferretti et al., 1986) as previously described (Sakmar et al., 1989). Recombinant opsin and mutants were expressed in COS cells (Oprian et al., 1987), regenerated with 11-*cis*-retinal, and purified by an immunoaffinity procedure in dodecyl maltoside detergent buffer (Franke et al., 1992; Sakmar et al., 1989; Zvyaga et al., 1993). Transducin was purified from bovine rod outer segments essentially as described (Fung et al., 1981).

UV-Visible Absorption Spectroscopy. Spectroscopy was performed on a λ-19 Perkin-Elmer spectrophotometer at 10 °C in a cuvette with a 1-cm path length (Chan et al., 1992; Zvyaga et al., 1993). Pigment concentrations were determined on the basis of the absorbance at the visible λ_{max} value (Sakmar et al., 1989).

Controlled Photoactivation of Pigments. Photoactivation efficiency was tested as a function of wavelength at 20-nm increments ranging from 340 to 580 nm. A mixture of recombinant pigment (5–30 nM) and transducin (110 nM) in detergent buffer (10 mM Tris–maleate, pH 6.2, 100 mM NaCl, 2 mM $MgCl_2$, and 0.01% dodecyl maltoside) was kept on ice in the dark. For each wavelength tested, a 1.5-mL aliquot of the mixture was transferred to a quartz cuvette (4 mm × 10 mm cross section). The solution was thermostated to 10 °C, continuously stirred at maximal speed, and allowed to equilibrate in the dark in the sample compartment of a specially modified SPEX-Fluorolog II spectrofluorometer used for monitoring transducin activation (see below). Samples were illuminated using the excitation optics of the instrument. Thus, any handling of samples between light activation and the subsequent fluorescence assay was avoided. The excitation wavelength and the duration of illumination were defined by use of the "time based scan" option of the instrument software. A KG2 300-nm long-pass filter (Melles-Griot) was positioned in front of the mirror that reflects the excitation light into the cuvette in order to block any second-order refracted light.

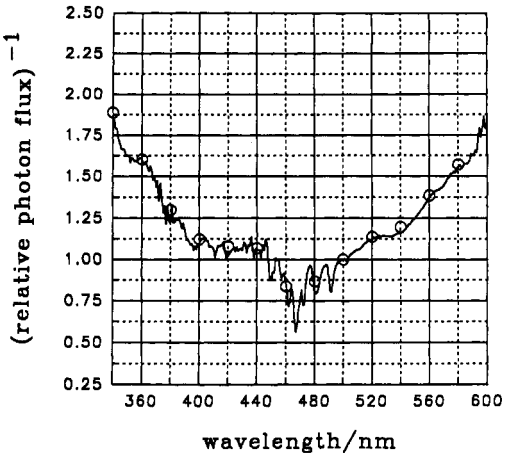

FIGURE 1: Plot of photoactivation correction factor (inverse of relative photon flux reaching the sample) as a function of wavelength. The curve was obtained by measuring the ratio between the absorbance spectrum and the excitation spectrum of rhodamine B, both measured with a 2-nm band width and normalizing to unity for 500-nm light. A wider band width of 15 nm was employed for the photoactivation of the pigments. This was taken into account as described under Materials and Methods. Final corrected values are given by open circles.

The intensity of the excitation beam was attenuated by mounting two Glan–Thompson polarizers back-to-back in front of the sample cuvette stage. One polarizer was kept in a fixed position. Setting the second polarizer to 90° resulted in a transmittance low enough to prevent complete photoconversion of rhodopsin to R* during light activation. The angle of 90° refers to an arbitrarily chosen fixed axis within the instrument. The actual angle between the polarization planes of both polarizers in this position was approximately 80°. The light intensity thus adjusted allowed an illumination time long enough for efficient mixing of the solution. This was important because only part of the total sample volume lies within the excitation beam. To allow illumination of as large a volume as possible, a slit width of 4 mm was chosen for excitation. The corresponding band width of 15 nm was small enough to record an action spectrum with a desired "spectral resolution" of 20 nm. Each sample was exposed to light for 150 s at a given wavelength. Under these conditions, illumination with 500-nm light for 150 s converted about 50% of rhodopsin to R*.

Correction for Different Relative Illumination Intensities. The excitation spectrum of rhodamine B was measured with a band width of 2 nm using the same modified excitation optics used for light activation (polarizers and KG2 filter), and was ratioed against the absorbance spectrum of the dye measured with the same band width (and maximal absorbance below 0.15 absorbance unit). This resulted in a measure of the relative quantum flux reaching the sample (Figure 1). The effect of the larger band width used in the actual light activation of rhodopsin was accounted for by convoluting the relative quantum flux spectrum with a triangular function of 15-nm "full-width at half-height" that approximated the resolution of the monochromator grating. Such a correction determines the relative quantum flux reaching the sample by taking the contribution of the wavelength that is maximally transmitted through the monochromator as unity. The contribution of neighboring wavelengths was linearly decreased by the convolution with the triangular function. This yields a weighing factor of 0.5 for wavelengths 7.5 nm longer or shorter than the selected one and neglects any intensity from light of wavelengths differing by more than 15 nm from the monochromator setting. Therefore, the mathematical treat-

ment simulated the effect which a triangular slit function typical of a grating monochromator exerts on the spectral composition at any given monochromator setting with a 15-nm band width.

The described correction of the relative illumination intensity as a function of wavelength affects mainly the value of 480-nm light intensity since at this position the Xe arc lamp spectrum is more structured as compared with other wavelengths chosen for pigment photoactivation. The action spectra thus obtained were not further corrected for the different rise times of the nonlinear time courses of R* formation during the controlled illuminations.

Fluorescence Assay of Transducin Activation. The fluorsesecence assay employed was similar to those described previously (Antonny et al., 1991, 1992; Fahmy & Sakmar, 1993; Higashijima et al., 1987; Guy et al., 1990; Phillips & Cerione, 1988). A dark period of 150 s followed each light activation. The movable polarizer was set to 0° during this time to increase the excitation beam transmittance to allow efficient fluorescence exitation at 290 nm. Thus, during the fluorescence assay, the KG2 filter was the attenuating component in the excitation optics and warranted a low rhodopsin activation rate during measurement of the nucleotide-induced increase of transducin α-subunit fluorescence. The slit width of the excitation monochromator was not changed in order to maintain identical conditions for the next light activation. A "time based scan" was started, and the emission signal was recorded at 345 nm with 15-nm band width. After 30 s, 50 μL of GTPγS solution was injected into the cuvette through a 1-mm opening in the lid of the sample compartment, and the time course of the fluorescence was recorded for another 5–10 min. In the case of rhodopsin, an additional saturating illumination was carried out 2 min after nucleotide injection to completely convert rhodopsin to R* (Figure 2). The 543-nm light of a He,Ne laser was guided through a fiber optic bundle into the sample compartment, and the tip of the bundle was positioned just above the top of the cuvette. Since the emission double monochromator blocks any stray light from monochromatic activation, the recording of the fluorescence signal could be done simultaneously with sample illumination.

Under the conditions of the assay, the lifetime of the photoactivated pigment was on the order of hours. Therefore, decay of R* during the time course of the experiment could be neglected.

RESULTS

The primary aim of this work was to determine whether or not the UV-absorbing form of rhodopsin mutant E113Q, which contains an unprotonated Schiff base, could be activated by UV illumination. Activation was defined as the ability of photolyzed pigment to catalyze guanine nucleotide exchange by transducin. Transducin nucleotide exchange was monitored by measuring the increase of the intrinsic tryptophan fluorescence of the α-subunit of transducin as a function of time. Transducin α-subunit intrinsic fluorescence increases markedly upon GTP (or GTPγS) binding (Fahmy & Sakmar, 1993; Guy et al., 1990; Phillips & Cerione, 1988).

Mutant pigment E113Q was purified at pH 6.2 so that approximately equal amounts of protonated and unprotonated Schiff base species were present. Illuminations of separate identical samples of E113Q were carried out with either 500-nm or 380-nm light so that the wavelength of illumination roughly corresponded to the λ_{max} value of the protonated or unprotonated Schiff base form, respectively. The results of

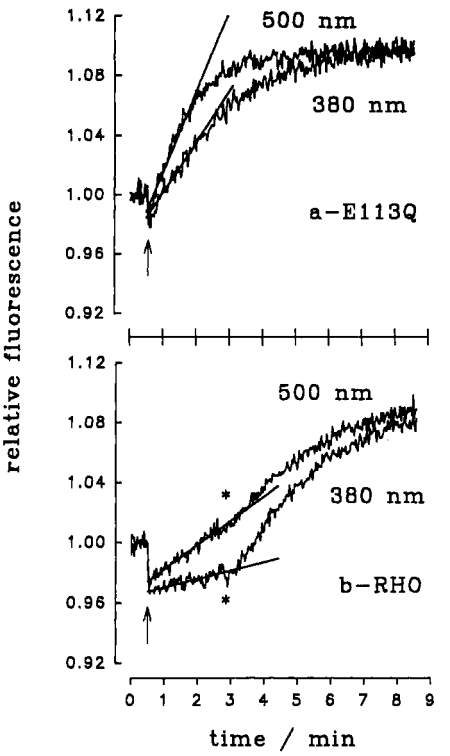

FIGURE 2: Time courses of transducin tryptophan fluorescence. The efficiency of transducin activation by a recombinant pigment was dependent on the wavelength of pigment photoactivation. Each sample, consisting of purified pigment and transducin (110 nM), was illuminated for 150 s with light of the indicated wavelength (380 or 500 nm). After a dark period of another 150 s, the sample was excited with 290-nm light, and the intrinsic fluorescence signal was recorded. GTPγS was added 30 s later as indicated by the arrow. The slope of the linear regression line indicated on the fluorescence trace defines the efficiency of transducin activation by photoactivated pigment. The traces are normalized to the total fluorescence increase reached after complete nucleotide uptake. Measurements were done at 10 °C. (a) The results of two separate experiments are shown superimposed for mutant pigment E113Q activated by 380-nm light or by 500-nm light (the concentration of 500-nm species was 16.5 nM). (b) The results of two separate experiments are shown superimposed for rhodopsin activated by 380-nm light or by 500-nm light (the concentration of pigment was 5.7 nM). A second illumination with intense green light (543 nm) was performed 2 min after nucleotide addition (*) to show that the preilluminations were not saturating.

E113Q pigment photoactivation at these two illumination wavelengths are shown in Figure 2a. The slope of the fluorescence rise after addition of nucleotide is a direct measure of the concentration of active pigment produced by the controlled illumination. Linear regression lines for data points corresponding to 30% of the total fluorescence increase after nucleotide addition (30–120 s) are shown. It is clear that significant pigment photoactivation occurred at both 500-nm and 380-nm illumination. The ratio of the transducin activation rate after 380-nm illumination to that obtained with 500-nm illumination was 0.64. The traces shown in Figure 2a are uncorrected data and do not account for different light intensities of illumination. However, the relative quantum fluxes of the 380- and 500-nm light reaching the sample are similar (within 25%) as shown in Figure 1.

As a control, recombinant rhodopsin was prepared in parallel with mutant E113Q and subjected to the same experiment (Figure 2b). Linear regression lines for data points corresponding to the first 2 min after nucleotide addition (30–150 s) are shown. It is clear that much lower pigment photoactivation occurred at 380-nm illumination when compared with that at 500 nm, the λ_{max} value of the pigment. The ratio of

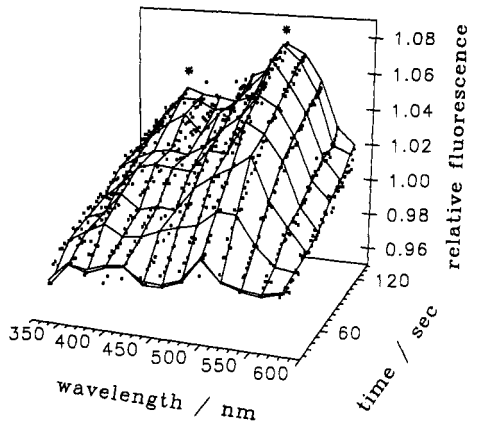

FIGURE 3: Time courses of transducin tryptophan fluorescence as a function of the wavelength of mutant pigment E113Q photoactivation. A set of 13 experiments analogous to that shown in Figure 2a is presented. The traces as shown are not corrected for different relative intensities of activating light. Fluorescence maxima are noted at about 380 and 500 nm (*).

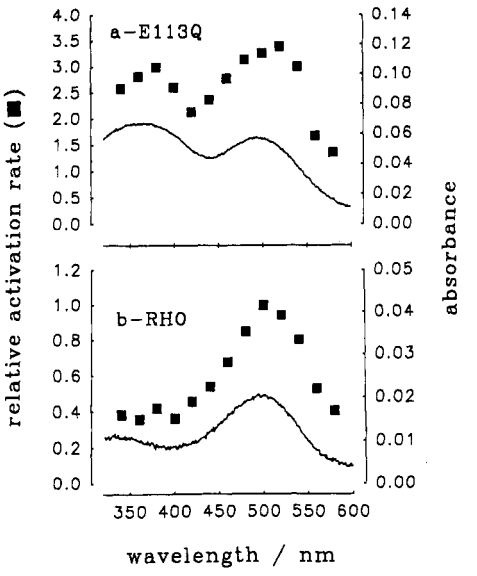

FIGURE 4: Quantum flux corrected action spectra of R* formation. Symbols represent the relative transducin activation rates as calculated from the slopes of linear regressions through data points of fluorescence time courses as shown in Figure 2. The values obtained were multiplied by the correction factors plotted in Figure 1. Solid lines show the absorption spectra of the recombinant pigment preparations before they were added to the transducin solution in which they were diluted 60-fold. (a) Mutant pigment E113Q; (b) rhodopsin. For each pigment, the wavelength action spectrum closely resembles the absorption spectrum. The activation rate of transducin by the 500-nm form of the mutant is 3.3 times larger than that of rhodopsin mainly due to the higher concentration of pigment used (see Results).

the transducin activation rate after 380-nm illumination to that obtained with 500-nm illumination was 0.33. The rhodopsin control samples were additionally illuminated with saturating 543-nm light from a He,Ne laser approximately 2.5 min after nucleotide addition (a complete bleach of the remaining 500-nm absorbance was achieved within 15 s, data not shown). The corresponding acceleration of the fluorescence increase demonstrates that the controlled preillumination with light of defined wavelength did not completely convert rhodopsin to R*. This control was necessary to show that the preillumination limited the activation rate so that an action spectrum could eventually be calculated from the data.

A comparison of the respective linearized fluorescence signals in Figure 2 reveals that the mutant E113Q was activated by 380-nm illumination to a larger degree than rhodopsin by 500-nm illumination. Two reasons account for the higher level of maximal photoactivation by mutant E113Q. First, a higher concentration of mutant pigment was used. Second, the 290-nm excitation light caused a slow activation of the UV form of E113Q during the fluorescence recording. This background activity does not contribute to the rates measured with rhodopsin. In addition, the mutant E113Q was found in earlier studies to be slightly more active than rhodopsin (Sakmar et al., 1989). This hyperactivity was also apparent under the particular conditions employed in the present study. The key comparison in Figure 2 is the higher UV light-dependent transducin activation rate relative to the rate after 500-nm light activation of the mutant E113Q as compared with rhodopsin. This finding shows that an R*-like species fully capable of transducin activation can be formed by direct photoconversion of a pigment containing an unprotonated Schiff base.

A set of transducin activation time courses as a function of the wavelength of preillumination for the mutant E113Q is shown in Figure 3. Local maxima of transducin activation rates are present at about 380 and 500 nm of photoactivating light. This data collection is not corrected for any relative differences in light intensity of illumination. However, the ensemble of traces clearly demonstrates light-dependent activation of transducin by the mutant E113Q irrespective of the protonation state of the Schiff base in the unphotolyzed pigment. A corresponding set of data for rhodopsin exhibited a single maximum at 500 nm (not shown). By calculation of the slopes of the fluorescence rises after nucleotide addition and by correction for relative quantum flux, plots of the action spectra of mutant E113Q and rhodopsin are obtained. The relative transducin activation rates as a function of photoactivation wavelength are plotted with the absorption spectrum of each pigment under the conditions of the assay in Figure 4. In both cases, the obtained spectral transducin activation efficiency is essentially proportional to the UV–visible absorption of the respective pigment. This result for rhodopsin is completely expected, but is presented as the control for the mutant pigment E113Q. The absorption of the 380-nm species of the mutant is slightly overestimated relative to the 500-nm form due to light scatter in the absorption spectrum. The results for mutant E113Q prove that photoactivation of the UV-absorbing form of the pigment activates transducin, and that its activation efficiency is comparable to that of the visible-absorbing form.

DISCUSSION

We have shown that the rhodopsin mutant E113Q can be photoactivated irrespective of the protonation state of the Schiff base. Therefore, two light-dependent activation processes can be characterized in the mutant. Both involve 11-cis to all-trans photoisomerization of the chromophore as judged by the fact that the 11-*cis*-retinal-regenerated visible and UV-absorbing forms of the mutant do not activate transducin in the dark in contrast to the all-trans-regenerated mutant opsin (Sakmar et al., 1989). However, only the photoactivation of the form containing the protonated Schiff base parallels spectral and chemical changes described for native rhodopsin, i.e., deprotonation of the Schiff base and a concomitant shift of the absorption maximum from 500 to 380 nm (Sakmar et al., 1989). The UV-absorbing form of the mutant does not exhibit either of these changes. Nevertheless, the active conformation of the mutant opsin is achieved. This demonstrates that the light-induced net loss of the Schiff base proton in the 500-nm species of mutant E113Q is not a necessary

trigger for the formation of an R*-like conformation of the mutant opsin. In the following discussion, the possible implications of this result for the mechanistic role of the Schiff base proton in native rhodopsin will be considered separately from the implications for models dealing with chromophore isomerization and energetics of R* formation.

Implications for Chromophore Isomerization Models and Thermal Photoreceptor Dark Noise. The UV-absorbing pigment activates transducin with comparable efficiency to that of the visible-absorbing pigment, as shown by the similarity between the action spectrum and the absorption spectrum of mutant pigment E113Q (Figure 4). Since the MII-like intermediates of the mutant contain an unprotonated Schiff base and an *all-trans*-retinal chromophore, we assume that the active conformations obtained by photoactivation of the 500-nm and the 380-nm forms are identical. On this basis, the comparable relative nucleotide exchange rates obtained after photoactivation of the UV- and visible-absorbing species suggest that the quantum efficiencies of both forms of the pigment must be similar.

The similarity of quantum efficiencies for activation of the protonated and unprotonated Schiff base forms is striking, since a lower quantum yield for photoisomerization of the unprotonated Schiff base might be expected due to less π-electron delocalization in the ground state. Semiempirical calculations and experiments on protonated and unprotonated Schiff base model compounds have shown that photoisomerization occurs in the $^1\pi$–π^* state of the chromophore and is facilitated by mixing or reversal of level-ordering of the "$^1Bu^+$" and "$^1Ag^-$" states [Birge et al., 1987; Palmer et al., 1982; for a review, see Birge (1990)]. Schiff base protonation causes this mixing to be relatively independent of the other physical parameters of the chromophore environment (Becker & Freedman, 1985; Freedman & Becker, 1986). In contrast, an unprotonated Schiff base of 11-*cis*-retinal renders the quantum yield for photoisomerization to the all-trans form very sensitive to the polarizability of the chromophore environment and to the presence of protic molecules. The salient result of these studies is that the quantum yield for cis–trans isomerization of an unprotonated Schiff base of 11-*cis*-retinal can be essentially the same or even greater than that of a protonated Schiff base, provided that the chromophore environment allows H-bonding, or has high polarizability. Obviously, these requirements are met in the case of the UV-absorbing mutant pigment E113Q.

In addition to state mixing or reversed-level-ordering of ionic and covalent $^1\pi$–π^* states, electrostatic repulsion between the Schiff base of the chromophore in the excited singlet state and the negatively charged counterion has been suggested to promote photoisomerization in rhodopsin (Birge, 1990). Notwithstanding the possible importance of this mechanism in rhodopsin, it is not a prerequisite for photoisomerization *per se*, because in the mutant E113Q the counterion has been neutralized by the amino acid replacement.

The abolishment of strong electrostatic interaction between the Schiff base and the mutant opsin also bears on the mechanism of R* formation during the thermal relaxation after photoisomerization. On the order of 10–15 kcal out of a total of ~35 kcal (Cooper, 1979; Schick et al., 1987) is stored as electrostatic energy in bathorhodopsin after photoisomerization due to the concomitant charge separation of the protonated Schiff base from the counterion (Birge & Hubbard, 1980, 1981). Other investigators emphasize larger contributions to energy storage from charge separation as compared to conformational energy (Honig et al., 1979; Rosenfeld et al., 1977). The transducin activation by the UV form of the mutant E113Q pigment demonstrates that an active conformation similar or identical to R* of rhodopsin can be attained without participation of this particular electrostatic driving force.

Photoisomerization of the unprotonated Schiff base chromophore in the mutant E113Q proceeds with quantum efficiency similar to that of the protonated Schiff base chromophore, and stores enough conformational energy to drive thermal relaxation to an R*-like state that activates transducin. This contradicts an earlier suggestion that a protonated Schiff base linkage is required for all visual pigments including those with absorption maxima in the UV (Pande et al., 1987). Therefore, the physiological role of the protonation state of the Schiff base has to be reconsidered (see below).

Since it has been shown that a net proton transfer reaction of the Schiff base need not accompany the photoactivation of the mutant pigment, the question arises as to whether chromophore isomerization and Schiff base deprotonation can occur independently in the activation process of native rhodopsin. In particular, the results suggest that it should be possible to activate native rhodopsin by chromophore isomerization alone if the Schiff base proton were transferred to an adequate acceptor group prior to the isomerization. In this hypothetical activation process, chromophore isomerization and Schiff base deprotonation would be decoupled from each other and would proceed in the inverse order as compared with the normally observed photoactivation. Such a prediction is based on the assumption that Glu^{113} is the proton acceptor for the Schiff base deprotonation in native rhodopsin. Therefore, proton transfer to Glu^{113} prior to chromophore isomerization would create a chromophore-binding site which lacks the electrostatic protein chromophore interactions as is the case with the UV-absorbing from of mutant E113Q. In analogy to the mutant UV pigment, chromophore isomerization within such a rhodopsin species would be expected to produce an R*-like conformation without a concomitant net proton transfer reaction.

It has been hypothesized that such a process may be responsible for the thermally activated dark noise in photoreceptor cells (Birge, 1990). The rationale for this suggestion was the similarity between the activation energy determined from electrical dark noise in toad retinal rod outer segments and *Limulus* photoreceptor cells and the activation energy for thermal isomerization of the unprotonated Schiff base of 11-*cis*-retinal (Baylor et al., 1980; Barlow & Kaplan, 1989; Barlow & Silbaugh, 1989). The energy barriers for these processes are all on the order of 20–30 kcal/mol, considerably less than the estimated thermal activation energy of 45 kcal/mol for isomerization of the protonated Schiff base (Birge, 1990). The suggested participation of small amounts of unprotonated Schiff base species in the generation of thermal receptor noise is supported by the present data since isomerization of an unprotonated retinal Schiff base chromophore is indeed able to switch from an inactive to an active opsin conformation in mutant E113Q. Thermally-induced deprotonation of the Schiff base without subsequent thermal isomerization does not seem to be sufficient to cause receptor dark noise. In such a case, the UV form of the mutant pigment would be expected to activate transducin in the dark, which is not observed.

Implications for the Role of the Schiff Base Proton in Photoreception. The data show that the presence of a protonated Schiff base is not a prerequisite for the light-

dependent formation of R* in visual pigments. The protonated Schiff base may function primarily to provide spectral tuning to the range of wavelengths represented by the family of rhodopsin and iodopsin photoreceptors. Only in the protonated Schiff base state can the chromophore absorption be widely regulated in the visible range, depending on the electrostatic environment of the retinal in addition to other factors (Blatz et al., 1972; Honig et al., 1976, 1979; Irving et al., 1969, 1970, Kropf & Hubbard, 1958; Mathies & Stryer, 1976). Assuming that breakage of the salt bridge between the Schiff base and the counterion Glu^{113} is important for activation of rhodopsin (Cohen et al., 1992; Robinson et al., 1991), the Schiff base proton used for tuning of the ground-state absorption has to be removed after photoisomerization, presumably by net proton transfer from the Schiff base to Glu^{113}. Therefore, deprotonation of the Schiff base is likely to be a prerequisite for R* formation in photoreceptors tuned to the 400–600-nm range because it provides a mechanism to neutralize the respective counterions which correspond to Glu^{113} in bovine rhodopsin.

Near-UV absorption by a retinal Schiff base pigment can be achieved in its unprotonated state. In such a pigment, a 380-nm ground-state absorption peak results from a chromophore that is relatively unaffected by the protein environment. However, protein–chromophore interactions are important in allowing efficient photoisomerization as discussed above. In mutant pigment E113Q, a high quantum efficiency is displayed, and the active conformation is achieved by photoisomerization without net transfer of a Schiff base proton. We assume that the determinants of the active state, R*, in the mutant are nevertheless the same as in rhodopsin. One determinant of R* is a neutral amino acid side chain at position 113, which is occupied by glutamine in the mutant. Therefore, there is no need for a neutralizing mechanism, and light activation can be triggered by isomerization alone. Whether or not a transient short-lived protonation of the Schiff base occurs during this process cannot be answered by this set of experiments.

As discussed above, the general features of the photoactivated state of the mutant E113Q seem to be unaffected by the initial protonation state of the Schiff base. Therefore, a mechanism that couples chromophore isomerization to "activating" conformational changes on the cytoplasmic surface must exist independently of the initial Schiff base protonation state. UV photoreception apparently can use the same activating machinery as long-wavelength photoreception by employing a neutral amino acid at the position homologous to the counterion in rhodopsin.

The discussed features of the UV form of the mutant pigment E113Q make it a suitable model system for naturally occurring UV photoreception. UV sensitivity has been demonstrated in a variety of invertebrate organimsms (Stark & Tan, 1982), and sequence information is available for several pigments. Interestingly, the amino acid sequences of the Drosophila R7 pigments reveal a phenylalanine at the position homologous to Glu^{113} in bovine rhodopsin (Montell et al., 1987; Zuker et al., 1987). Vertebrate animals such as rodents and fishes have also been reported to show UV visual photosensitivity (Harôsi & Hashimoto, 1983; Hawryshyn, 1991; Jacobs et al., 1991). The deduced amino acid sequence of the zebrafish UV-absorbing pigment shows a glutamic acid residue at position 113, but interestingly has a lysine residue, which is not found in visible-absorbing pigments, at position 126 (Robinson et al., 1993). In contrast to the role of amino acid side chains for spectral tuning in the visible range in protonated Schiff base pigments (Chan et al., 1992; Merbs & Nathans, 1992; Neitz et al., 1991), we expect that polarizable side chains near the chromophore in UV-absorbing pigments should be present to facilitate mixing between the "$^1Ag^-$" and "$^1Bu^+$" states in the excited $^1\pi$–π^* state of the unprotonated Schiff base chromophore.

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REFERENCES

Antonny, B., & Chabre, M. (1992) *J. Biol. Chem. 267*, 6710–6718.
Antonny, B., Chardin, P., Roux, M., & Chabre, M. (1991) *Biochemistry 30*, 8287–8295.
Barlow, R. B., Jr., & Kaplan, E. (1989) *Biol. Bull. 177*, 323.
Barlow, R. B., Jr., & Stilbaugh, T. H. (1989) *Invest. Ophthalmol. Visual Sci., Suppl. 30*, 61.
Baylor, D. A., Matthews, G., & Yau, K.-W. (1980) *J. Physiol. 309*, 591–621.
Becker, R. S., & Freedman, K. A. (1985) *J. Am. Chem. Soc. 107*, 1477–1485.
Birge, R. R. (1990) *Biochim. Biophys. Acta 1016*, 293–327.
Birge, R. R., & Hubbard, L. M. (1980) *J. Am. Chem. Soc. 102*, 2195–2204.
Birge, R. R., & Hubbard, L. M. (1981) *Biophys. J. 34*, 517–534.
Birge, R. R., Murray, L. P., Zidovetzki, R., & Knapp, H. M. (1987) *J. Am. Chem. Soc. 109*, 2090–2101.
Blatz, P. E., Mohler, J. H., & Navangul, H. V. (1972) *Biochemistry 11*, 848–855.
Chan, T., Lee, M., & Sakmar, T. P. (1992) *J. Biol. Chem. 267*, 9478–9480.
Cohen, G. B., Oprian, D. D., & Robinson, P. R. (1992) *Biochemistry 31*, 12592–12601.
Cooper, A. (1979) *Nature 282*, 531–533.
Doukas, A. G., Aton, B., Callender, R. H., & Ebrey, T. G. (1978) *Biochemistry 17*, 2430–2435.
Dratz, E. A., & Hargrave, P. A. (1983) *Trends Biochem. Sci. 8*, 128–131.
Fahmy, K., & Sakmar, T. P. (1993) *Biochemistry 32*, 7229–7236.
Ferretti, L., Karnik, S. S., Khorana, H. G., Nassal, M., & Oprian, D. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 599–603.
Franke, R. R., Sakmar, T. P., Graham, R. M., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 14767–14774.
Freedman, K. A., & Becker, R. S. (1986) *J. Am. Chem. Soc. 108*, 1245–1251.
Fung, B. K.-K., Hurley, J. B., & Stryer, L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 75*, 152–156.
Guy, P. M., Koland, J. G., & Cerione, R. A. (1990) *Biochemistry 29*, 6954–6964.
Hárosi, F. I., & Hashimoto, Y. (1983) *Science 222*, 1021–1023.
Hawryshyn, C. W. (1991) *Visual Neurosci. 6*, 293–301.
Higashijima, T., Ferguson, K. M., Smigel, M. D., & Gilman, A. G. (1987) *J. Biol. Chem. 262*, 757–761.
Honig, B., Greenberg, A. D., Dinur, U., & Ebrey, T. G. (1976) *Biochemistry 15*, 4593–4599.
Honig, B., Ebrey, T. G., Callender, R. H., Dinur, U., & Ottolenghi, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2503–2507.
Irving, C. S., Byers, G. W., & Leermakers, P. A. (1969) *J. Am. Chem. Soc. 91*, 2141–2143.
Irving, C. S., Byers, G. W., & Leermakers, P. A. (1970) *Biochemistry 9*, 858–864.
Jacobs, G. H., Neitz, J., & Deegan, J. F. (1991) *Nature 353*, 655–656.
Khorana, H. G. (1992) *J. Biol. Chem. 267*, 1–4.
Kropf, A., & Hubbard, R. (1958) *Ann. N.Y. Acad. Sci. 74*, 266–280.

Liebman, P. A., Parker, K. R., & Dratz, E. A. (1987) *Annu. Rev. Physiol. 49*, 765–791.
Lin, S. W., Sakmar, T. P., Franke, R. R., Khorana, H. G., & Mathies, R. A. (1992) *Biochemistry 31*, 5105–5111.
Longstaff, C., Calhoon, R. D., & Rando, R. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4209–4213.
Mathies, R. A., & Streyer, L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2169–2173.
Merbs, S. L., & Nathans, J. (1992) *Science 258*, 464–466.
Montell, C., Jones, K., Zuker, C. S., & Rubin, G. M. (1987) *J. Neurosci. 7*, 1558–1566.
Nathans, J. (1990) *Biochemistry 29*, 9746–9752.
Nathans, J. (1992) *Biochemistry 31*, 4923–4930.
Neitz, M., Neitz, J., & Jacobs, G. H. (1991) *Science 252*, 971–974.
Oprian, D. D., Molday, R. S., Kaufman, R. J., & Khorana, H. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8874–8878.
Oseroff, A. R., & Callender, R. H. (1974) *Biochemistry 13*, 4243–4248.
Palmer, B., Jumper, B., Hagan, W., Baum, J. C., & Christensen, R. L. (1982) *J. Am. Chem. Soc. 104*, 6907–6913.
Pande, C., Deng, H., Rath, P., Callender, R. H., & Schwemer, J. (1987) *Biochemistry 26*, 7426–7430.
Phillips, W. J., & Cerione, R. A. (1988) *J. Biol. Chem. 263*, 15498–15505.
Robinson, J., Schmitt, E. A., Hárosi, F. I., Reece, R. J., & Dowling, J. E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6009–6012.
Robinson, P. R., Cohen, G. B., Zhukovsky, E. A., & Oprian, D. D. (1992) *Neuron 9*, 719–725.
Rosenfeld, T., Honig, B., Ottolenghi, M., Hurley, J. B., & Ebrey, T. G. (1977) *Pure Appl. Chem. 49*, 341–351.
Sakmar, T. P., Franke, R. R., & Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8309–8313.
Sakmar, T. P., Franke, R. R., & Khorana, H. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3079–3083.
Schick, G. A., Cooper, T. M., Holloway, R. A., Murray, L. P., & Birge, R. R. (1987) *Biochemistry 26*, 2556–2562.
Stark, W. S., & Tan, K. E. W. P. (1982) *Photochem. Photobiol. 36*, 371–380.
Steinberg, G. M., Ottolenghi, M., & Sheves, M. (1993) *Biophys. J.* (in press).
Zhukovsky, E. A., & Oprian, D. D. (1989) *Science 246*, 928–930.
Zuker, C. S., Montell, C., Jones, K., Laverty, T., & Rubin, G. M. (1987) *J. Neurosci. 7*, 1550–1557.
Zvyaga, T. A., Min, K. C., Beck, M., & Sakmar, T. P. (1993) *J. Biol. Chem. 268*, 4661–4667.