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On the molecular origins of thermal noise in vertebrate and invertebrate photoreceptors

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Abstract

Retinal photoreceptors generate discrete electrical events in the dark indistinguishable from those evoked by light and the resulting dark signals limit visual sensitivity at low levels of illumination. The random spontaneous events are strongly temperature dependent and in both vertebrate and invertebrate photoreceptors require activation energies usually in the range of 23 to 28 kcal mol^{-1}. Recent molecular orbital studies and pH experiments on horseshoe crabs (*Limulus*) suggest that the thermal isomerization of a relatively unstable form of rhodopsin, one in which the Schiff-base linkage between the chromophore and protein is unprotonated, is responsible for thermal noise. This mechanism is examined in detail and compared to other literature models for photoreceptor noise. We conclude that this two-step process is likely to be the principal source of noise in all vertebrate and invertebrate photoreceptors. This model predicts that the rate of photoreceptor noise will scale in proportion to $10^{-\xi}$, where ξ is the pK_a of the Schiff base proton on the retinyl chromophore. Nature minimizes photoreceptor noise by selecting a binding site geometry which shifts the pK_a of the Schiff base proton to > 16, a value significantly larger than the pK_a of the chromophore in bacteriorhodopsin ($pK_a \approx 13$) or model protonated Schiff bases in solution ($pK_a \approx 7$).

Keywords: Photoreceptor noise; Rhodopsin; Visual sensitivity

1. Introduction

The human visual system can detect faint stars at night and distinguish objects in direct sunlight for an effective operating range of about 10 log units of light intensity. At high light intensities the key limitation of visual function is the bleaching of photoreceptor pigments. At low light levels the key limiting factor is noise in the photoreceptors. We can reliably detect pulses of light that send roughly 100 photons through the pupil and activate approximately 10–20 rhodopsin molecules in as many rod photoreceptors. We see such dim flashes of light against a background of noise caused by each photoreceptor eliciting false signals ("noise") which are indistinguishable from the signals triggered by single photon absorptions [1]. The origin of this photoreceptor noise remains a subject of debate and a number of recent articles have examined this subject from di-

Abbreviations: R, rhodopsin; R^* or $R^*_{h\nu}$, photoactivated rhodopsin; R^{**} or $R^*_{thermal}$, thermally activated rhodopsin; R_d, rhodopsin with a deprotonated chromophore; R_{FC}, rhodopsin activated into the vertically excited (Franck–Condon) state; B, bathorhodopsin; B_d, bathorhodopsin with a deprotonated chromophore; An, arrestin; PDE, phosphodiesterase; PP, pyrophosphate; $T_{\alpha\beta\gamma}$, transducin with α, β and γ subunits; INDO, intermediate neglect of differential overlap; MNDO, modified neglect of differential overlap; PSDCI, partial single and double configuration interaction.

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verse perspectives [2–9]. In this review we examine the origin of photoreceptor noise and demonstrate that the available evidence supports a two-step molecular process inside the protein binding site of rhodopsin as the primary source. We also discuss how nature has optimized the active site of rhodopsin

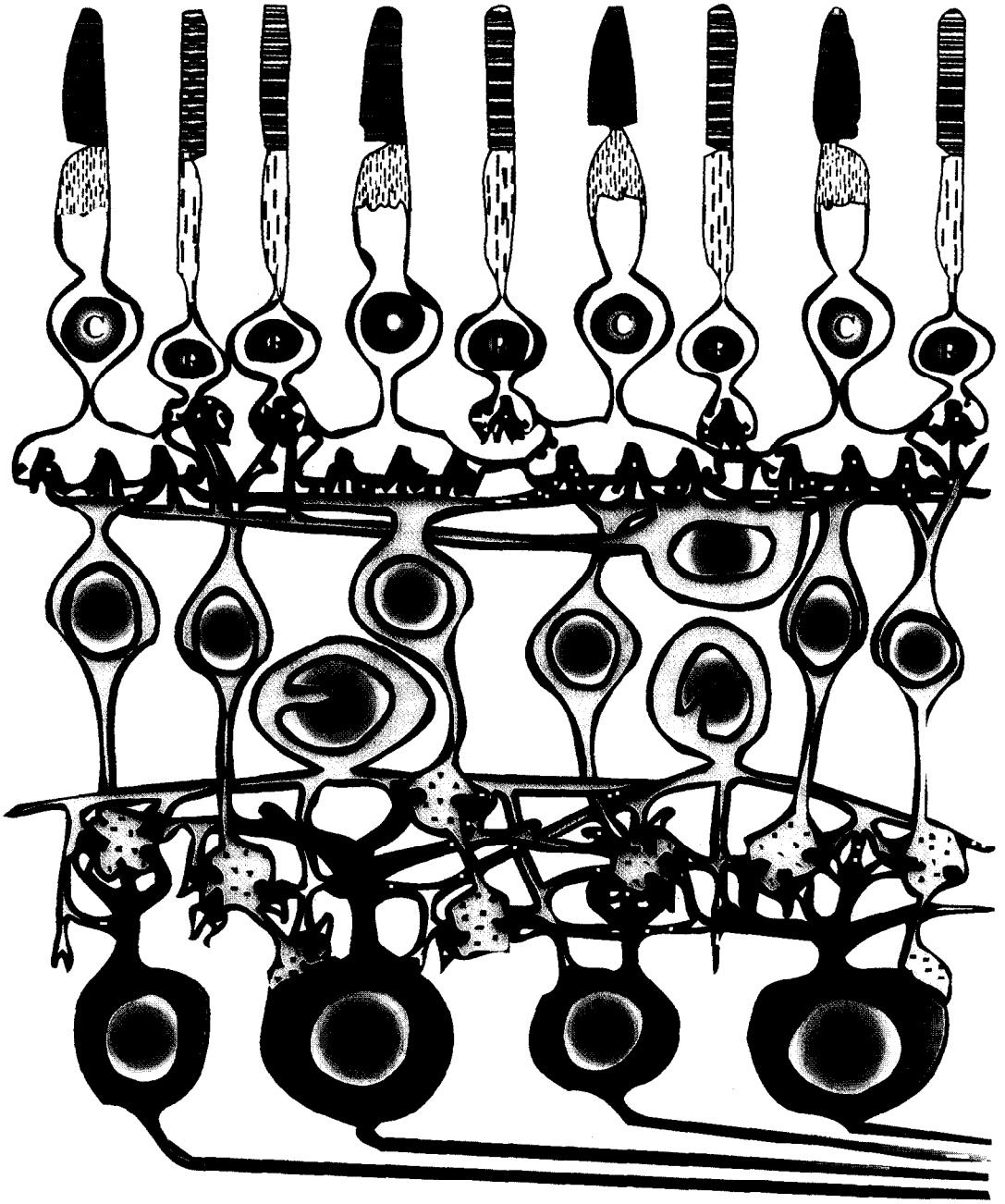


Fig. 1. Schematic diagram of the neural components in the primate retina based on the electron microscopy studies of Dowling and Boycott [22]. The symbols are: R, rod; C, cone; MB, midget bipolar nerve cell; RB, rod bipolar; FB, flat bipolar; H, horizontal cell; A, amacrine cell; MG, midget ganglion; DG, diffuse ganglion. Light enters the retina from the bottom of the figure and must pass through the neural complex prior to striking the photoreceptor cells.

to minimize the thermal reactions that are responsible for generating the false signals.

2. Rhodopsin is the source of photoreceptor noise

Although the origin of photoreceptor thermal noise may remain a subject of debate, investigators agree that thermal and light activated photoreceptor signals are identical with respect to intensity and temporal profile [1–5,9,10]. Thus, it is important to understand the nature of the light activation and amplification process. The thermal noise phenomenon must involve either a side reaction or corruption of this process prior to amplification or a side reaction that undergoes nearly identical amplification. The light absorbing chromophore in the visual pigment rhodopsin is 11-*cis* retinal covalently bound to the opsin protein via a protonated Schiff base linkage to lysine 296 [6]. The primary photochemical event is an 11-*cis* to 11-*trans* photoisomerization of the protonated Schiff base chromophore [11,12] and recent studies indicate that it involves photochemistry along a barrier-less excited state potential surface [6,13–17]. A series of dark reactions then occur which ultimately deprotonate the chromophore and activate the protein. The activated protein initiates a complex biochemical process that hyperpolarizes the plasma membrane of the rod cell in the retina [18–21]. A single molecule of photoactivated rhodopsin (R^*) catalyzes the activation of up to 1000 transducin molecules ($T_{\alpha\beta\gamma}$-GDP + $R^* \rightarrow R^*$-$T_{\alpha\beta\gamma}$-GDP + GTP $\rightarrow R^*$-$T_{\alpha\beta\gamma}$-GTP + GDP), and represents the initial stage in the amplification process [20,21]. The second stage of amplification involves a splitting off of the α subunit of transducin from the β and γ subunits, and activation of phosphodiesterase (PDE) by the α subunit (PDE + R^*-$T_{\alpha\beta\gamma}$-GTP $\rightarrow$ PDE + T_α-GTP + R^* + $T_{\beta\gamma} \rightarrow$ PDE*-T_α-GTP + R^* + $T_{\beta\gamma}$). The binding of GTP to transducin releases activated rhodopsin (R^*) for continued catalytic activity via the initial stage. As noted below, this catalytic activity of the activated protein places important constraints on the mechanism of thermal noise. The activated phosphodiesterase complex (PDE*-T_α-GTP) hydrolyzes cyclic GMP (c-GMP) to 5′-GMP which closes the sodium ion channels (c-GMP + H_2O + open channel(s) $\rightarrow$ 5′-GMP + H^+ + closed channel(s)) [18,19]. The transducin cycle returns to the starting point through deactivation of phosphodiesterase via hydrolysis of GTP bound to T_α and recombination of the β and γ subunits with T_α (PDE*-T_α-GTP + $T_{\beta\gamma} \rightarrow$ PDE + $T_{\alpha\beta\gamma}$-GDP). Two mechanisms operate to close down the cascade and regenerate the resting state in preparation for reactivation by a subsequent photon absorption event. Activated rhodopsin (R^*) is removed through phosphorylation followed by the binding of arrestin (An) (R^* + ATP + An $\rightarrow R^*$-P + ADP + An $\rightarrow$ *A* n-R^*-P). Arrestin is an inhibitory protein that blocks the binding of transducin to photoactivated rhodopsin. The second mechanism involves restoration of the open channels via catalysis of GTP with guanylate cyclase (GC) followed by hydrolysis of pyrophosphate (PP) (closed channels + GTP + GC + $H_2O \rightarrow$ c-GMP + PP + $H_2O \rightarrow$ c-GMP + 2P + open channels) [20,21].

An analysis of the above mechanism coupled with the observation that the intensity and duration of photoreceptor noise signals are identical to light-induced signals leads to the conclusion that if the source of the noise involves a thermal corruption of the above process, rhodopsin must be the source of the noise. That is, activated rhodopsins being generated by a thermal process, and the resulting R^{**} (i.e. $R^*_{thermal}$) is identical with R^* (i.e. $R^*_{h\nu}$) with respect to the catalytic amplification process described above. There is, however, one other possibility that needs to be considered. As is schematically shown in Fig. 1, the connections among the neural elements in the retina are complex and are responsible for extensive signal processing prior to transferring the photoreceptor signals to the brain [22]. Because the bipolar cells that mediate the photoreceptor signals prior to transfer to the ganglion network have a signal leveling effect, the observation that dark signals have the same shape and intensity may indicate that the bipolar cells have mediated the signal. Thus, the origin of photoreceptor noise may be within the neural network rather than the photoreceptor system. There are two observations that argue against this interpretation. First, the nature of the photoreceptor noise in humans is invariably perceived as point source noise [23]. This observation supports a model in which thermal activation involves individual photoreceptors rather than mediating neural elements. Perhaps the

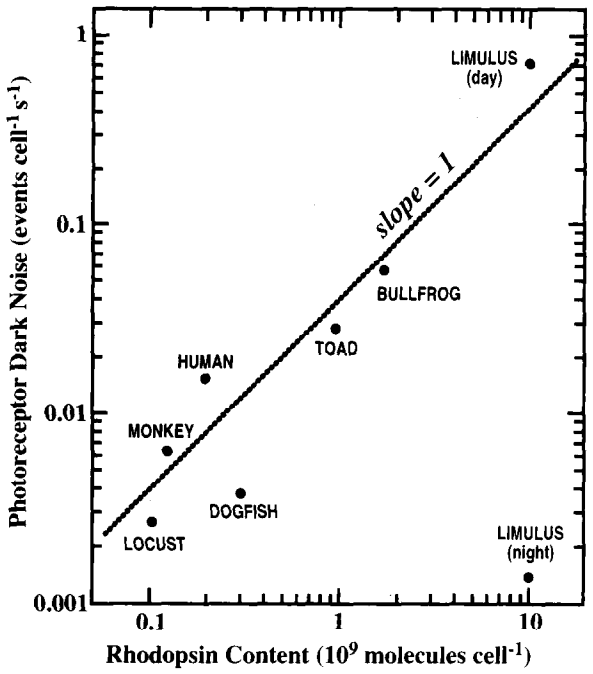


Fig. 2. Dark noise and rhodopsin content of photoreceptors. Measured or inferred photoreceptor dark noise is plotted as a function of rhodopsin content, both on log scales. Photoreceptor noise has been measured directly from locust retinular cells [24], *Limulus* retinular cells [25], toad rods [1], and primate rods [10]. The level of *Limulus* photoreceptor noise was measured in the dark from 09:00 to 15:00 h during the day and 21:00 to 22:00 h at night. Photoreceptor noise has been inferred in dogfish from bipolar cell recordings [26] and in humans from psychophysical measurements [27]. Dark noise for these various visual systems is approximately proportional to rhodopsin content (slope of dashed line = 1) supporting the concept that the rate constants for noise are about equal and that rhodopsin is the source of the thermal noise.

strongest argument in favor of rhodopsin is the observation that there is a near linear proportionality between dark noise and the rhodopsin content of photoreceptors in a range of animals (Fig. 2). Because the animals analyzed in Fig. 2 have significantly different neural architectures, the observed linear relationship suggests that photoreceptors and not neural elements are responsible. The data of Fig. 2 coupled with the observed invariance between light-induced and thermal photoreceptor signals points to rhodopsin as the source.

There are many possible mechanisms that could be responsible for the thermal activation of rhodopsin. In the following section, we examine and compare the various possibilities with the goal of eliminating those which are inconsistent with thermodynamic, kinetic or spectroscopic observation.

3. Possible mechanisms of thermal activation of rhodopsin

Baylor and coworkers have carried out a detailed analysis of electrical dark noise in toad retinal rod outer segments, and assigned the thermodynamic properties of the thermally activated dark processes: ($E_a = 21.9 \pm 1.6$ kcal mol^{-1}, $\Delta G^{\ddagger} = 31.9 \pm 0.13$ kcal mol^{-1}, $\Delta H^{\ddagger} = 21.6 \pm 1.6$ kcal mol^{-1}, $\Delta S^{\ddagger} = -35.3 \pm 5.6$ e.u.) [1]. The activation energies measured for the horseshoe crab (*Limulus*) agree within experimental error ($E_a = 26.3 \pm 7.8$ kcal mol^{-1} (day), 27.9 ± 6.5 kcal mol^{-1} (night), 26.5 ± 7.5 kcal mol^{-1} (in vitro)) [4,5]. We will discuss the *Limulus* data in more detail below, because this animal is capable of dramatically decreasing photoreceptor noise at night (see Fig. 1). Dogfish rod cells appear to possess a somewhat larger Arrhenius activation energy of 36 kcal mol^{-1} [26].

A comparison of these data with denaturation activation energies measured by Hubbard for cattle rhodopsin ($E_a = \sim 100$ kcal mol^{-1}), frog rhodopsin ($E_a = \sim 45$ kcal mol^{-1}) and squid rhodopsin ($E_a = \sim 72$ kcal mol^{-1}) indicates that protein denaturation is not the origin of the dark signal [28]. Measurements of thermal isomerization of 11-*cis* retinal, however, appear to offer a much more compatible set of thermodynamic properties ($E_a = 22.4$ kcal mol^{-1}, $\Delta G^{\ddagger} = 29.3$ kcal mol^{-1}, $\Delta H^{\ddagger} = 21.7$ kcal mol^{-1}, $\Delta S^{\ddagger} = -21.4$ e.u.) (1-propanol solution) [29]. Comparison of the latter measurements on 11-*cis* retinal with those observed by Baylor on rod segments has prompted some investigators to propose that thermal isomerization of "the chromophore" is responsible for dark activation of rhodopsin [1–3]. However, this hypothesis is not consistent with the energetics of ground state isomerization of the protein bound chromophore [6]. The protein bound chromophore is not 11-*cis* retinal, but the protonated Schiff base of 11-*cis* retinal. The ground state barrier to isomerization of the protein bound chromophore is estimated to be $\Delta H^{\ddagger} = 45 \pm 3$ kcal mol^{-1} [6]. We can establish a lower limit of $\Delta H^{\ddagger} \geq 42 \pm 3$ kcal mol^{-1}

based on the relative enthalpy of bathorhodopsin ($\Delta H_{RB} = 32.2 \pm 0.9$ kcal mol^{-1}) [30] plus the activation enthalpy of the bathorhodopsin → lumirhodopsin dark reaction ($\Delta H^{\ddagger} = 10 \pm 2$ kcal mol^{-1}) [31] and assuming additive errors. In contrast, the activation energies for thermal activation are all less than 36 kcal/mol, and a majority are less than 30 kcal/mol. Thus, thermal (ground state) isomerization of the native (protonated) chromophore cannot be responsible for thermal activation of the protein.

Rhodopsin might have sufficient conformational flexibility to undergo a conformational change ($R \xrightarrow{\Delta} R^{**}$) that is interpreted (incorrectly) by transducin ($T_{\alpha\beta\gamma}$-GDP) to represent photochemically activated rhodopsin (R^*). Thus the initial step in the amplification process takes place involving this thermally activated rhodopsin ($T_{\alpha\beta\gamma}$-GDP + R^{**} → R^{**}-$T_{\alpha\beta\gamma}$-GDP + GTP → R^{**}-$T_{\alpha\beta\gamma}$-GTP + GDP). The key problem with this mechanism, however, involves the observation that the thermal noise signals have amplitudes identical to the light activated signals. Thus, R^{**} (thermally activated rhodopsin) must have a lifetime nearly identical to R^* (photochemically activated rhodopsin). This observation precludes enthusiasm for spontaneous conforma-

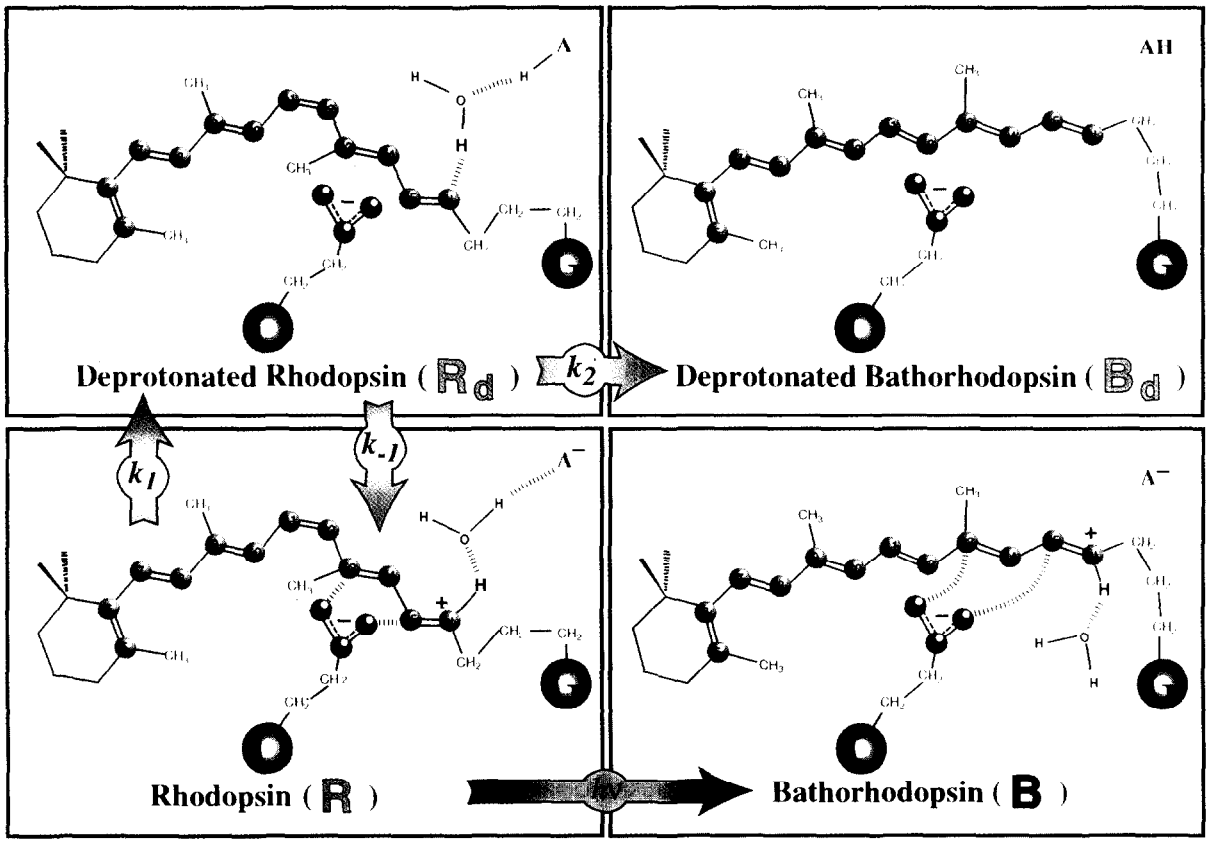
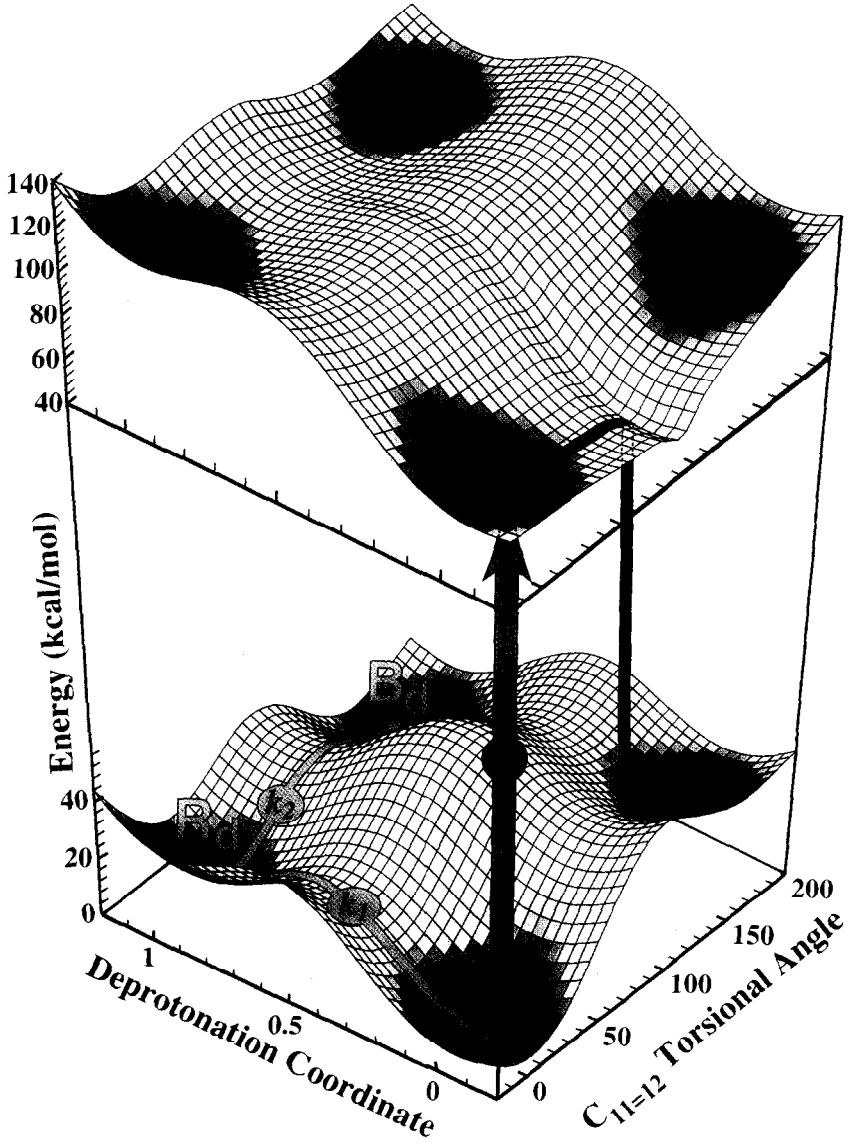


Fig. 3. Molecular schematics of the photochemical and thermal pathways of activation of rhodopsin. The membrane spanning helix to which the chromophore is attached via LYS_{297} is labeled G. The primary counterion, GLU_{113}, is attached to helix C [35,36]. Only one water molecule is shown for simplicity, and the symbol A represents one or more negatively charged amino acids outside of the binding site which ultimately accepts the proton. (Note that the rhodopsin (R) binding site is neutral [37], and thus the acceptor group must be separated from the chromophore by a larger distance than inferred in this Figure). Spectroscopic and theoretical studies indicate that the glutamic acid counterion interacts primarily with the chromophore in the region C12–C13 = C14–C15 region of the chromophore and that at least one water molecule is hydrogen bonded to the imine proton [6,37–40].

tional distortion, because the spontaneous process would either generate a very short-lived R^{**}, or would require denaturation of the protein. The latter observation follows from studies that indicate that even in the absence of a chromophore, opsin is incapable of activating transducin until the salt bridge between LYS_{296} and GLU_{113} that forms within the apoprotein has been broken [32].

Another possibility is that there is an equilibrium within the rhodopsin binding site coupling protonated versus unprotonated chromophores. The experiments of Longstaff and Rando have demonstrated that deprotonation of the Schiff base of retinal is *obligate* for rhodopsin activation [33]. It is thus possible that deprotonation of the Schiff base is *sufficient* for activation. More precisely, deprotonation generates a form of rhodopsin that is interpreted by transducin as activated (i.e. R^*), and the cascade is initiated. (Deprotonation would break the above mentioned salt bridge). The observation that the



thermal noise signals have amplitudes identical to the light activated signals requires that the rhodopsin molecule with an unprotonated chromophore have a lifetime identical to R^*. This is unlikely due to the instability of the R_d species. Recent site directed mutagenesis studies by Fahmy and Sakmar provide more explicit evidence that deprotonation is not sufficient for activation [34]. These investigators replaced the primary counterion of rhodopsin, the glutamate (E) residue at position 113, with glutamine (Q) and regenerated the mutant opsin with 11-*cis* retinal. The E113Q substitution dramatically decreases the pK_a of the Schiff base proton and generates a pigment containing a mixture of protonated and unprotonated retinyl chromophores with absorption maxima at 490 and 380 nm, respectively. The key observation is that the unprotonated (380 nm) species does not activate transducin which indicates that deprotonation is not sufficient to generate R^{**}.

The above observations coupled with the theoretical and experimental studies described below led us to conclude that the most likely mechanism for thermal activation of rhodopsin is a two step process [6,8,9]. The first step is deprotonation of the 11-*cis* protonated Schiff base chromophore. The second step is thermal 11-*cis* to 11-*trans* isomerization of the chromophore. This mechanism is schematically diagrammed in Fig. 3 and the adiabatic potential surfaces generated by using MNDO/AM1 and INDO-PSDCI molecular orbital theory are shown in Fig. 4. The calculated adiabatic activation energies from Fig. 4 are given below:

$$R \underset{\Delta E_{-1}\,\text{ca. 10 kcal/mol}}{\overset{\Delta E_1\,\text{ca. 27 kcal/mol}}{\rightleftharpoons}} R_d \xrightarrow{\Delta E_2\,\text{ca. 23 kcal/mol}} B_d$$

The above energetics would produce an apparent (experimental) Arrhenius-like activation energy of approximately 24–25 kcal mol^{-1} (see Section 5 below) which is consistent with the experimental activation energies (see above). We note that the B_d species generated via the above mechanism would be virtually identical to the R^* generated via the light induced photobleaching sequence, and that both species would decay to form all-*trans* retinal and opsin. We conclude that the above two step mechanism is viable with respect to observed energetics as well as observed thermal noise photoreceptor signals.

If the above mechanism is correct, and the binding site is accessible for titration from the aqueous bulk medium, then changes in extracellular pH should affect the ratio of unprotonated versus protonated chromophores within the binding site. Recent experiments by R. Callender and coworkers [40] on bovine rhodopsin are consistent with the studies of Lisman and Strong [41] on *Limulus* and indicate that the binding site of rhodopsin in both vertebrate and invertebrate photoreceptors is accessible to changes in extracellular pH. If the pH were decreased, then the equilibrium would favor the protonated species, and the rate of thermal activation processes would

Fig. 4. Theoretical analysis of the photochemical and thermal pathways of activation of rhodopsin. Shown are the ground and first excited singlet state adiabatic potential surfaces of the protein bound chromophore of rhodopsin based on MNDO/AM1 and INDO-PSDCI molecular orbital theory [9,17]. The model of the binding site (minus water) and the molecular orbital procedures are described and examined in Refs. [6,17]. The surfaces are calculated by minimizing the geometry of the chromophore and three water molecules as a function of two coordinates, the $C_{11}=C_{12}$ double bond torsional angle (0° = 11-*cis*, 180° = 11-*trans*) and the deprotonation coordinate (0 = covalently bonded; 1 = deprotonated). The key ground state minima and calculated relative energies (kcal/mol) are as follows: R (rhodopsin, $\Delta E = 0$); B (bathorhodopsin, $\Delta E = 31$); R_d (rhodopsin with a deprotonated 11-*cis* chromophore, $\Delta E = 17$); B_d (bathorhodopsin with a deprotonated 11-*trans* chromophore, $\Delta E = 23$). The corresponding excited state potential surface is shifted vertically to facilitate viewing of the ground state surface. The symbol R^{FC} designates the Franck Condon excited state of rhodopsin. The photochemical activation of rhodopsin proceeds by excitation into R^{FC}, partial isomerization in the excited state to a $C_{11}=C_{12}$ distorted geometry (ca. 90°), crossing into the ground state and continued rotation about the $\Phi_{11=12}$ to form bathorhodopsin (B_d) (for details, see Ref. [17]). The thermal activation of rhodopsin involves a two-step process involving deprotonation of the chromophore (rate $= k_1 = A_1 \exp(-27/RT)$) followed by isomerization of deprotonated chromophore (rate $= k_2 = A_2 \exp(-23/RT)$), where simple Arrhenius rate equations are assumed with prefactors (unknown) of A_1 and A_2 and activation energies in kcal/mol. Activation to the first excited singlet state of R_d requires absorption of higher energy photons (λ_{max} = 380 nm, 73 kcal mol^{-1}) than for native rhodopsin, but the ground state barrier for isomerization of 23 kcal mol^{-1} is considerably lower than for native rhodopsin making the unprotonated form relatively less stable.

decrease. The following experiments on *Limulus* provide evidence in support of our proposed mechanism.

4. The effect of extracellular pH on limulus photoreceptor noise

Photoreceptor noise exhibits a circadian rhythm in the lateral eyes of *Limulus*. At night, a circadian clock in the brain transmits efferent optic-nerve activity to the lateral eyes and other visual organs of the animal [27]. The efferent input reduces the rate of "noise" events generated in the dark by photoreceptors and second-order cell without affecting the events evoked by light [25,42]. In photoreceptors the noise events take the form of discrete voltage signals called "quantum bumps", and in second-order cells they are efferent optic-nerve impulses the eye transmits to the brain. We found that changes in the temperature of the retina affected both types of noise equally day and night [9], that is, the Arrhenius activation energies were the same day and night. The "day" function in Fig. 5 yields an apparent activation energy of 25.5 kcal/mol for both types of noise and the "night" function yields an activation energy of 21.2 kcal/mol for spontaneous optic-nerve activity. Over all experiments we found average daytime activation energies of 26.3 ± 7.8 kcal/mol ($n = 10$) for nerve impulses and 26.5 ± 7.5 kcal/mol for quantum bumps ($n = 8$). At night the average activation energy was 27.9 ± 6.5 kcal/mol for optic-nerve activity. Because the Arrhenius activation energies do not change with time of day, we conclude that the circadian clock does not lower photoreceptor noise at night by increasing the energy required to generate it.

We propose that the clock lowers photoreceptor noise by first lowering retinal pH which in turn reduces the small ($< 0.01\%$) population of rhodopsin molecules with unprotonated Schiff base chromophores. Because the protonated species have a thermal barrier to isomerization of ca. 45 kcal mol^{-1} versus ca. 22 kcal mol^{-1}, the former do not contribute to an observable thermal activation reaction. Lowering extracellular pH might further push the equilibrium towards the protonated species by protonating the acceptor group (A in Fig. 3) which would

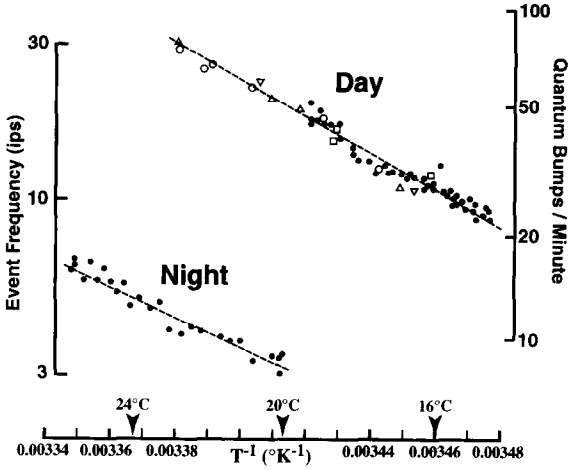


Fig. 5. Temperature influence on the frequency of occurrence of dark events in the *Limulus* retina. Figure shows Arrhenius plots of event frequencies on log scales against reciprocal absolute temperature. Filled circles give the spike activity of ca. 25 optic nerve fibers recorded in vivo from an animal immersed in an sea water aquarium located in a darkened, shielded cage. Lateral-eye temperature was shifted in the range of 14° to 26° C. During the day spontaneous optic-nerve activity was 28 impulses s^{-1} (or ca. 1 impulse/s/optic nerve fiber) with eye temperature set at 20° C. A 6° C decrease to 14° C reduced spontaneous spike activity from 28 to 12 impulses s^{-1} (left ordinate). At night the clock's efferent input reduced spontaneous activity to 3 impulses s^{-1}, and increasing eye temperature from 20° to 26° C about doubled the rate to 6.5 impulses s^{-1}. Unfilled symbols give the results of four measurements from as many eyes of the temperature effect on the frequency of occurrence of quantum bumps recorded in darkness from single photoreceptor cells with glass microelectrodes. We studied the thermal properties of quantum bump noise in single photoreceptor cells in vitro because changing the temperature of the eye in an animal elicited muscular movements which disrupted intracellular recordings from single cells. Excising the eye removed the clock's influence and yielded high levels of spontaneous activity characteristic of the daytime state of the eye. At 20° C the spontaneous activity was ca. 1 bump/s/cell. Note that the rates of quantum bump production and daytime optic-nerve activity have nearly the same steep temperature dependence. Refer to the text for measures of the Arrhenius activation energies.

block or at least hinder deprotonation of the chromophore by removing the primary proton acceptor. We tested the above two-step molecular mechanism by determining whether the clock's efferent input lowers the extracellular pH of the retina. Fig. 6a shows an experiment in which activating the efferent input to the retina with current shocks delivered to the optic nerve decreased retinal pH by 0.14 units.

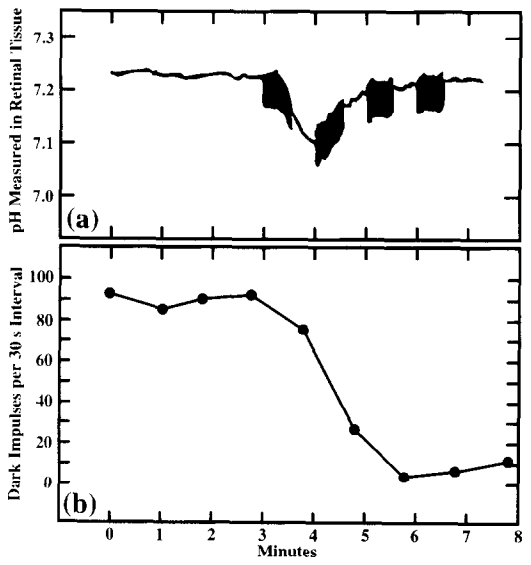


Fig. 6. Efferent optic-nerve activity reduces both pH and dark noise of the *Limulus* retina. (a) Voltage trace shows retinal pH recorded in vivo with an H^+-selective microelectrode inserted into retinal tissue via an access hole cut in the cornea. The technique follows that developed for intracellular recordings of retinal cells in vivo except that the tip of the pH electrode was located in extracellular retinal space in our experiments. The optic nerve trunk was exposed, cut, and its distal end was pulled into a suction electrode. The optic-nerve trunk was stimulated through the access hole with a 30-s train of current pulses (2 s^{-1}) repeated three times to mimic the clock's efferent input to the eye [9]. The current pulses (dark band artifacts) decreased retinal pH from 7.23 to 7.09, but subsequent volleys had no effect. The time course of pH changes for six other experiments was similar to that shown here. (b) Effect of efferent input on the spontaneous impulse activity of three lateral optic nerve fibers of an animal maintained in darkness. Condition of the animal and temporal sequence of efferent activity match those of the pH experiment in (a) above. Ordinate gives the number of impulses recorded in 30-s bins every min. Note that the reduction of retinal noise follows by ca. 1 min. the transient decrease in pH in the top trace.

Fig. 6b shows that the same regime of current shocks to an optic nerve of another animal reduced the optic-nerve activity recorded in darkness. In this and other experiments the changes in retinal noise appear to outlast pH changes for reasons that are not clear. Changes in retinal pH were detected in 6 out of 22 experiments. The remaining 16 experiments exhibited no detectable changes in pH perhaps because of buffering by the retinal tissue and variability in positioning the tip of the pH microelectrode within the tissue. For the same reasons the small change in pH detected in Fig. 6a probably does not reflect the actual pH changes in the vicinity of rhodopsin-containing membrane on which efferent terminals synapse [43]. In other experiments we found that injecting mildly acidic saline also reduces photoreceptor noise [9], which corresponds well with earlier studies of *Limulus* ventral photoreceptors by Lisman and Strong [41].

How does the clock's neural input lower retinal pH in *Limulus*? The mechanism is not known; however, studies in other animals show that neural activity can reduce pH in a variety of tissues [44]. In *Limulus* presynaptic activity itself may lower pH because agonists of the neural input to the eye that act postsynaptically do not strongly influence photoreceptor noise [45,46]. Whatever may be the precise mechanism for lowering retinal pH, the physiological results in Figs. 5 and 6 are consistent with the molecular mechanism we propose.

5. How nature minimizes intrinsic photoreceptor noise

The previous section presents the intriguing proposal that the visual system of the horseshoe crab may diminish photoreceptor noise at night by decreasing extracellular pH. It is likely that *Limulus* has developed a method of controlling photoreceptor noise in part due to the high content of rhodopsin and the necessity to minimize photoreceptor noise during night when vision is important for finding mates [52]. The more highly developed visual systems of land-based animals contain much less rhodopsin in the photoreceptor cells and appear to maintain low levels of dark noise at all times (see Fig. 2). Indeed, rhodopsin has a very low probability of thermal activation with a rate of approximately 10^{-11} thermal events per rhodopsin molecule per second. The question we address in this section is how nature has optimized the binding site of rhodopsin to minimize photoreceptor noise. We also provide a kinetic model of photoreceptor noise that provides a better understanding of how photoreceptor noise can be reduced significantly without a change in activation energy (Fig. 5).

Steinberg et al. recently measured the pK_a of the protonated Schiff base of rhodopsin to be 16 or greater by using a series of model retinal chromophores with electron-withdrawing substituents [47]. This pK_a value is significantly larger than corresponding values in model compounds or related proteins (see below). We conclude that the binding site has been designed to minimize noise by maximizing the pK_a of the chromophore. The implications are best analyzed in terms of the following kinetic scheme.

$$R \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} R_d \overset{k_2}{\rightarrow} B_d$$

Simple reaction rate theory suggests that the total rate of this process can be described approximately by the following equation [8]:

$$k_{tot} = \frac{k_1 k_2}{k_{-1}} \cong \frac{kT}{h}\left(\frac{10^{\Delta pK}}{1+10^{\Delta pK}}\right)\left(\frac{1-\exp\left(\frac{-h\nu_{isom}}{kT}\right)}{\exp\left(\frac{E_2}{kT}\right)}\right) \quad (1)$$

where $\Delta pK = pK_a^A - pK_a^{PSB}$, pK_a^A represents the pK_a of the principal proton acceptor group within the protein binding site, pK_a^{PSB} represents the pK_a of the protonated Schiff base chromophore, ν_{isom} is the frequency of the $C_{11}{=}C_{12}$ ground state torsional mode, E_2 is the activation energy of the isomerization step, h is Planck's constant, k is Boltzmann's constant and T is the temperature. If we assume $pK_a^{PSB} = 16$, $pK_a^A = 7$, $\nu_{isom} = 300\ cm^{-1}$, $E_2 = 22$ kcal mol^{-1} (see Ref. [29]) and $T = 310$ K (body temperature) we calculate $k_{tot} = 1.5 \times 10^{-12}\ s^{-1}$. Given the level of approximation inherent in Eq. (1) and the above assignments, the agreement with the observed dark noise rate of ca. 10^{-11} events rhodopsin^{-1} s^{-1} is encouraging.

One might anticipate based on the above rate equation that the observed activation energy would be significantly higher than E_2, the barrier to thermal isomerization of the unprotonated chromophore. In fact, for reasons outlined below, the observed activation energy should be only slightly larger than E_2. We can write Eq. (1) in the form of a simple Arrhenius-like equation as follows:

$$k_{tot} \cong A_0 \exp\left(\frac{-E_a}{kT}\right) \approx A_{eff}^{*} \exp\left(\frac{-E_a^{*}}{kT}\right) \quad (2)$$

where

$$E_a = E_2 + h\nu_{isom} - kT \ln\left(\frac{kT\,10^{\Delta pK}\left(\exp\left(\frac{-h\nu_{isom}}{kT}\right)-1\right)}{hA_0(1+10^{\Delta pK})}\right) \quad (3)$$

and

$$A_0 = \frac{kT\,10^{\Delta pK}\exp\left(\frac{(E_a - E_2 - h\nu_{isom})}{kT}\right)\left(\exp\left(\frac{h\nu_{isom}}{kT}\right)-1\right)}{h(1+10^{\Delta pK})} \quad (4)$$

We note, however, that the prefactor, A_0, is a function of E_a and that the activation energy, E_a, is a function of A_0. Thus, Eq. (1) cannot be formally separated into "pure" prefactor and activation energy terms. By using numerical methods, however, we can solve for E_a and A_0, and a series of calculations over a broad range of variable assignments indicates that the value of E_a never exceeds $E_2 + h\nu_{isom}$ for reasonable values of ν_{isom}. In fact, to a good approximation, we can equate the activation energy to $E_2 + \frac{1}{2}h\nu_{isom}$. This assumption leads to the following set of equations:

$$E_a^{*} = E_2 + \tfrac{1}{2}h\nu_{isom} \quad (5)$$

and

$$A_{eff}^{*} = \left(\frac{h\nu_{isom} + kT}{kT}\right) \times \left(\frac{kT\,10^{\Delta pK}\left(\exp\left(\frac{-h\nu_{isom}}{kT}\right)-1\right)}{h\exp\left(\frac{h\nu_{isom}}{kT}\right)(1+10^{\Delta pK})}\right) \quad (6)$$

We now have nearly pure prefactor and activation energy terms and this decomposition allows us to examine the effective (Arrhenius-like) activation energy in a straight-forward fashion. We note that in our above example, we assumed $\nu_{isom} = 300\ cm^{-1}$.

This equates to an energy contribution of $\frac{1}{2}h\nu_{isom}$ = 0.43 kcal mol^{-1}. The value of ν_{isom} is poorly defined for a complex molecule such as the chromophore in rhodopsin. In particular, there is no single ground state vibrational mode which is a pure torsional distortion mode involving the C_{11}=C_{12} double bond (see, for example, the vibrational assignment for the chromophore in bacteriorhodopsin [48]). Nevertheless, we can state with confidence that ν_{isom} must be less than 1000 cm^{-1}, which will lead to a value of $\frac{1}{2}h\nu_{isom}$ = 1.4 kcal mol^{-1}. Thus, we can conclude that the observed activation energy of photoreceptor noise will be less than 1.4 kcal mol^{-1} larger than the barrier to isomerization of the unprotonated Schiff base chromophore. This observation is fully consistent with the experimental activation energies, which tend to be larger, but not significantly larger, than the barrier for isomerization of 11-*cis* retinal in solution (E_a = 22 kcal mol^{-1}).

The effect of pH on the rate of photoreceptor noise is basically observed as an adjustment of the prefactor. We can write ΔpK approximately as $pH_{ex} - pK_a^{PSB}$, where pH_{ex} represents the extracellular pH. A decrease of 1 pH unit in extracellular pH will induce a decrease in rate of ca. 0.1 (see Eq. (6)). This will have no effect on the observed activation energy, however, which is consistent with the experimental measurements on *Limulus* (see above). We can offer no definitive explanation for why extracellular pH has a larger impact on photoreceptor noise in *Limulus* than is predicted by Eqs. (1) and (6) [9]. Extracellular buffering is one possibility, but protonation of an acceptor group (A in Fig. 3) would also effectively multiply the effect. Further work will be necessary to fully resolve the apparent enhancement effect.

What remains to be explained is how nature has designed the rhodopsin binding site in order to diminish intrinsic photoreceptor noise. For purposes of discussion, we can write the total rate as proportional to two factors:

$$k_{tot} = \frac{k_1 k_2}{k_{-1}} \propto k_2 10^{-pK_a^{PSB}} \qquad (7)$$

It follows from Eq. (7) that a high pK_a of the protonated Schiff base chromophore is important to the biological control of photoreceptor noise. Model protonated retinyl Schiff base chromophores in solution exhibit pK_a values around 7 [47,49–51]. The retinyl chromophore in bacteriorhodopsin, the light transducing protein in the purple membrane of *Halobacterium halobium*, exhibits a pK_a of ca. 13 [49]. An increase in pK_a of six units upon incorporation of the retinyl chromophore in bacteriorhodopsin is impressive. The corresponding increase of more than nine units in rhodopsin is extraordinary. Nature rarely explores the limits of physical phenomena without simultaneously improving the comparative advantage of the system. We propose that the adjustment of the pK_a in rhodopsin is intimately related to the natural selection of a photoreceptor protein exhibiting minimal dark noise. For comparison, if the chromophore of rhodopsin had the same pK_a as that observed in bacteriorhodopsin, the visual pigment would have a dark noise rate three to four orders of magnitude larger than observed.

Further experimental and theoretical work will be required before the mechanistic origins of the high pK_a of the rhodopsin chromophore can be established. Molecular orbital calculationsindicate that the strength of the Schiff base N–H bond increases as the counterion is moved down the chain from the C=N group towards atom C_{12} (see Fig. 3) [8]. Thus, the shift in pK_a appears to be associated at least in part with the specificity of the chromophore–counterion interaction. We anticipate that other amino acids near the binding site may establish a local field at the retinyl chromophore which further enhances the pK_a. This proposal, however, must remain an hypothesis pending the assignment of the tertiary structure of the rhodopsin protein.

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