Role of Retinal Hydrogen Bond Network in
Rhodopsin Schiff Base Stability and Hydrolysis.

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RUNNING TITLE: Hydrogen Bond Network Stabilizes the Rhodopsin Schiff Base Linkage.
SUMMARY

Little is known about the molecular mechanism of Schiff base hydrolysis in rhodopsin. We report here our investigation into this process focusing on the role of amino acids involved in a hydrogen bond network around the retinal Schiff base. We find conservative mutations in this network (T94I, E113Q, S186A, E181Q, Y192F and Y268F) increase the activation energy ($E_a$) and abolish the concave Arrhenius plot normally seen for Schiff base hydrolysis in dark-state rhodopsin. Interestingly, two mutants (T94I and E113Q) show dramatically faster rates of Schiff-base hydrolysis in dark-state rhodopsin, yet slower hydrolysis rates in the active MII form. We find deuterium effects the hydrolysis process in wild-type rhodopsin, exhibiting a specific isotope effect of $\sim 2.5$, and proton inventory studies indicate multiple proton transfer events occur during the process of Schiff base hydrolysis for both dark-state and MII forms. Taken together, our studies demonstrate the importance of the retinal hydrogen bond network both in maintaining Schiff base integrity in dark-state rhodopsin, as well as in catalyzing the hydrolysis and release of retinal from the MII form. Finally, we note that the dramatic alteration of Schiff base stability caused by mutation T94I may play a causative role in Congenital Night Blindness, as has been suggested by the Oprian and Garriga laboratories.
INTRODUCTION

Rhodopsin, the dim light photoreceptor of rod cells is arguably the best-characterized member of the class A superfamily of G-protein coupled receptors (GPCRs\(^1\)), (1-7). A transmembrane receptor, it has evolved into an efficient photoreceptor by covalently binding its chromophore, 11-\textit{cis}-retinal, to lysine-296 via a protonated Schiff-base linkage within the helical bundle (8,9). Dim light vision begins when the 11-\textit{cis}-retinal chromophore absorbs a photon and isomerizes to the all-\textit{trans}-retinal form. This change in retinal initiates a series of photo-intermediates and conformational changes in the protein, resulting in the formation of metarhodopsin II (MII), the active conformation of rhodopsin that is able to bind and activate the G-protein transducin. The MII photoprodut is in dynamic equilibrium with its predecessor MI and this MI/MII pool is thought to decay through two processes (10). The MII product may be directly hydrolyzed and release all-\textit{trans}-retinal from the binding pocket, or the MI pool may undergo an addition thermal isomerization along the chromophore C=N double bond (all-\textit{trans} 15-\textit{syn}) giving rise to the MIII storage product (10). This MIII intermediate also decays to opsin and all-\textit{trans}-retinal (albeit at a slower rate) either through the MI/MII pool or possibly direct retinal Schiff base hydrolysis of the MIII intermediate.

Rhodopsin deactivation ultimately requires hydrolysis of the all-\textit{trans}-retinal Schiff base linkage and release of retinal from the binding pocket. Recycling the receptor and returning it to a photosensitive conformational state completes the recovery process (11). The retinoid cycle accomplishes this task by converting the released all-\textit{trans}-retinal back to the 11-\textit{cis} conformation through a series of enzymatic reactions – eventually resulting
in the reformation of the retinal Schiff base linkage and regeneration of the photosensitive receptor (12,13). While extensive research into Schiff base formation and the retinoid cycle has resulted in a wealth of knowledge (12,13), little is known about the molecular mechanism of Schiff base hydrolysis and the subsequent retinal release both in the dark-state, or during the decay of the functional MII state (12,14).

Although both rod and cone opsins bind their retinal chromophores through a Schiff base linkage (1,15), this linkage is markedly less stable in cone opsins, as a result, the turnover and regeneration rates for cone cells are significantly faster than for rod cells (16-19). Furthermore, while the retinal Schiff base linkage is quite stable in dark-state rhodopsin, it hydrolyzes quickly in the active MII signaling state of the protein (1,20-22), suggesting that some change likely occurs in the vicinity of the Schiff base attachment site to account for this disparity. Clearly the apoprotein plays a significant role in stabilizing the retinal Schiff base linkage.

Recently, a high resolution structure of rhodopsin suggested the presence of a hydrogen bond network encompassing the retinal Schiff base attachment site near extracellular loop E-2 and the “retinal plug” domain (23). This network consists of both the carbonyl backbone and side chains of amino acids lining the retinal binding pocket, as well as water molecules within the pocket that surround the retinal Schiff base linkage. This network was recently demonstrated to play a key role in the protonated Schiff base counter ion switch mechanism proposed to occur upon formation of the MI photointermediate subsequent to light activation (24). However, the role this retinal
hydrogen bond network plays in stabilizing the retinal Schiff base and in potentially participating in the mechanism of Schiff base hydrolysis remains as yet unexplored.

In this manuscript we report our investigation into the role of the retinal hydrogen bond network in relation to the retinal Schiff base linkage in rhodopsin. Through site-directed mutagenesis, we find that disruption of this network results in compromised stability of the Schiff base linkage in the dark-state, and a loss of concave Arrhenius plots for all of the mutant proteins (especially for residues T94 and E113). In addition, we find that mutation of this network also affects Schiff base stability in the MII state, but only at some sites. Paradoxically, mutation of these sites, (T94, E113 and S186), actually slows MII decay, suggesting that these residues act as participants in Schiff base hydrolysis and retinal release process. Finally, through deuterium isotope studies, we provide evidence that multiple proton transfer events occur during the process of Schiff base hydrolysis and subsequent retinal release and that this process proceeds through a carbinolamine intermediate. Our results further illustrate how the rhodopsin structure maintains Schiff base integrity and provides insight to the mechanism of Schiff base hydrolysis and retinal release.
EXPERIMENTAL PROCEDURES

Materials and Buffers. Except where noted, all buffers and chemicals were purchased from either Fisher (Pittsburgh, PA) or Sigma (St. Louis, MO). Dodecyl maltoside (DM) was purchased from Anatrace (Maumee, OH), Polystyrene columns (2-ml bed volume) were purchased from Pierce. Frozen bovine retinas were from J.A. Lawson Co. (Lincoln, NE). Transducin was purified from rod outer segments as previously described (25). Restriction endonucleases were from New England Biolabs (Beverly, MA). 11-cis-retinal was a generous gift from Dr. R. Crouch (Medical University of South Carolina and the National Eye Institute). The 1D4 antibody was purchased from the National Cell Culture Center (Minneapolis, MN). The nonapeptide corresponding to the C-terminus of rhodopsin was acquired from the Emory University Microchemical Facility (Atlanta, GA). Cuvettes were purchased from Uvonics (Plainview, NY). Deuterium oxide was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Centrifugal filter devices were purchased from Millipore (Billerica, MA), 0.5 µM volume, 10 kDa cut off. Definitions for the buffers used are as follows: PBSSC [0.137 M NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8 mM Na₂HPO₄ (pH 7.2)], buffer A [1% DM and PBSSC (pH 7.2)], buffer B [2 mM ATP, 0.1% DM, 1 M NaCl, and 2 mM MgCl₂ (pH 7.2)], buffer C [0.05% DM and PBSSC (pH 7.0)], buffer D [0.05% DM and 5 mM MES (pH 6.0)].

Construction, Expression and Purification of Rhodopsin Mutants. Site-directed mutagenesis was performed using overlap extension PCR to generate fragments containing the mutation of interest (T94I, E113Q, E181Q, S186A, Y192F and Y268F) in the synthetic bovine rhodopsin gene (26) essentially as described previously and
subcloned into the pMT4 plasmid (27,28) for expression. All mutations were confirmed by the dideoxynucleotide sequencing method.

The mutant rhodopsin proteins were transiently expressed in COS-1 cells using the DEAE-dextran method, and cells were harvested 56 to 72 h after transfection as previously described (27,29). The harvested opsin mutants were subsequently regenerated with 10 µM 11-cis-retinal at 4 °C for 1 h, followed by an additional 5 µM of 11-cis retinal and 1 h incubation (30). The purification of the rhodopsin mutants proceeded essentially as the original procedure (29), with modifications as previously described (28,31). A spectrum of each elution fraction was recorded and the purified samples were either used immediately or snap frozen in liquid N2 and stored at -80 °C.

**UV-Vis Absorption Spectroscopy.** All UV-Vis absorption spectra were recorded with a Shimadzu UV-1601 spectrophotometer at 20 °C using a bandwidth of 2 nm, a response time of 1 s, and a scan speed of 500 nm/min unless otherwise noted. For concentration calculations, the molar extinction coefficient value ($\epsilon_{500}$) for WT rhodopsin was taken to be 40 600 M$^{-1}$ cm$^{-1}$ (32). The samples were photobleached in buffer A by illumination for 30 s (at a 6 Hz flash rate) with a Machine Vision Strobe light source (EG&G) equipped with a wavelength > 490 nm long-pass filter. This light treatment was found to be adequate for full conversion of all samples. The presence of a protonated Schiff-base (PSB) in the MII state for each mutant was verified by adding H$_2$SO$_4$ to a pH of 1.9 immediately following photobleaching then measuring the absorbance spectrum to assay the presence of a spectral species at 440 nm (which indicates an intact retinal PSB) (33).
Photobleaching time course analysis on mutant Y268F was performed as above but with bleaching time reduced to 15 s and results compared to a WT rhodopsin irradiated in the exact same manner.

**Determination of Transducin (G\(_T\)) Activation Rates.** Activation of G\(_T\) by rhodopsin was monitored using fluorescence spectroscopy at 10 °C as described previously (34-36) using a Photon Technologies QM-1 steady state fluorescence spectrophotometer (37). The excitation wavelength was 295 nm (2 nm bandwidth), and fluorescence emission was monitored at 340 nm (12 nm bandwidth). Briefly, photobleached mutant rhodopsin (final concentration of 5 nM) was added to the reaction mixture consisting of 250 nM G\(_T\) in 10 mM Tris (pH 7.2), 2 mM MgCl\(_2\), 100 mM NaCl, 1 mM DTT, and 0.01% DM. Thus the mixture was allowed to stir for 300 s, then the reaction was initiated by the addition of GTP\(_\gamma\)S to a final concentration of 5 µM, and the increase in fluorescence was followed for an additional 1500 s. To calculate the activation rates, the slopes of the initial fluorescence increase following GTP\(_\gamma\)S addition were determined through the data points covering the first 60 s using linear regression analysis. Mutant rhodopsin activation values are reported as percentages relative to WT, which was taken to be 100%.

**Thermal Bleaching of Rhodopsin Samples.** Thermal decay rates were followed by UV-Vis spectroscopy in buffer A essentially as previously described (28,38). The thermal stability of each mutant was determined by first monitoring the absorbance of each sample from 650 nm to 250 nm at 1 minute intervals at a given temperature. Thermal decay rates were subsequently obtained by monitoring the decrease of the 500 nm
absorbing dark-state species from these measurements over time (39-41). Baseline drift was corrected for by normalizing all spectra to an absorbance of zero at 650 nm. Thermal decay rates were also measured by monitoring the increase in tryptophan fluorescence at 330 nm, caused by the release of retinal from the chromophore-binding pocket (37) as previously described (28,38). The experimental setup for these assays is similar to that of the retinal release assay (described below) except that the samples were not photobleached during the assay. All thermal decay data was analyzed using mono-exponential decay (absorbance experiments) or mono-exponential rise to maximum (fluorescence experiments) fitting algorithms in Sigma Plot (Jandel Scientific Software). Activation energies ($E_a$) for mutant thermal decay assays were determined by applying the rate data to the Arrhenius equation: $k = Ae^{-Ea/(RT)}$, as previously described (28).

Measurement of the Rate of Retinal Release and/or MII Decay by Fluorescence Spectroscopy. The MII stability was assessed by measuring the time course of retinal release coinciding with the rate of decay for the active MII state, using the PTI fluorimeter described above. Each measurement was carried out using 100 µL of a 0.25 µM mutant sample in buffer A, and sample temperature was maintained as described above. After the samples were photobleached to the MII state (see above), the retinal release measurements were carried out at the appropriate temperature by exciting the sample for 3 s (excitation wavelength = 295 nm, 1/4 nm bandwidth slit setting) and then blocking the excitation beam for 42 s, to avoid further photobleaching the samples. Tryptophan fluorescence emission was monitored at 330 nm (12-nm bandwidth slit setting), and this cycle repeated till a plateau in the signal was achieved. To determine
the t_{1/2} values for retinal release, experimental data was analyzed using a mono-
exponential rise to maximum fit in Sigma Plot (Jandel Scientific Software). In this
manner series of MII decay rates were obtained at 5, 10, 15, 20, 25, 30, and 35 °C, and
their rates were applied to the Arrhenius equation, \( k = Ae^{-E_a/(RT)} \), to determine the
activation energy (\( E_a \)) of the retinal release process for each mutant rhodopsin.

**Hydroxylamine Reactivity.** Hydroxylamine reactivity of the dark-state was determined
for purified WT and mutant rhodopsins by monitoring the rate of 500 nm absorbance
decrease after the addition of hydroxylamine (pH 6.0) to the samples in buffer A to a
final concentration of 50 mM at the indicated temperatures (28,42). Baseline drift was
corrected as described above (see Thermal Bleaching of Rhodopsin Samples).

**Solvent isotope effects on dark-state stability and MII decay rates.** A 10% DM stock and
a 500 mM MES pH 6.0 stock buffer were made up fresh in deuterium oxide (D_2O) and
were then used to make a 0.05% DM, 5 mM MES pH 6.0 (buffer D) D_2O exchange
buffer. The pH of the D_2O experiments ranged between 6.0 – 6.4, since the pH for the
deuterium buffer is expected to be off by + 0.4 pH units. However, as the process of MII
decay has been shown to be independent over a pH range of 5.0 to 8.0 (38), this slight pH
change should not alter interpretation of the results. Using a concentrated stock of
purified rhodopsin (to use a small volume for dilution) 2 equal molar stocks of both D_2O
and H_2O rhodopsin were prepared. These samples (500 nM) were then buffer exchanged
using Millipore Ultrafree 0.5 centrifugal filter devices at low spin speeds. The D_2O
sample was washed 8 times with 500 µL of D_2O containing buffer, while the H_2O sample


was washed in the exact same method using H₂O buffer to ensure that any detergent concentration that may have taken place during the exchange would be equal for both samples. The samples were then assayed for dark-state thermal decay (55 and 37 °C) as well as MII decay rates at 20 °C as described above. The procedure was repeated and values obtained from 3 separate experiments from 2 different stock preparations.

Proton inventory studies on dark-state retinal stability and MII decay/retinal release rates. The stock solutions were prepared as described above, and combined in various proportions to give different mole fractions (n) of deuterium ranging from 0 to 1.0. The dark-state thermal decay rate or the MII decay rate of WT rhodopsin was monitored as described above over the range of different mole fractions of D₂O. Data was analyzed by plotting the ratio of $k_n/k_H$ versus the mole fraction of D₂O, where $k_n$ is the rate in the molar fraction of D₂O and $k_H$ is the rate in 100% H₂O.
RESULTS

Rational for choice of mutants. The site-directed rhodopsin mutants analyzed in this study were generated to assess their individual roles in maintaining retinal Schiff base integrity. Specifically, mutants were investigated to evaluate their role in a hydrogen bond network that has previously been proposed based on crystallographic data (23), functional studies (24,38,43-47) and molecular modeling. Each mutant was designed to disrupt the hydrogen bond capability of that individual residue, yet preserve the size of the amino acid side chain as much as possible. Toward this end, we made and analyzed the following series of retinal binding pocket mutants: T94I, E113Q, E181Q, S186A, Y192F and Y268F (Figure 1). Mutant T94I was investigated not only because of its potential role in this hydrogen bonding network, but also because mutant T94I is associated with the disease Congenital Night Blindness (CNB) (44-46).

Characterization of rhodopsin mutants. All mutants expressed to levels similar to WT rhodopsin in a COS cell expression system (10 to 15 µg/15 cm plate), were capable of binding the chromophore 11-cis-retinal and could be purified following standard procedures to homogeneity. All mutants exhibited spectral ratios ($\lambda_{280}/\lambda_{\text{max}}$) between 1.6 and 1.8, with the exception of the counter ion-mutant E113Q, which shows a pH dependence in its absorbance profile (42,43,48). Many of the mutants exhibited shifted dark-state absorption maximum values ($\lambda_{\text{max}}$), (Figure 2). Mutant T94I, which is in close proximity to the counter-ion residue E113, showed a $\lambda_{\text{max}}$ of 478 nm in agreement with previous results (44-46). Interestingly, we also found that mutant T94I could be regenerated with all-trans-retinal, exhibiting a $\lambda_{\text{max}} = 464$ nm as reported earlier (44).
Additionally, mutant E181Q showed a slight red-shift in its absorbance profile (47), while the dark-state $\lambda_{\text{max}}$ of mutant S186A was similar to that of WT rhodopsin (24). Notably, the tyrosine to phenylalanine mutations at residues 192 and 268 resulted in blue-shifted $\lambda_{\text{max}}$ values in the dark-state of 491 and 495 nm, respectively.

Photobleaching experiments showed that all mutants could form a MII-like photointermediate upon illumination, with $\lambda_{\text{max}}$ values not deviating substantially from that of WT rhodopsin ($\lambda_{\text{max}} = 381$ nm, see Table I). In addition, acid denaturation of this species confirmed the presence of an intact protonated retinal Schiff base linkage ($\lambda_{\text{max}} = 440$ nm), (Figure 2). However, while capable of forming a MII-like intermediate, mutant Y268F (and to a lesser extent Y192F) also showed a “shoulder” species centered at ~ 480 nm immediately following illumination (Figure 2). Interestingly, this species slightly decayed over time, but was still present up to 500 min following bleaching (Figure 3A). Similar results were obtained for mutant Y192F (data not shown). We do not have an explanation for these observations, but similar results have been previously noted for other rhodopsin point mutants, including G90S and L226C (27,28,49,50). The spectral properties for all of the mutants are presented in Table I.

Transducin activation by hydrogen bond network mutants. Mutants were tested for their ability to catalytically activate transducin using a fluorescence-based assay that measures the increase in tryptophan fluorescence of the $\text{G}_{\text{Tt}}$-GTP$_{\gamma}$S species (34-36). The slight decreases in initial rates for mutants T94I, E113Q and E181Q were in agreement with values previously reported (43,45,47). In addition, our results indicate that T94I
regenerated with all-\textit{trans}-retinal (T94I-ATR) is able to activate transducin to near WT levels, and mutation S186A also retains its ability to activate transducin. Interestingly, we find that transducin activation of T94I-ATR occurs in a light dependent manner. However, we do detect a slight level (~15\%) of constitutive activity in the dark-state for this mutant (not shown). Furthermore, our results demonstrate the new mutants Y192F and Y268F are much less able to activate transducin compared to WT rhodopsin. This is perhaps most striking for mutant Y268F, which only exhibits ~20\% of the functional activity of WT rhodopsin (Figure 3B). The results for transducin activation are compiled in Table I.

\textit{Effects on retinal Schiff base integrity in the dark-state.} All of the mutants tested showed some increased rate of dark-state thermal decay in comparison to WT rhodopsin, as judged by their loss in absorbance at 500 nm, (or increase in fluorescence at 330 nm for mutant E113Q). These measurements were carried out over 37 – 55 °C, and the results are compiled in Table II. Activation energy (E\textsubscript{a}) values for the dark-state thermal decays were obtained from Arrhenius analysis of the decay data, as previously described (28,38). These plots reveal several interesting findings: i) at higher temperatures, all of the mutants have expedited rates of thermal decay in comparison to WT rhodopsin, ii) except for mutants T94I and E113Q, the magnitude of rate increase is substantially less for the mutants at lower, more physiologically relevant temperatures, and iii) all of the mutants tested exhibit linear plots in contrast to WT rhodopsin, which displays a concave Arrhenius plot (Figure 4). Taken together, these results suggest that mutations of the retinal hydrogen bond network residues affect both the kinetics and energetics of the
dark-state thermal decay process. Further, this effect on dark-state hydrolysis is most pronounced for mutation sites in close proximity to the Schiff base attachment site – T94I and E113Q. Rate data and $E_a$ values for the dark-state thermal decay experiments are presented in Table II.

**Effects on retinal Schiff base integrity in the MII state.** MII stability was measured over a range of temperatures to determine the kinetic rates and activation energies for the retinal release process (27,28). Under our conditions, the $t_{1/2}$ of retinal release for WT rhodopsin was 13.5 min at 20 °C in buffer D, comparable to values previously reported (27,36,41,47). These studies show E181Q, Y192F and Y268F only exhibit slightly increased rates of retinal release during MII decay (see Table II). In contrast, mutants T94I, E113Q and S186A all show dramatically decreased rates of retinal release (see Table II). Arrhenius analysis of these measurements carried out from 5 – 35 °C indicates a temperature-dependent linear relationship for all mutants (Figure 5). These plots again illustrate that mutants E181Q, Y192F and Y268F show minimal effect on the process of Schiff base hydrolysis and retinal release during decay of the MII state, whereas mutants T94I, E113Q and S186A are all substantially more stable than WT rhodopsin (Figure 5B). The Schiff base hydrolysis and retinal release rates as well as $E_a$ values for these processes are presented in Table II.

**Solvent accessibility measured using hydroxylamine.** The small molecule hydroxylamine (NH$_2$OH) was used to measure the solvent accessibility of the retinal in each of the mutants, as it rapidly cleaves retinal Schiff base linkages (8). WT rhodopsin is
effectively non-reactive to NH$_2$OH in the dark-state at 37 °C (28,38), thus dark-state hydroxylamine reactivity is often used to probe the solvent accessibility of the retinal Schiff base in rhodopsin mutants (43,48). Hydroxylamine reactivity experiments illustrate that relative to WT rhodopsin, all of the mutants are more susceptible to hydroxylamine cleavage of the retinal Schiff base in the dark-state. Kinetic parameters for dark-state hydroxylamine reactivity are presented in Table II.

Deuterium isotope effects on the process of Schiff base hydrolysis. Deuterium isotope exchange experiments were conducted on WT rhodopsin to further investigate the role of water molecules and potential proton exchange occurring during the chemical process of retinal Schiff base hydrolysis. In these experiments, dark-state thermal decay assays were performed on D$_2$O buffer exchanged samples and the rate values compared to samples prepared in an identical manner using H$_2$O. As shown in Figure 6A, the rate of Schiff base hydrolysis in the dark-state at 37 °C is significantly slower in D$_2$O buffer than that of the H$_2$O control ($k_{H2O} = 5120$ min, $k_{D2O} = 12,232$ min), exhibiting a solvent isotope effect of 2.4. Interestingly, proton inventory analyses of these dark-state decay reactions results exhibit a nonlinear relationship between rate and molar amount of D$_2$O present (Figure 6B). A slowed rate in D$_2$O was also observed when the experiments were conducted at 50 and 55 °C (data not shown).

Possible isotope effects occurring during the chemical process of retinal Schiff base hydrolysis in the MII state of WT rhodopsin were also investigated. In these experiments, retinal release assays were performed following D$_2$O buffer exchange and
rate values compared to samples prepared in an identical fashion using H$_2$O buffers (see above). The rate of Schiff base hydrolysis and subsequent retinal release at 20 °C is also substantially slower in deuterium buffer ($k_{H_2O} = 15.3 \pm 0.4$ min, $k_{D_2O} = 38.6 \pm 0.3$ min, $n = 3$ for both), exhibiting a solvent isotope effect of 2.5 (Figure 6C). Proton inventory analysis of these MII decay reactions results also exhibits in a nonlinear relationship between rate and molar amount of D$_2$O present (Figure 6D). The interpretation of these finding are discussed further below.
DISCUSSION

Extracellular loop E-2 in rhodopsin folds back into the core of the protein and forms a “plug” surrounding the retinal binding pocket (9). Numerous amino acid side chains in loop E-2 make contact with the retinal, and thus may be important for both Schiff base stability as well as signal transduction (24,28,47). Recently, a rhodopsin structure was published which suggested water molecules also lie within the retinal binding pocket (23), in agreement with previous studies (51,52), and suggested a hydrogen bond network surrounds the retinal Schiff base linkage (23). The present report details the structural and functional implications this retinal hydrogen bond network has on the stability of the retinal Schiff base as well as rhodopsin signaling.

Expression and spectral characteristics of hydrogen bond network mutants. All of the mutants examined in this study express well, are properly folded and bind 11-cis-retinal (Figure 2). Since all of these mutants are part of, or in close proximity to, the retinal binding pocket it is not surprising that many show altered dark-state absorption λ_{max} values. We find the absorbance properties for mutants E113Q, T94I, E181 and S186A are as previously described (24,43-45,47), and that two previously unexamined mutants, Y192F and Y268F, also result in dark-state absorbance blue-shifts (Figure 2, Table I). The absorbance shift in these two mutants may be due to alterations in their contacts with the retinal. Alternatively, they may disrupt the retinal hydrogen bond network, as both Y192 and Y268 appear to align E181 (23,24,53). Disruption of this alignment may alter the manner in which the hydrogen bond network interacts with the retinal, and effects photobleaching as well as receptor signaling properties (discussed further below).
Most of the mutants showed WT-like photobleaching properties, and could form a MII absorbing species and a PSB upon acid denaturation (Figure 2). However, mutant Y268F (and to a lesser extent Y192F) demonstrate a residual absorbing species with a $\lambda_{\text{max}}$ of $\sim$ 480 nm following illumination (Figure 2), which slightly decays over time, yet still retain some residual $\sim$ 480 nm absorbance (see Figure 3A for an example). In contrast, WT rhodopsin under identical conditions exhibits only a slow, slight increase in $\sim$ 480 nm absorbance, presumably due to the formation of the MIII storage intermediate (54,55). The exact reason for this altered photoproduct in mutant Y268F and Y192F is not clear. These residues may play a role in the recently proposed counter-ion switch mechanism of rhodopsin activation (24), which proposes the Schiff base counter-ion switches from residue E113 in the dark-state to residue E181 in the MI state, in a process mediated by the hydrogen-bonding network of residue side chains and water molecules. The Y268F and Y192F mutations may misalign residue E181 and thus result in inefficient counter-ion transfer and possible build up or trapping of photointermediate species MI or MIII. Further studies, particularly FTIR, may help shed light on the identity of this species. Whatever the mechanism, it is interesting to note that these residues are conserved in all almost all visual rhodopsins.

*Functional characteristics of hydrogen bond network mutants.* The ability of each of the hydrogen bond network mutants to activate transducin varied. We find mutations T94I, E113Q and E181Q do not drastically effect initial rates of transducin activation, in agreement with previous studies (43,45,47). Interestingly, T94I-ATR activates transducin in a light-dependent manner; however, we find it also exhibits some
constitutive activity, judged from modified fluorescence activation assays we conducted (not shown). This finding suggests that mutant T94I binds ATR in a manner that may cause isomerization of the retinal to the photoactive cis-species. Further experiments are necessary to more fully investigate this anomaly. We also find that mutation S186A has little effect on the ability of rhodopsin to activate transducin. In contrast, mutations Y268F and Y192F show significantly reduced ability to activate transducin (Figure 3B, Table I). This reduction in transducin activation is similar to other rhodopsin mutants that exhibit comparably perturbed photobleaching patterns (50).

Why might Y268F and Y192F show impaired ability to activate transducin? Notably, residue Y268F makes contact with the retinal 9-methyl and 13-methyl groups. Rhodopsin regenerated with retinal analogues lacking the 9-methyl group have previously been shown to be greatly impaired in their ability to activate transducin (56,57), These findings have lead to the “Steric Trigger” hypothesis, which suggest that movement of the retinal 9-methyl group acts as a mechanical switch to induce active MII rhodopsin formation (58), although it appears not to do so in cone pigments (59). Thus, mutation to Y268 (and possibly Y192) may alter interactions with the retinal 9-methyl (and 13-methyl groups), and in turn may result in less efficient coupling between retinal isomerization and rhodopsin activation. Consistent with this hypothesis, recent NMR studies suggest the 9-methyl group on retinal rotates >90° upon conversion to the MII state (60). Alternatively, these mutations may disrupt the counter-ion switch mechanism (as described above) and thus reduce the efficiency of MII formation and subsequent transducin activation. Finally, it is possible these mutations alter the ability of MII
rhodopsin to effectively couple with the C-terminal tail of the transducin α-subunit, thereby reducing functional activity (61).

*Dark-state rhodopsin is stabilized by the cooperative action of the hydrogen bond network.* All of the hydrogen bond mutants exhibited some decrease in dark-state thermal stability (Table II). Two mutants, T94I and E113Q were notably less stable. In addition, the Arrhenius plots of the thermal decay rates were linear for all mutants – in sharp contrast to the concave plot shown for WT rhodopsin (Figure 4). Previously, we suggested the concave Arrhenius plot exhibited for Schiff base hydrolysis by WT rhodopsin may be attributed to the presence of at least 2 different rate-limiting steps occurring during dark-state decay, suggesting some type of cooperative involvement (28,38). The present study shows that mutations in the retinal hydrogen bond network appear to abrogate this cooperative effect, and result in dark-state decay processes dependent upon only one rate-limiting step. Interestingly, at physiological temperatures (37 °C), the mutants also show higher activation energies ($E_a$) for hydrolysis than WT rhodopsin (Table II), which may indicate the disruption of an event (such as proton tunneling), which can occur more efficiently at lower temperatures in WT rhodopsin (28). Note that we put less emphasis on the $E_a$ values calculated at higher temperatures (55 °C). At these higher temperatures, denaturation of the protein may also be occurring, and thus these values may not really reflect the process that occurs in the folded protein.

The Arrhenius analysis of dark-state hydrolysis establishes that the hydrogen bond network mutations effect both the kinetics and energetics of dark-state Schiff base
The increased reactivity to hydroxylamine reactivity (Table II), also show the hydrogen bond network mutations result in increased exposure of the retinal linkage to the bulk solvent in the dark-state. Taken together, the data indicate that the hydrogen-bond network helps stabilize the retinal Schiff base in dark-state rhodopsin, both by affecting the chemistry of Schiff base hydrolysis and by limiting the accessibility of the Schiff base linkage to solvent.

*MII hydrolysis is facilitated by three key residues.* Arrhenius analysis of MII Schiff base hydrolysis and retinal release indicate three mutants (T94I, E113Q and S186A) show substantially slower rates of MII Schiff base hydrolysis. In contrast, mutants E181Q, Y192F and Y268F have only minor effects, both on the rates of hydrolysis and $E_a$ values (Figure 5). Why is the hydrolysis and release so much slower for mutants T94I, E113Q and S186A? Given the close proximity of these residues to the Schiff base attachment site and interaction with water molecules in the binding pocket, it is reasonable to suggest these residues are at least partially involved in the chemical event of Schiff base hydrolysis. Consistent with this interpretation, these mutants all exhibit altered $E_a$ for MII hydrolysis.

*Deuterium isotope studies support a tetrahedral carbinolamine intermediate is formed during hydrolysis.* The significantly slower rates of Schiff base hydrolysis in D$_2$O that we observe for both dark-state and MII (solvent isotope effect of 2.4 and 2.5 respectively) indicate that proton transfer events occur during the hydrolysis process (Figure 6). Furthermore, our proton inventory studies (the rate of hydrolysis plotted as a function of
D$_2$O concentration) generate a nonlinear plot(s). Non-linear proton inventory plots indicate that more than one time of flight proton is involved in the rate-limiting step of the hydrolysis reaction (62). The complexity in the process of dark-state retinal Schiff base hydrolysis indicated by the deuterium isotope studies is in good agreement with our concave Arrhenius plots for WT rhodopsin (discussed above), previous estimates (20), as well as the vibrational spectroscopy experiments, which clearly show that the Schiff base proton itself undergoes D→H exchange (63,64).

*Proposed mechanism of retinal Schiff base hydrolysis.* Taken together, our data clearly indicate proton exchange occurs during the rate limiting step for dark-state and MII Schiff base hydrolysis. Previous studies of model Schiff base compounds show a specific isotope effect of 2.3 (64,65), and from these studies, it was proposed that the transition state of the Schiff base hydrolysis reaction involves a protonated carbinolamine intermediate. The close similarity of our specific isotope effects (2.4 and 2.5) lend strong support that the same proposed transition state mechanism occurs during the hydrolysis of both forms of rhodopsin. Cooper and colleagues have previously carried out an extensive study of retinal Schiff base compounds, and proposed that base catalyzed water molecules may initiate Schiff base hydrolysis in rhodopsin, in a process that also proceeds through a protonated carbinolamine intermediate (20). Our new results are in agreement with those studies, and enable us to discuss an updated mechanistic scheme for retinal Schiff base hydrolysis in rhodopsin.
For the case of dark-state rhodopsin, we speculate that one of the water molecules within the retinal binding pocket (possibly Wat2b, see Figure 1) initiates hydrolysis by attacking the Schiff base linkage. This step is likely mediated by base catalysis from residue E113 and possibly orientated in part by residue T94. The resulting tetrahedral carbinolamine intermediate thus formed would then either be transiently protonated and then deprotonated by one of the neighboring water molecules, or by a neighboring residue. After the Schiff base linkage breaks, the noncovalently bound retinal could then either re-form the linkage in the reverse reaction, or be released, due to sterically unfavorable conditions in the opsin-binding pocket. We propose loop E-2 may function to shield the Schiff base linkage from solvent, as well as act as a “kinetic trap” that hinders release of retinal in dark-state rhodopsin, and encourage re-formation of the Schiff base linkage (28). It appears that such a function for loop E-2 can be perturbed by introducing mutations into the R177/D190 ion-pair at the ends of this loop (28), or by altering residues that interact with loop E-2, such H21 (66). Interestingly, mutations at either of these sites can result in Retinitis Pigmentosa. Conversely, it appears Schiff-base stability can be enhanced by “tying-down” the retinal plug with a K2C-D282C disulfide bond (67). Note that in the above hydrolysis mechanism, all of the steps with the exception of retinal leaving the binding pocket should be reversible. As such, similar interactions from residues participating in the hydrolysis mechanism may serve to catalyze this reaction. It is noteworthy that Oprian and colleagues have recently shown that the two residues we identified in our studies here as key players in Schiff base hydrolysis also play a key role in retinal Schiff base formation (46).
In the absence of structural data for MII rhodopsin, it is difficult to discern the precise role of the hydrogen bond network in catalyzing Schiff base hydrolysis, although recent NMR results suggest that the network does remains intact in the MII state (60). Note that our results clearly exclude a role for E181, Y192 and Y268 in MII Schiff base hydrolysis, whereas they suggest residues T94, E113 and S186 play some role in facilitating MII hydrolysis. It is tempting to speculate these latter residues act in catalysis as water activators or proton donors/acceptors, since the hydrolysis reaction requires Schiff base protonation to occur efficiently. This point leads to an interesting question – how can Schiff base hydrolysis even occur in MII rhodopsin, since a protonated Schiff base is not detected for rhodopsin by vibrational spectroscopy (22,68,69)? There are two likely possibilities: i) protonation is transient and followed by rapid hydrolysis, or ii) a small population of MII rhodopsin may exists in the protonated form that is not resolved by the above-mentioned techniques. This issue at the moment remains unresolved.

Conclusions. The present work indicates that a hydrogen bond network surrounding the retinal Schiff base works to regulate Schiff base hydrolysis in rhodopsin. In dark-state rhodopsin, the residues involved in this network act cooperatively to stabilize this linkage by slowing the rate of hydrolysis and retinal release, and by limiting the accessibility of the linkage to bulk solvent. Our mutagenesis studies suggest that two residues in this network, T94 and E113, play a key role in slowing the rate of hydrolysis in dark-state rhodopsin. In contrast, T94I and E113 (along with S186) help accelerate Schiff base hydrolysis and retinal release in the MII form of rhodopsin. Interestingly, our studies clearly indicate the process of hydrolysis is complex with multiple proton transfer events.
occur during hydrolysis in both the dark-state and MII rhodopsin forms. Future studies
will focus on further elucidating the mechanism of this fascinating and complex process
that plays a key role in the visual cycle.
REFERENCES


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FOOTNOTES

1 The Abbreviations used are: CNB, Congenital Night Blindness; D$_2$O, deuterium oxide; DM, $n$-dodecyl-β-maltoside; DS, dark-state rhodopsin; $E_a$, Energy of Activation; G-protein, guanine nucleotide-binding regulatory protein; GPCR, G-protein coupled receptor; $G_T$, heterotrimeric G-protein transducin; $G_{T\alpha}$, alpha subunit of transducin G-protein; GTP$_\gamma$S, guanosine 5'–3-O-(thio)triphosphate; $\lambda_{max}$, absorbance maxima; MES, 2-(N-Morpholino)-ehanesulfonic acid Monohydrate; MII, metarhodopsin II; nd, not determined; PMSF, phenylmethylsulfonyl fluoride; PBS, protonated Schiff-base; ROS, rod outer segment; TM, transmembrane; Tris, 2-Amino-2-hydroxymethyl-1,3-propanediol; W, water molecule; WT, wild-type rhodopsin.
Table I: Spectral and Functional Properties of Hydrogen Bond Network Mutants

(absorbance maxima, t_{1/2} of retinal release, transducin activation).

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Dark-State λ_{max} (nm)^a</th>
<th>MII State λ_{max} (nm)^a</th>
<th>MII Decay t_{1/2} (min)^b</th>
<th>G_T activation rate (%)^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>500</td>
<td>381</td>
<td>13.5</td>
<td>100</td>
</tr>
<tr>
<td>T94I</td>
<td>478</td>
<td>381</td>
<td>95.4</td>
<td>97</td>
</tr>
<tr>
<td>T94I-ATR^d</td>
<td>464</td>
<td>380</td>
<td>66.6</td>
<td>98</td>
</tr>
<tr>
<td>E113Q</td>
<td>385/495^e</td>
<td>378</td>
<td>126.2</td>
<td>96</td>
</tr>
<tr>
<td>E181Q</td>
<td>508</td>
<td>381</td>
<td>6.8</td>
<td>78</td>
</tr>
<tr>
<td>S186A</td>
<td>500</td>
<td>384</td>
<td>51.4</td>
<td>95</td>
</tr>
<tr>
<td>Y192F</td>
<td>491</td>
<td>386</td>
<td>5.8</td>
<td>38</td>
</tr>
<tr>
<td>Y268F</td>
<td>495</td>
<td>386</td>
<td>6.8</td>
<td>19</td>
</tr>
</tbody>
</table>

^a All λ_{max} values are determined from the 1st derivative of the raw spectral data and estimated to within ± 1 nm.

^b MII decay (retinal release) assays performed at 20 °C in buffer A as described in Experimental Procedures.

^c The relative initial rate of G_T activation is represented by the rate of fluorescence increase obtained from the slope of the fluorescence measurements in the first 60 s after addition of GTPγS relative to that of WT rhodopsin. The averages of n = 3 experiments are shown except for T94I-ATR (n = 1).

^d T94I-ATR is the T94I opsin mutant regenerated with all-trans-retinal.

^e The λ_{max} of mutant E113Q is pH dependent (see Results).
Table II: Kinetic and thermodynamic parameters of Schiff base stability studies on hydrogen bond network rhodopsin mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>DS hydrolysis $t_{1/2}$ 37 ºC (min)$^a$</th>
<th>DS hydrolysis $E_a$ 37 ºC (kcal/mol)$^b$</th>
<th>DS hydrolysis $t_{1/2}$ 55 ºC (min)$^a$</th>
<th>DS hydrolysis $E_a$ 55 ºC (kcal/mol)$^b$</th>
<th>MII hydrolysis $E_a$ (kcal/mol)$^b$</th>
<th>Hydroxyl-amine reactivity $t_{1/2}$ 37 ºC (min)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3100 ± 45</td>
<td>16.1</td>
<td>38.5</td>
<td>103</td>
<td>20.5</td>
<td>3100</td>
</tr>
<tr>
<td>T94I</td>
<td>103</td>
<td>53.5</td>
<td>0.9</td>
<td>53.5</td>
<td>25.4</td>
<td>19.5</td>
</tr>
<tr>
<td>T94I-ATR$^d$</td>
<td>66</td>
<td>nd</td>
<td>0.8</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>E113Q</td>
<td>29</td>
<td>32.1</td>
<td>1.8</td>
<td>32.1</td>
<td>39.3</td>
<td>4.8</td>
</tr>
<tr>
<td>E181Q</td>
<td>2150</td>
<td>58.5</td>
<td>12.1</td>
<td>58.8</td>
<td>21.7</td>
<td>58</td>
</tr>
<tr>
<td>S186A</td>
<td>1032</td>
<td>49.5</td>
<td>2.4</td>
<td>89.2</td>
<td>33.4</td>
<td>14.9</td>
</tr>
<tr>
<td>Y192F</td>
<td>958</td>
<td>51.2</td>
<td>10.5</td>
<td>49.3</td>
<td>14.7</td>
<td>21</td>
</tr>
<tr>
<td>Y268F</td>
<td>1152</td>
<td>80.8</td>
<td>1.1</td>
<td>74.5</td>
<td>21.7</td>
<td>13.9</td>
</tr>
</tbody>
</table>

$^a$ Dark-state thermal decay rates obtained from mono-exponential fits of dark-state thermal hydrolysis absorbance measurements at respective temperatures.

$^b$ Activation energies ($E_a$) obtained from linear regression of Arrhenius plots.

$^c$ Hydroxylamine reactivity rates determined from mono-exponential fits of dark-state thermal hydrolysis experiments performed at pH 6.0, 37 ºC in the presence of 50 mM pH buffered hydroxylamine. For further details see Experimental Procedures.

$^d$ T94I-ATR is the T94I opsin mutant regenerated with all-trans-retinal.
FIGURE LEGENDS

FIGURE 1: Residues involved in forming a hydrogen bond network around the retinal Schiff base linkage in rhodopsin. (A) Two-dimensional diagram of rhodopsin illustrating residues investigated in this study. Hydrogen bond network residues are indicated (T94, Y192 and Y268 in green with E113 and E181, in red). The attachment site for the retinal Schiff base linkage is shown in blue (K296). Residues C110 and C187 that form a disulfide bond in the extracellular region are shaded gray. (B) Three-dimensional model of rhodopsin illustrating residues and water molecules involved in the formation of a hydrogen bond network that encompasses the retinal Schiff base linkage. Individual amino acids are labeled and waters are shown as blue spheres. The retinal Schiff base (SB) attachment site at residue K296 is shown in blue. The backbone of residue C187 also participates in this network (not shown). Portions of the protein surrounding the retinal binding pocket have been removed for clarity. The model is based on the rhodopsin crystal structure (Protein Data Bank accession #: 1L9H, (23)) and generated using the program Weblab.

FIGURE 2: UV/vis absorption profiles of purified rhodopsin hydrogen bond network mutants. Photobleaching properties are shown for each mutant relative to WT: dark-state (solid), following 30 s bleaching (Meta II state - dotted), and immediately following acid denaturation of the photobleached species to a pH 1.9 (dashed). All spectra recorded in buffer D, pH 6.0, at 20 °C. The ~ 480 nm shoulder species present in mutant Y268F following photobleaching is followed as a function of time in Figure 3. All absorbance values are presented in Table I.
FIGURE 3: Spectral and functional properties of mutant Y268F in comparison with WT rhodopsin. (A) Photobleaching properties of mutant Y268F relative to WT rhodopsin. Samples were photobleached for 15 s in buffer D, pH 6.0 at 20°C. Upon illumination, Y268F (top) exhibits a spectral shoulder centered around ~480 nm. This species persists even 500 min post-illumination. In contrast, WT rhodopsin (bottom) exhibits no ~480 nm species under these conditions immediately following photobleaching. The arrow in the lower panel indicates a slow rise over time of a 480 nm species – the MIII intermediate. (B) Transducin activation by mutant Y268F and WT rhodopsin. Transducin activation was monitored by following the increase in tryptophan fluorescence, which occurs upon MII stimulation of the transducin-GTPγS complex formation. Relative to WT rhodopsin, the functional activity of Y268F is impaired. The relative rates of transducin activation for this and all mutants compared to WT rhodopsin are shown in Table I.

FIGURE 4: Arrhenius plots of dark-state thermal decay rates. Rates were obtained from absorbance decay or fluorescence retinal release assays (E113Q) and plotted as a function of temperature. All mutations show accelerated rates of dark-state decay suggesting a kinetic effect. Additionally, Arrhenius analysis of the rates indicate that all of the mutants show linear plots (contrast to the concave plot WT) suggesting the reaction energetics have changed as well. All measurements were carried out in buffer D at pH 6.0 over a temperature range of 36 to 55°C. Kinetic and thermodynamic parameters are provided in Table II.
FIGURE 5: Hydrogen bond network rhodopsin mutations show location dependent effects on Schiff base stability in the MII state. Arrhenius plots of retinal release rates for hydrogen bond network mutations in buffer D at pH 6.0 over a temperature range of 5 to 35 °C. (A) Mutants E181Q, Y192F and Y268F show no dramatic effect on MII Schiff base linkage stability compared to other hydrogen bond network mutation sites. (B) In contrast to their diminished dark-state stabilities in the MII state (see Figure 4) the Schiff base linkage of mutants T94I, E113Q and S186A are all significantly more stable than WT rhodopsin. Kinetic and thermodynamic parameters for these retinal release experiments are provided in Table II.

FIGURE 6: Kinetic isotope effects of retinal Schiff base hydrolysis and retinal release. Dark-state thermal decay assays and MII retinal release assays were conducted in D₂O buffer D (■, solid line) and rates compared to samples prepared in an identical fashion in H₂O buffer D (●, dashed line). (A) The rate of dark-state thermal decay for WT rhodopsin at 37 °C is substantially slower in D₂O relative to H₂O buffer D and exhibits a solvent isotope effect of 2.4. (B) Proton inventory of deuterium solvent isotope effect on the dark-state thermal decay process (●). The data were best fit to a cubic equation (solid line), the linear fit is shown as a dotted line for comparison purposes. (C) Retinal release assay on WT, MII rhodopsin at 20 °C, pH 6.0, in buffer D prepared in either H₂O (●, dashed line) or D₂O (black). (D) Proton inventory of deuterium solvent isotope effect on retinal release process in MII rhodopsin (■). The data fit best to a cubic equation (solid line) the linear fit is shown as a dotted line. For further details see Experimental Procedures and Discussion.
FIGURE 1

(A) Cytoplasmic side

(B) Retinal binding site

- K296
- SB
- W2b
- W2a
- E181
- Y268
- S186
- E113
- Y192
- T94
FIGURE 5

(A) 

\[ \ln k \ (s^{-1}) \]

-12
-10
-8
-6

\[ \frac{1}{T} \times 1000 \ (K^{-1}) \]

WT
E181Q
Y192F
Y268F

(B) 

\[ \ln k \ (s^{-1}) \]

-12
-10
-8
-6

\[ \frac{1}{T} \times 1000 \ (K^{-1}) \]

WT
T94I
E113Q
S186A

43
FIGURE 6

(A) Absorbance 500 nm (normalized) over time (h).

(B) Ratio of $k_D/k_H$ as a function of mole fraction $D_2O$.

(C) Change in fluorescence at 330 nm ($\Delta F$) with time (s) for $H_2O$ and $D_2O$.

(D) Ratio of $k_D/k_H$ as a function of mole fraction $D_2O$.
Role of retinal hydrogen bond network in rhodopsin Schiff base stability and hydrolysis
Jay M. Janz and David L. Farrens

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