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

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Jay M. Janz‡ and David L. Farrens§
From the Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, Oregon 97239

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 affects the hydrolysis process in wild-type rhodopsin, exhibiting a specific isotope effect of $\sim$2.5, and proton inventory studies indicate that multiple proton transfer events occur during the process of Schiff base hydrolysis for both dark state and MII forms. Taken together, our study demonstrates 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.

Rhodopsin, the dim light photoreceptor of rod cells, is arguably the best characterized member of the class A superfamily of GPCR (1–7). A transmembrane receptor, it has evolved into an efficient photoreceptor by covalently binding its chromophore, 11-cis-retinal, to lysine 296 via a protonated Schiff base linkage within the helical bundle (8, 9). Dim light vision begins when the 11-cis-retinal chromophore absorbs a photon and isomerizes to the all-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-trans-retinal from the binding pocket, or the MI pool may undergo an addition thermal isomerization along the chromophore C=N double bond (all-trans 15-syn) giving rise to the MIII storage product (10). This MIII intermediate also decays to opsin and all-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-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-trans-retinal back to the 11-cis conforma- tion 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). Although 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, and as a result the turnover and regeneration rates for cone cells are significantly faster than for rod cells (16–19). Furthermore, although 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 probably 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 that 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 the this study 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 Thr114 and Glu115). 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 (Thr114, Glu115 and Ser186) actually slows MII decay, suggesting that these residues act as participants in Schiff base hydrolysis and subsequent 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 intermediae. 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 or Sigma. n-Dodecyl β-D-maltoside (DM) was purchased from Anatrace (Maumee, OH). Polystyrene columns (2-m bed volume) were purchased from Pierce. Frozen bovine retinas were obtained from A. Lawson (University of California, NE). Trichloroacetic acid was purified from rod outer segments as described previously (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 (0.5 μm volume, 10-kDa cut off) were purchased from Millipore (Billericia, MA). 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 mM 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 (7941, E113Q, E181Q, S186A, Y192F, and Y268F) in the synthetic bovine rhodopsin gene (26) essentially as described previously and subcloned into the E. coli expression vector pMT4 (27, 28). 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 the cell harvests were made 56–72 h after transfection as described previously (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 μl 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-visible Absorption Spectroscopy—**All UV-visible absorption spectra were recorded with a Shimadzu UV-1800 spectrophotometer at 20 °C using a 1-cm path length, of 2 nm, a 1-nm bandwidth and a scan speed of 500 nm/min unless otherwise noted. For concentration calculations, the molar extinction coefficient value (ε₅₅₀) 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 activation of WT rhodopsin. The samples were then exchanged into the PBSD buffer for the MII state of each mutant was verified by adding H₂SO₄ 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 protonated Schiff base) (33). Photobleaching time course analysis on mutant Y268F was performed as above but with bleaching time reduced to 15 s, and results compared with a WT rhodopsin irradiated in the exact same manner.

Determination of Transducin (GT) Activation Rates—Activation of Gₛ 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 μM) was added to the reaction mixture consisting of 250 nM Gₛ in 10 mM Tris (pH 7.2), 2 mM MgCl₂, 100 mM NaCl, 1 mM diithiothreitol, and 0.01% DM. Thus the mixture was allowed to stir for 300 s; then the reaction was initiated by the addition of Gₛ in 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 Gₛ injection were determined through the data points covering the first 60 s using linear regression analysis. Mutant rhodopsin activation values were reported as percentages relative to WT, which was taken to be 100%.

**Thermal Bleaching of Rhodopsin Samples—**Thermal decay rates were followed by UV-visible spectroscopy in buffer A essentially as described previously (28, 38). The thermal stability of each mutant was determined by first monitoring the absorbance of each sample from 650–250 nm at 1-min intervals as at a given temperature. Thermal decay rates were subsequently obtained by monitoring the decrease of the 500-nm absorbance at each time point from these measurements (29–41). Baseline drift was corrected for by normalizing all spectra to an absorbance of zero at 650 nm. For E113Q, thermal decay rates were measured by monitoring the increase in tryptophan fluorescence at 330 nm, which was caused by the release of retinal from the chromophore-binding pocket (37) as described previously (28, 38). The experimental setup for these assays is similar to that of the retinal release assay (described above) except that the samples were not photobleached during the assay. All thermal decay data was analyzed using monoe-xponential decay (absorbance experiments) or monoe-xponential rise to maximum (fluorescence experiments) fitting algorithms in Sigma Plot (Jandel Scientific Software). E₅₀ values for mutant thermal decay assays were determined by applying the rate data to the Arrhenius equation: k = (E₅₀)⁻¹ = Ae⁻(Ea/(RT)), as described previously (39).

**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 the sample temperature was maintained as described above. After the samples were photobleached to the MII state (see above), 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 was repeated until a plateau in the decay was achieved. The temperature dependence of the rate decay, experimental data were analyzed using a monoe-xponential rise to maximum fit in Sigma Plot. 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⁻(Ea/(RT)), to determine the E₅₀ value 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”).

**Effect on Dark 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 (D₂O) and were then used to make a 0.05% DM, 5 mM MES (pH 6.0) (buffer D) D₂O exchange buffer. The pH of the D₂O experiments ranged between 6.0 and 6.4 because 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 5.0–7.0, the Emötsch slight pH change should not alter interpretation of the results. Using a concentrated stock of purified rhodopsin (to use a small volume for dilution) two equal molar stocks of both D₂O and H₂O rhodopsin were prepared. These samples (500 μl) were then buffer-exchanged using Millipore Ultrafree 0.5 centrifugal filter devices at low spin speeds. The D₂O sample was washed eight times with 500 μl of D₂O-containing buffer, whereas the H₂O sample was washed in the exact same method using H₂O buffer to ensure that any detergent

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concentration that may have taken place during the exchange was 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 were obtained from three separate experiments from two 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 0–1.0. The dark state thermal decay rate or the MII decay rate of WT rhodopsin was moni-

Fig. 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 (Thr94, Tyr192, and Tyr268 in green with Glu113 and Glu181 in red). The attachment site for the retinal Schiff base linkage is shown in blue (Lys296). Residues Cys110 and Cys187 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 Lys296 is shown in blue. The backbone of residue Cys187 also participates in this network (data 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 number 1L9H, (23)) and generated using the program Weblab. Note that the proposed Glu113 to Thr94 hydrogen bond is based on molecular dynamic studies rather than crystal structures (71).
with the disease congenital night blindness (44–46).

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 potential role in this hydrogen bond network but also because mutant T94I is associated with linear plots in contrast to WT rhodopsin, which displays a "shoulder" species centered at ~480 nm immediately following illumination (Fig. 2). Interestingly, this species slightly decayed over time but was still present up to 500 min following bleaching (Fig. 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 G_{T,\text{ATR}}-GTP-S species (34–36). The slight decreases in initial rates for mutants T94I, E113Q, and E181Q were in agreement with values reported previously (43, 45, 47). In addition, our results indicate that T94I regenerated with all-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 did detect a slight level (~15%) of constitutive activity in the dark state for this mutant (not shown). Furthermore, our results demonstrate that the new mutants Y192F and Y268F are much less able to activate transducin compared with WT rhodopsin. This is perhaps most striking for mutant Y268F, which exhibits only ~20% of the functional activity of WT rhodopsin (Fig. 3B). The results for transducin activation are compiled in Table I.

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. $E_a$ values for the dark state thermal decays were obtained from Arrhenius analysis of the decay data as described previously (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 (Fig. 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 sites, 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

![FIG. 2. UV-visible absorption profiles of purified rhodopsin hydrogen bond network mutants.](http://www.jbc.org/)
Deuterium isotope exchange experiments were conducted on WT rhodopsin to further investigate the role of water 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 that suggested water molecules also lie within the retinal binding pocket (23) (in agreement with previous studies (51, 52)) and suggested that a hydrogen bond network surrounds the retinal Schiff base (23). The present report details the structural and functional implications that 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 (Fig. 2). Because 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 λmax values. We find the absorbance properties for mutants E113Q, T94I, E113Q, 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 (Fig. 2, Table I). The absorbance shift in these two mutants may be caused by 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 protonated Schiff base upon acid denaturation (Fig. 2). However, mutant Y268F (and to a lesser extent Y192F) demonstrate a residual absorbing species with a λmax of ~480 nm following illumina-

**Spectral and functional properties of hydrogen bond network mutants (absorbance maxima, t1⁄2 of retinal release, transducin activation)**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Dark state λmaxa</th>
<th>MII state λmaxa</th>
<th>MII decayb t1⁄2</th>
<th>Gα activation ratec</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>
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<td>464</td>
<td>380</td>
<td>66.6</td>
<td>98</td>
</tr>
<tr>
<td>E113Q</td>
<td>385/495</td>
<td>378</td>
<td>128.2</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 λmax values are determined from the first 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 under "Experimental Procedures.”
c The relative initial rate of photo-induced Gα 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 GTP-γ-S 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 λmax of mutant E113Q is pH dependent (see “Results”).

**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).
Comparison with WT rhodopsin. Y268F is impaired. The relative rates of transducin activation for this complex formation. Relative to WT rhodopsin, the functional activity of rescence, which occurs upon MII stimulation of the transducin-GTP transition (Fig. 2), which slightly decays over time, yet still retain some residual ~480 nm absorbance (see Fig. 3A for an example). In contrast, WT rhodopsin under identical conditions exhibits only a slow, slight increase in ~480 nm absorbance, presumably because of the formation of the MII 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 Glu181 in the dark state to residue Glu181 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 Glu181 and thus result in inefficient counter-ion transfer and possible build up or trapping of photointermediate species MI or MIII. Further studies, particularly Fourier transform infrared spectroscopy, 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 that 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 as judged from modified fluorescence activation assays we conducted (data not shown). This finding suggests that mutant T94I binds all-trans-retinal in a manner that may cause isomerization of the retinal to the phototoxic 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 a significantly reduced ability to activate transducin (Fig. 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 suggests 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 Tyr268 (and possibly Tyr192) 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 (Fig. 4). Previously, we suggested that the concave Arrhenius plot exhibited for Schiff base hydrolysis by WT rhodopsin may be attributed to the presence of at least two 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 $E_a$ values 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 occur, 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 hydrolysis. The increased hydroxylamine reactivity (Table II) also show that 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 that 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 (Fig. 5).

### Table II

<table>
<thead>
<tr>
<th>Mutant</th>
<th>DS hydrolysis$^a$ ($t_{1/2}$ 37 °C)</th>
<th>DS hydrolysis$^b$ ($t_{1/2}$ 55 °C)</th>
<th>DS hydrolysis$^c$ ($E_a$ 37 °C)</th>
<th>MII hydrolysis$^d$ ($E_a$ 55 °C)</th>
<th>Hydroxylamine reactivity$^e$ ($t_{1/2}$ 37 °C)</th>
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<tr>
<td>WT</td>
<td>3100 ± 45</td>
<td>16.1</td>
<td>38.5</td>
<td>103</td>
<td>20.5</td>
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<tr>
<td>T94I</td>
<td>103</td>
<td>53.5</td>
<td>0.9</td>
<td>53.5</td>
<td>25.4</td>
</tr>
<tr>
<td>T94I-ATR</td>
<td>66</td>
<td>ND$^f$</td>
<td>0.8</td>
<td>ND$^f$</td>
<td>ND$^f$</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>
</tr>
<tr>
<td>E181Q</td>
<td>2150</td>
<td>58.5</td>
<td>12.1</td>
<td>58.8</td>
<td>21.7</td>
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<tr>
<td>S186A</td>
<td>1032</td>
<td>49.5</td>
<td>2.4</td>
<td>89.2</td>
<td>33.4</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>
</tr>
<tr>
<td>Y268F</td>
<td>1152</td>
<td>80.8</td>
<td>1.1</td>
<td>74.5</td>
<td>21.7</td>
</tr>
</tbody>
</table>

$^a$ The $t_{1/2}$ for dark state (DS) thermal decay was obtained from monoeponential 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 values determined from monoeponential fits of dark state thermal hydrolysis experiments performed at pH 6.0, 37 °C 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.

$^e$ ND, not determined.

**FIG. 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 indicates that all of the mutants show linear plots (contrast to the concave plot WT) suggesting that the reaction energetics have changed as well. All measurements were carried out in buffer D at pH 6.0 over a temperature range of 36–55 °C. Kinetic and thermodynamic parameters are provided in Table II.

**FIG. 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 5–35 °C. A, mutants E181Q, Y192F, and Y268F show no dramatic effect on MII Schiff base linkage stability compared with other hydrogen bond network mutation sites. B, in contrast to their diminished dark state stabilities in the MII state (see Fig. 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.

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 that 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 That A Tetrahedral Carbosilamine 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 (Fig. 6). Furthermore, our proton inventory studies (the rate of hydrolysis plotted as a function of D$_2$O concentration) generate non-linear 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
Rhodopsin at 37 °C is substantially slower in D2O relative to H2O buffer were compared with samples prepared in an identical fashion in H2O.

The data were best fit to a cubic equation; the linear solvent isotope effect on the dark state thermal decay process 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. C, retinal release assay on WT, MII rhodopsin at 20 °C (pH 6.0) in buffer D prepared in either H2O (solid line) or D2O (dotted line). 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.”

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 that 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 carbazolamine intermediate. The close similarity of our specific isotope effects (2.4 and 2.5) lend strong support for the idea that the same proposed transition state mechanism occurs during the hydrolysis of both forms of rhodopsin. Cooper et al. (20) 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 carbazolamine intermediate. 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 Wat2h, see Fig. 1) initiates hydrolysis by attacking the Schiff base linkage. This step is likely mediated by base catalysis from residue Gly113 and possibly orientated in part by residue Thr94.

The resulting tetrahedral carbazolamine intermediate thus formed would then 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 because of sterically unfavorable conditions in the opsin-binding pocket. We propose that 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 reformation of the Schiff base linkage (28). It appears that such a function for loop E-2 can be perturbed by introducing mutations into the Arg177/Asp190 ion pair at the ends of this loop (28) or by altering residues that interact with loop E-2, such His211(66).

Interestingly, mutations at either of these sites can result in retinitis pigmentosa. Conversely, it appears that 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 co-workers 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 remains intact in the MII state (60). Note that our results clearly exclude a role for Glu181, Tyr192, and Tyr268 in MII Schiff base hydrolysis, whereas they suggest that residues Thr24, Glu113, and Ser186 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 because 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 exist 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, Thr24 and Glu113, play a key role in slowing the rate of hydrolysis in dark state rhodopsin. In contrast, Thr24 and Glu113 (along with Ser186) help accelerate Schiff base hydrolysis and retinal release in the MII form of rhodopsin. Furthermore, our studies clearly indicate that 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.

Acknowledgments—We thank Dr. Mike Jackson (Oregon Health and Science University) and Dr. John Denu (University of Wisconsin-Madison) for helpful discussions regarding kinetics and isotope experiments.

Note Added in Proof—Two new rhodopsin structures have been published since the acceptance of this manuscript (70, 71). Importantly, both structures support the hydrogen bonding network and water molecules discussed in the present work.

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

Jay M. Janz and David L. Farrens

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