Chemical Kinetic Analysis of Thermal Decay of Rhodopsin Reveals Unusual Energetics of Thermal Isomerization and Hydrolysis of Schiff Base

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The thermal properties of rhodopsin, which set the threshold of our vision, have long been investigated, but the chemical kinetics of the thermal decay of rhodopsin has not been revealed in detail. To understand thermal decay quantitatively, we propose a kinetic model consisting of two pathways: 1) thermal isomerization of 11-cis-retinal followed by hydrolysis of Schiff base (SB) and 2) hydrolysis of SB in dark state rhodopsin followed by opsin-catalyzed isomerization of free 11-cis-retinal. We solve the kinetic model mathematically and use it to analyze kinetic data from four experiments that we designed to assay thermal decay, isomerization, hydrolysis of SB using dark state rhodopsin, and hydrolysis of SB using photoaivated rhodopsin. We apply the model to WT rhodopsin and E181Q and S186A mutants at 55 °C, as well as WT rhodopsin in H2O and D2O at 59 °C. The results show that the hydrogen-bonding network strongly restrains thermal isomerization but is less important in opsin and activated rhodopsin. Furthermore, the ability to obtain individual rate constants allows comparison of thermal processes under various conditions. Our kinetic model and experiments reveal two unusual energetic properties: the steep temperature dependence of the rates of thermal isomerization and SB hydrolysis in the dark state and a strong deuterium isotope effect on dark state SB hydrolysis. These findings can be applied to study pathogenic rhodopsin mutants and other visual pigments.

Although rhodopsin is known to have single-photon sensitivity, more photons are required to elicit a sensation of light in humans (11). Visual sensitivity can be limited by two factors that cause aberrant, false visual signaling: discrete dark noise and constitutive activation of rhodopsin. Discrete dark noise was first observed in 1972 by Srebro and Behbehani (12), who recorded electrophysiological signals generated by thermally induced chemical changes of visual pigments. The signals were identical to those generated by single photons. Baylor and coworkers (11, 13–15) further demonstrated that discrete dark noise of rhodopsin originates from spontaneous thermal isomerization of 11-cis-retinal. On the other hand, in some rhodopsin mutants, the SB linking retinal to opsin hydrolyzes, resulting in opsin that can constitutively activate transducin, causing partial or complete saturation of the rod response and desensitizing dim light vision. Rao et al. (16) first demonstrated constitutive activation in the G90D mutant, which is implicated in congenital stationary night blindness. These previous studies suggest that rhodopsin must minimize thermal isomerization and hydrolysis of SB to function as a sensitive photoreceptor. Hence, the contributions of thermal isomerization and SB hydrolysis in the thermal decay process of rhodopsin are crucial to understanding dim light vision.

The thermal properties of rhodopsin have been studied since the 1950s, when Hubbard (17) observed that rhodopsin thermally bleaches at 75 °C in the dark. This raised the question of the mechanism of thermal bleaching. Del Valle et al. (18) observed thermal isomerization in purified bovine rhodopsin and detected an increase of all-trans-retinal extracted from rhodopsin undergoing thermal decay at 55 °C in the dark. Around the same time, Janz et al. (19, 20) measured the rate of thermal decay of purified rhodopsin and rhodopsin mutants by monitoring the decrease of the 500-nm absorption and increase of 380-nm absorption. They attributed the thermal decay not to thermal isomerization as Del Valle proposed but rather to hydrolysis of the protonated SB linkage, which yields unbound 11-cis-retinal and opsin. Finally, Lórenz-Fonfría et al. (21) studied the temperature dependence of hydrogen-deuterium exchange in rhodopsin. They proposed a two-step model to illustrate the dark activation of rhodopsin, whereby the retinal-binding pocket opens transiently followed by thermal isomerization. They concluded that the sensitivity of rhodopsin is limited by structural fluctuations of the binding pocket rather than of 11-cis-retinal. Based on these studies, we wished to elucidate the mechanism behind the thermal decay of rhodopsin, specif-
ically the contributions of thermal isomerization and hydrolysis of SB.

We designed experiments to study the thermal processes of rhodopsin and showed that thermal decay consists of both thermal isomerization of retinal and hydrolysis of the SB (22, 23). What molecular properties control the kinetics of these thermal processes? Crystal structures suggested the presence of an extensive hydrogen-bonding network involving ~20 conserved structural water molecules, hydrophilic residues, and the protein backbone in the transmembrane region (7, 24–28). We hypothesized that this network might stabilize the dark state, preventing thermal decay (22, 23). We discovered that at 59 °C rhodopsin undergoes thermal decay, isomerization, and hydrolysis of SB two or three times more slowly in D2O than in H2O, consistent with the fact that stronger hydrogen bonds form in D2O (22). We also introduced the mutations E181Q and S186A to disrupt hydrogen bonds in the binding site of rhodopsin (23). At 55 °C, the mutations increase the rates of thermal decay, isomerization, and hydrolysis of SB by almost 2 orders of magnitude compared with WT rhodopsin. Thus, we demonstrated the importance of the hydrogen-bonding network in stabilizing dark state rhodopsin and preventing thermal activity, which allows for dim light vision.

Still, questions remain. We previously analyzed the data from thermal decay, isomerization, and hydrolysis experiments using single-exponential fitting (22, 23). However, this method does not account for overlaps between the thermal processes measured by individual experiments, which precludes the solution of discrete rate constants. Our aim now is to achieve a quantitative description of all thermal processes involved in the thermal decay of rhodopsin, which will enable the quantitative analysis of effects on individual processes under various conditions, such as different temperatures, mutations, and isotope exchange.

To this end, we have built a kinetic model containing two pathways of thermal decay (Fig. 1). In the first pathway, 11-cis-retinal isomerizes in the binding site of rhodopsin, thermally activating the photoreceptor; then the SB hydrolyzes to yield all-trans-retinal and opsin. In the second pathway, the protonated SB dark state rhodopsin hydrolyzes; then opsin catalyzes the isomerization of the free 11-cis-retinal to all-trans-retinal.

This kinetic model can be solved to analyze kinetic data obtained in the following four experiments that assay thermal processes (Fig. 1). First, we acquired the rate of hydrolysis of SB in Meta II independently by acid denaturation of Meta II followed by UV-visible spectroscopy. Second, using UV-visible absorption spectra, we measure the rate of thermal decay of dark state rhodopsin, which includes hydrolysis of the SB and isomerization of retinal. Next, using HPLC analysis, we measure the rates of thermal isomerization of the retinal, which includes the isomerization of both dark state rhodopsin and 11-cis-retinal free in solution in the presence of opsin. Finally, using UV-visible absorption spectra following acid denaturation of the sample, we obtain the rates of hydrolysis processes, which include hydrolysis of SB in both dark state rhodopsin and Meta II.

In this paper, we describe our kinetic model, corresponding experiments, and the mathematical solution to the model. We perform experiments to measure the rate of SB hydrolysis in Meta II and apply our model to our previously reported data obtained for WT rhodopsin and the E181Q and S186A mutants at 55 °C and for WT rhodopsin in H2O and D2O at 59 °C (22, 23). Using global fitting, we determine the rate constants for all thermal processes, which allows a quantitative study of the
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effect of mutations, D₂O, and temperature on the kinetics of individual chemical reactions involved in the thermal decay process (Fig. 1). Our analyses reveal two unique kinetic properties of rhodopsin: an unusually high isotopic effect on the hydrolysis of SB in the dark state and an extremely steep temperature dependence of rates for both SB hydrolysis and thermal isomerization. The results point to a sophisticated molecular mechanism behind the thermal stability of rhodopsin, which enables the protein to function as a sensitive photoreceptor. A better understanding of the thermal properties of rhodopsin at the molecular level could provide insight into the mechanism of visual diseases and the molecular evolution from cone to rod visual pigments.

EXPERIMENTAL PROCEDURES

Preparation of Rhodopsin—Recombinant WT bovine rhodopsin and the E181Q and S186A mutants were expressed in HEK293 stable cell lines and purified using the immunoaffinity method, as previously described (22, 23, 29). The rhodopsin samples were concentrated to ~20 μM in Buffer A (50 mM sodium phosphate, 0.1% n-dodecyl-β-D-maltopyranoside, pH 6.5) for the kinetic measurements. For the D₂O experiments, the sample was exchanged to Buffer B (50 mM sodium phosphate in D₂O, 0.1% n-dodecyl-β-D-maltopyranoside, pH 6.5) using a Biomax-30K NMWL centrifugal tube (0.5 ml; Millipore) as previously described (22).

Hydrolysis of the SB in Meta II—We monitored the hydrolysis of the deprotonated SB in the active state of rhodopsin, Meta II, by UV-visible absorption spectroscopy (22). A volume of 0.1 ml of concentrated rhodopsin sample (20 μM) was photobleached by a 30-W illuminator for 30 s at 20 °C so that 11-cis-retinal was completely converted to all-trans-retinal by light, forming the active Meta II state. Immediately after bleaching, at time t = 0, the Meta II sample was added into 0.9 ml of Buffer A, which was equilibrated at 55 or 59 °C. At various time points, approximately ten 100-μl samples were collected from the cuvette, transferred immediately to precooled vials, and stored on ice to quench further thermal processes. Each sample was denatured by mixing with 4 μl of 1 M HCl. The pH of the solution was confirmed to be 1–2. The denatured samples were analyzed by UV-visible spectroscopy. Retinal covalently linked to denatured opsin protein with a protonated SB absorbs at 440 nm, whereas free retinal in solution, the product of hydrolysis of the SB, absorbs at 380 nm. Hence, the amount of hydrolyzed SB can be monitored by the decrease of the optical density at 440 nm (A₄₄₀). The measured A₄₄₀ was normalized to the initial value, A₄₄₀ (t = 0), and plotted as a function of time. The decrease of the 440-nm peak reveals the hydrolysis of SB.

Thermal Decay—Thermal decay was monitored using UV-visible absorption spectroscopy as described previously (22). In brief, to initiate the thermal decay process, at t = 0 a volume of 0.3 ml of concentrated rhodopsin sample (20 μM) was added into 2.7 ml of Buffer A, which was equilibrated at 55 or 59 °C. UV-visible absorption spectra were taken at various time points. The peaks at 280 and 500 nm represent the absorption of opsin and rhodopsin, respectively. Each spectrum was normalized to A₅₀₀ to account for solvent evaporation. Over time, the 500-nm absorbance decreases. The accompanying increase in absorbance at 380 nm could represent formation of Meta II, the active state of rhodopsin, and/or free 11-cis- or all-trans-retinal after hydrolysis of the SB. Because Rho is the only species absorbing 500-nm light in the system, the concentration of Rho is proportional to the optical density at 500 nm (A₅₀₀). The measured A₅₀₀ was normalized to the initial value, A₅₀₀ (t = 0), and plotted as a function of time. The decrease of the 500-nm peak reveals the thermal decay of rhodopsin.

Thermal Isomerization—During the measurement of thermal decay, approximately twelve 200-μl samples were collected from the cuvette at various time points, transferred immediately to precooled vials, and stored on ice. These samples were divided into two parts for thermal isomerization and SB hydrolysis experiments. The extent of the thermal isomerization of the sample was monitored by extracting retinal in the form of retinaloximes from the samples and analyzing the isomeric forms using HPLC, as described elsewhere (22). Six peaks were observed in the HPLC chromatograms, corresponding to 11-cis-15-syn-, all-trans-15-syn-, 13-cis-15-syn-, 13-cis-15-anti-, 11-cis-15-anti-, and all-trans-15-anti-retinaloximes (30). To determine the fraction of 11-cis-retinal at each time point, the area of the 11-cis peak was normalized to the sum of all six peak areas and the corresponding extinction coefficients for each isomeric form at 360 nm (30). The normalized fraction of 11-cis-retinal was plotted as a function of time. The decrease of the normalized fraction of 11-cis-retinal in the samples indicates thermal isomerization.

Hydrolysis of the SB Using Rhodopsin—Turning to the second component of thermal decay, we monitored the hydrolysis of the SB in dark state samples by UV-visible absorption spectroscopy (22). The samples taken at various time points during thermal decay were denatured with 4 μl of 1 M HCl and analyzed by UV-visible spectroscopy. As discussed previously, the A₄₄₀ is characteristic of an intact protonated SB, whereas the A₄₄₀ is characteristic of free 11-cis- or all-trans-retinal. The decrease of the 440-nm peak reveals the hydrolysis of SB in dark state rhodopsin.

Solution of the Kinetic Model—All of the above experiments yield kinetic data that can be fitted into the kinetic model (Fig. 1) to obtain individual rate constants. To do this, we need to solve the kinetic model. Here, we first define the chemical species and the rate constants in our kinetic model (Fig. 1). The model consists of two pathways. First, dark state rhodopsin (Rho) undergoes thermal isomerization at a rate kᵢₛₑ to yield all-trans-retinal bound to opsin via an unprotonated SB (Tᵢₑ), a Meta II-like state. The SB in Tᵢₑ breaks at a rate kᵢₑ to yield all-trans-retinal free in solution (Tᵢₑ). Second, Rho undergoes hydrolysis of the protonated SB at a rate kₜₑ to form 11-cis-retinal free in solution (Cᵣₑ), along with opsin protein. Then Cᵣₑ isomerizes in the presence of opsin protein at a rate kᵣᵢₛₑ to yield Tᵢₑ.

We are able to solve this kinetic model and apply it to obtain all of the rate constants (kᵢₛₑ, kᵢₑ, kᵢₛₑ, kᵢₑ, and kᵣᵢₛₑ) governing the thermal processes of rhodopsin by performing four experiments: kinetic measurements of hydrolysis of SB using photoactivated rhodopsin (Meta II) and kinetic measurements of thermal decay, thermal isomerization, and hydrolysis of SB using dark state rhodopsin. Here, we relate the experimental
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Hydrolysis of the SB in Meta II—First, we independently obtained the rate constant \( k^\text{hyd}_T \). The experiment measures a single thermal process, represented in our kinetic model as the hydrolysis of the SB of \( T^b \) at a rate \( k^\text{hyd}_T \) to form \( T^\ell \). This is a first-order reaction, so the rate can be expressed as follows,

\[
- \frac{d[T^b]}{dt} = k^\text{hyd}_T [T^b]
\]

(Eq. 1)

where \([T^b]\) is the concentration of \( T^b \). The solution can be written as follows.

\[
[T^b] = [T^b]_0 e^{-k^\text{hyd}_T t}
\]

(Eq. 2)

and

\[
\frac{[T^b]}{[T^b]_0} = e^{-k^\text{hyd}_T t}
\]

(Eq. 3)

Because \([T^b]/[T^b]_0\) is directly proportional to the normalized \( A_{440} \). Equation 3 can be used to fit the experimental data of SB hydrolysis in Meta II, the normalized \( A_{440} \) as a function of time. The fitting parameter in Equation 3 is \( k^\text{hyd}_T \).

Thermal Decay—Next, we considered the thermal decay of dark state rhodopsin (Rho). According to our kinetic model, Rho can undergo thermal decay through two pathways: 1) thermal isomerization at a rate \( k^{\text{iso}}_C \) to form \( C^\ell \) and 2) hydrolysis of the protonated SB at a rate \( k^\text{hyd}_T \) to form \( C^\ell \). Thus, the rate of thermal decay is the sum of the rates of thermal isomerization and hydrolysis of protonated SB and can be expressed as follows,

\[
- \frac{d[Rho]}{dt} = (k^{\text{iso}}_C + k^\text{hyd}_T) [Rho]
\]

(Eq. 4)

where \([Rho]\) is the concentration of Rho. The solution can be written as follows.

\[
[Rho] = [Rho]_0 e^{- (k^{\text{iso}}_C + k^\text{hyd}_T) t}
\]

(Eq. 5)

and

\[
\frac{[Rho]}{[Rho]_0} = e^{- (k^{\text{iso}}_C + k^\text{hyd}_T) t}
\]

(Eq. 6)

Because \([Rho]/[Rho]_0\) is directly proportional to the normalized \( A_{500} \). Equation 6 can be used to fit the experimental data of thermal decay, the normalized \( A_{500} \) as a function of time. The fitting parameters in Equation 6 are \( k^{\text{iso}}_C \) and \( k^\text{hyd}_T \).

Thermal Isomerization—Thermal isomerization of retinal measured in the experiment includes two processes presented in our kinetic model: 1) thermal isomerization of Rho at a rate \( k^{\text{iso}}_C \) to form \( C^\ell \) and 2) thermal isomerization of \( C^\ell \) at a rate \( k^\text{iso}_C \) to form \( T^\ell \). Thus, the total concentration of retinal in the 11-cis conformation, [11-cis], is the sum of [Rho] and [C^\ell],

\[
[11-cis] = [Rho] + [C^\ell]
\]

(Eq. 7)

and the rate of thermal isomerization can be expressed as follows.

\[
- \frac{d[11-cis]}{dt} = k^\text{iso}[Rho] + k^\text{iso}_C[C^\ell]
\]

(Eq. 8)

Substituting [Rho] from Equation 5 and integrating Equation 8 (see supplemental materials), we obtain the following.

\[
\frac{[11-cis]}{[Rho]_0} = \left( \frac{k^\text{iso}_C}{k^\text{iso}_C - (k^\text{hyd}_T + k^\text{iso}_C)} \right) e^{- (k^{\text{iso}}_C + k^\text{hyd}_T) t} + \frac{-k^\text{hyd}_T}{k^\text{iso}_C - (k^\text{hyd}_T + k^\text{iso}_C)} e^{-k^\text{hyd}_T t}
\]

(Eq. 9)

Equation 9 can be used to fit the experimental data of thermal isomerization, the fraction of 11-cis-retinal as a function of time. The fitting parameters in Equation 9 are \( k^{\text{iso}}_C \), \( k^\text{hyd}_T \), and \( k^\text{iso}_C \).

Hydrolysis of the SB Using Rhodopsin—In the kinetic model (Fig. 1), two thermal processes involve in hydrolysis of the SB: 1) hydrolysis of the protonated SB of Rho at a rate \( k^\text{hyd}_C \) to form \( C^\ell \) and 2) hydrolysis of the deprotonated SB of \( T^b \) at a rate \( k^\text{hyd}_T \) to form \( T^\ell \). Thus, the total concentration of all species with an intact SB linkage at time \( t \), \([\text{link}]\), is the sum of [Rho] and \([T^b]\).

\[
[\text{link}] = [Rho] + [T^b]
\]

(Eq. 10)

According to the model (Fig. 1), the rate of hydrolysis of the SB of rhodopsin can be expressed as follows.

\[
- \frac{d[\text{link}]}{dt} = k^\text{hyd}_C [Rho] + k^\text{hyd}_T [T^b]
\]

\[
= k^\text{hyd}_C [Rho] + k^\text{hyd}_T ([\text{link}] - [Rho])
\]

\[
= (k^\text{hyd}_C - k^\text{hyd}_T) [Rho] + k^\text{hyd}_T [\text{link}]
\]

(Eq. 11)

Substituting [Rho] from Equation 5 and integrating Equation 11 (see supplemental materials), we obtain the following.

\[
\frac{[\text{link}]}{[Rho]_0} = \left( \frac{k^\text{hyd}_T}{k^\text{hyd}_C - (k^\text{hyd}_T + k^\text{iso}_C)} \right) e^{- (k^{\text{iso}}_C + k^\text{hyd}_T) t} + \frac{-k^\text{hyd}_C}{k^\text{iso}_C - (k^\text{hyd}_T + k^\text{iso}_C)} e^{-k^\text{hyd}_C t}
\]

(Eq. 12)

Because \([\text{link}]/[Rho]_0\) is directly proportional to the normalized \( A_{440} \). Equation 12 can be used to fit the experimental data of SB hydrolysis, the normalized \( A_{440} \) as a function of time. Because \( k^\text{hyd}_T \) is determined independently, the fitting parameters in Equation 12 are \( k^{\text{iso}}_C \) and \( k^\text{iso}_C \).

Summary—Thus, we obtain four sets of data from four different experiments. One data set is independent: the rate constant \( k^\text{hyd}_T \) is found from the hydrolysis of SB in Meta II by fitting the normalized plot of \( A_{440} \) versus time into Equation 3. The other three data sets are interrelated: the data obtained from thermal decay experiment are fitted to Equation 6; the data obtained from thermal isomerization experiment are fitted to Equation 9; and the data obtained from hydrolysis of the SB are fitted to Equation 12 using the independently measured value for \( k^\text{hyd}_T \). Our three experiments, thermal decay, thermal isomerization, and hydrolysis of SB, probe kinetic processes
that are involved in both pathways: isomerization and hydrolysis. Hence, $k_{\text{hyd}}$ and $k_{\text{iso}}$ cannot be measured independently. To obtain $k_{\text{hyd}}$ and $k_{\text{iso}}$, it is necessary to globally fit all of the kinetic data to the solution of the whole kinetic model (Equations 6, 9, and 12). The global fitting can be carried out using commercial software, such as Igor Pro. Three sets of data from three experiments are fitted using the fitting parameters representing the rate constants, $k_{\text{hyd}}$, $k_{\text{iso}}$, and $k_{\text{iso}}$. Then we report the results for the E181Q and S186A mutants at 55 °C and WT in H$_2$O and D$_2$O at 59 °C.

The kinetic analysis of WT rhodopsin at 55 °C began with measuring $k_{\text{hyd}}$. Fig. 2A displays the UV-visible absorption spectra obtained in the Meta II hydrolysis experiment. The spectra were fit into two Gaussian functions centered at 440 and 380 nm. The fitted intensity for the 440-nm peak was plotted as a function of time (Fig. 2B). From single exponential fitting using Equation 3, $k_{\text{hyd}}$ was determined to be $0.77 \pm 0.12 \text{ min}^{-1}$.

Subsequently we used the measured $k_{\text{hyd}}$ and applied the solution of the kinetic model to analyze the data for thermal decay, isomerization, and hydrolysis of SB. The fitted intensity of the 440-nm peak was plotted as a function of time in Fig. 2B. From single exponential fitting using Equation 3, $k_{\text{hyd}}$ was determined to be $0.77 \pm 0.12 \text{ min}^{-1}$.

RESULTS

We now demonstrate the application of our kinetic model to analyze our previous data for WT rhodopsin and the E181Q and S186A mutants at 55 °C and WT rhodopsin in H$_2$O and D$_2$O at 59 °C. To achieve this, we needed to perform new experiments to determine the rate of hydrolysis of SB in Meta II, $k_{\text{hyd}}$, for each condition. We first focus on WT rhodopsin at 55 °C to illustrate how to use the kinetic model to obtain $k_{\text{hyd}}$, $k_{\text{hyd}}$, and $k_{\text{iso}}$. Then we report the results for the E181Q and S186A mutants at 55 °C and WT in H$_2$O and D$_2$O at 59 °C.

The kinetic analysis of WT rhodopsin at 55 °C began with measuring $k_{\text{hyd}}$. Fig. 2A displays the UV-visible absorption spectra obtained in the Meta II hydrolysis experiment. The spectra were fit into two Gaussian functions centered at 440 and 380 nm. Over time, the concentration of all-trans-retinal decreases, and the concentration of 11-cis-retinal increases. F, the fraction of 11-cis-retinal as a function of time and fit to Equation 9. G, UV-visible spectra of the hydrolysis of SB. Over time, the 440-nm peak decreases, and the 380-nm peak increases. H, $A_{\text{440}}$ as a function of time and fit to Equation 12.
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**TABLE 1**  
Summary of rate constants for the thermal processes of rhodopsin and the contribution of thermal isomerization and hydrolysis of SB to thermal decay

|                    | Rate constants | Contribution of each pathway |     |     |
|--------------------|----------------|-------------------------------|-----|-----|
|                    | \( k_{iso} \)   | \( k_{hyd} \)                 | \( k_{iso} \) | \( k_{hyd} \) | \% |
| WT \((H_2O)\) 55°C | 0.0055 ± 0.0008 | 0.0045 ± 0.0008 | 0.016 ± 0.003 | 0.77 ± 0.12 | 55 ± 0 |
| E181Q \((H_2O)\) 55°C | 0.21 ± 0.05   | 0.046 ± 0.018 | 0.045 ± 0.029 | 0.71 ± 0.04 | 82 ± 26 |
| S186A \((H_2O)\) 55°C | 0.64 ± 0.07   | 0.17 ± 0.05 | 0.052 ± 0.034 | 0.84 ± 0.09 | 79 ± 12 |
| WT \((H_2O)\) 59°C | 0.055 ± 0.010 | 0.043 ± 0.009 | 0.076 ± 0.021 | 0.72 ± 0.03 | 56 ± 13 |
| WT \((D_2O)\) 59°C | 0.041 ± 0.0018 | 0.0056 ± 0.0020 | 0.010 ± 0.006 | 0.59 ± 0.06 | 88 ± 6 |

**FIGURE 3.** Measurement of \( k_{iso} \) in A, E181Q at 55°C. B, S186A at 55°C. C, WT in \( H_2O \) at 59°C. D, WT in \( D_2O \) at 59°C.

Responding Equations 6, 9, and 12. The rate constants \( k_{iso} \), \( k_{hyd} \), and \( k_{iso} \) were found to be 0.0056 ± 0.0008, 0.0045 ± 0.0008, and 0.016 ± 0.003 min⁻¹, respectively (Table 1). Our results reveal that thermal isomerization of the chromophore (45 ± 9%) and hydrolysis of the SB linkage (55 ± 10%) contribute equally within experimental error to the thermal decay of rhodopsin, implying that neither pathway can be ignored.

Furthermore, we applied our kinetic model to solve all rate constants for E181Q and S186A mutants at 55°C and WT in \( H_2O \) and \( D_2O \) at 59°C. As far as we have previously reported data, we measured the SB hydrolysis in Meta II for E181Q and S186A at 55°C and WT rhodopsin in \( H_2O \) and \( D_2O \) at 59°C. Fig. 3 displays the fitted intensity for the 440-nm peak plotted as a function of time. Using Equation 3, \( k_{hyd} \) was determined to be 0.71 ± 0.04 and 0.84 ± 0.09 min⁻¹ for E181Q and S186A at 55°C, respectively. Moreover, \( k_{iso} \) was determined to be 0.72 ± 0.03 and 0.59 ± 0.06 min⁻¹ for WT in \( H_2O \) and WT in \( D_2O \) at 59°C, respectively (Table 1). These values were used to fit the data obtained from thermal decay, thermal isomerization, and hydrolysis experiments on E181Q and S186A mutants at 55°C and WT in \( H_2O \) and \( D_2O \) at 59°C (supplemental Fig. S1). The rate constants \( k_{iso} \), \( k_{hyd} \), and \( k_{iso} \) were obtained from global fitting and summarized in Table 1. The results reveal the effects of mutations, \( D_2O \), and temperature on the kinetics of the thermal processes of rhodopsin.

Our kinetic model includes four reactions (Fig. 1), and their backward reactions can be ignored on the basis of our experimental observations. First, the backward reaction of hydrolysis of the SB in dark state rhodopsin can be ignored, because no peak at 500 nm corresponding to the dark state rhodopsin was observed when 11-cis-retinal was incubated with opsin proteins at 55°C (23). Second, the backward reaction of thermal isomerization of rhodopsin can be ignored because Meta II cannot convert back to rhodopsin spontaneously. Indeed, in our experiments, when Meta II was incubated at 55°C, no 500-nm peak was observed. Third, the backward reaction of hydrolysis of the SB in the Meta II-like state can also be neglected based on the results of our control experiments. In the experiments (supplemental materials, Fig. S2), all-trans-retinal was incubated with opsin protein at 55°C, and the samples were taken out at various time points for the acid denaturation assay. The 440-nm peak was absent in the spectra of the acid-denatured samples, suggesting that the SB linkage did not form. Finally, the backward reaction of thermal isomerization of free 11-cis-retinal can be neglected also based on our control experiments (supplemental materials). All-trans-retinal was incubated with opsin protein at 55°C to examine the change of the retinal isomeric form. The concentration of all-trans-retinal was found to remain the same, suggesting that all-trans-retinal did not isomerize to 11-cis-retinal under our experimental conditions. Consequently, all backward reactions are not included in the kinetic model.

### DISCUSSION

We introduced a kinetic model to describe quantitatively the chemical kinetics of the thermal decay of rhodopsin. The kinetic model was successfully applied to analyze data collected from WT rhodopsin and E181Q and S186A mutants at 55°C and WT in \( H_2O \) and \( D_2O \) at 59°C. All of the rate constants are summarized in Table 1. The results show that the thermal decay of rhodopsin involves two pathways: 1) thermal isomerization of retinal inside the binding pocket followed by hydrolysis of the SB in Meta II and 2) hydrolysis of the SB between 11-cis-retinal and opsin followed by the thermal isomerization of free 11-cis-retinal in the presence of opsin protein. Thus, thermal isomerization and hydrolysis of the SB are involved in both pathways, and all of these processes must be taken into account when studying the thermal properties of rhodopsin.

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The rate constants of individual thermal processes were determined (Table 1). By performing the analysis on the E181Q and S186A mutations, WT rhodopsin in D2O, and WT rhodopsin at 55 °C and 59 °C, we reveal not only the contributions of thermal isomerization and hydrolysis to thermal decay but also the function of the hydrogen-bonding network in rhodopsin and the highly unusual energetics of thermal isomerization and hydrolysis of SB.

Decay Kinetics of E181Q and S186A Mutants—The rate constants $k_{\text{hyd}}^{b}$ and $k_{\text{iso}}^{b}$ of the E181Q and S186A mutants are 1–2 orders of magnitude greater than those of WT rhodopsin, and the S186A mutant increases these rates more than the E181Q mutant does. This indicates that the mutations dramatically accelerate both isomerization of the retinal chromophore and hydrolysis of the protonated SB. Because the mutations perturb hydrogen bonds, the results support our hypothesis that the extensive hydrogen-bonding network previously found throughout rhodopsin is crucial for stabilizing the dark state, preventing thermal isomerization and hydrolysis of SB. This also reveals the functional role of the conserved structural water molecules (7, 24–28) that help to coordinate the hydrogen-bonding network. Moreover, our results show that the ratio of the rate constants $k_{\text{iso}}^{b}$ to $k_{\text{hyd}}^{b}$ is 1.2 ± 0.2 for WT rhodopsin, 4.6 ± 0.5 for E181Q, and 3.8 ± 0.3 for S186A. Although the mutations increase the rate of both thermal isomerization and hydrolysis of SB, the mutations have more impact on the rate of thermal isomerization, which accounts for ~80% of the thermal decay of the mutants, compared with ~50% for WT rhodopsin.

Although the mutations dramatically increase the rates of thermal processes in dark state rhodopsin, $k_{\text{iso}}$ and $k_{\text{hyd}}$ for the E181Q and S186A mutants are the same as the rates for WT rhodopsin within experimental error, suggesting that thermal isomerization of free 11-cis-retinal with the presence of opsin and hydrolysis of SB in Meta II are not affected by perturbations of the hydrogen-bonding network (Table 1). These findings agree with our previous proposal that the hydrogen-bonding network has no significant effect on the stability of Meta II or on the ability of opsin to catalyze cis-to-trans isomerization of retinal. We propose that the dark state electrostatic interaction between the protonated SB and the Glu-113 counterion may serve as a switch for the hydrogen-bonding network. In the dark state, the presence of the counterion interaction switches on the hydrogen-bonding network involving Glu-181 and Ser-186, but when the counterion interaction is absent, as in opsin and Meta II, the hydrogen-bonding network is effectively switched off. Hence, these results imply that the hydrogen-bonding network is specialized for preventing thermal isomerization and hydrolysis of SB in the dark state, which suppresses discrete dark noise and constitutive activation of opsin, allowing rhodopsin to function as a highly sensitive photoreceptor.

Isotope Effects on Decay Kinetics—Table 1 shows the effect of D2O on the rates of thermal processes at 59 °C. We found that the rate of hydrolysis of SB of Meta II, $k_{\text{hyd}}^{C}$, is slower in D2O, exhibiting a solvent isotope effect of 1.2 ± 0.1 at 59 °C, compared with 2.5 at 20 °C previously reported by Janz and Farrens (20). Also, the rates of thermal isomerization and hydrolysis of SB of WT rhodopsin are slower in D2O than in H2O. The rate constant of thermal isomerization of dark state rhodopsin, $k_{\text{iso}}^{b}$, is 1.3 ± 0.3 times slower in D2O, and that of hydrolysis of SB, $k_{\text{hyd}}^{b}$, is 7.7 ± 3.2 times slower in D2O. These observations are consistent with the fact that hydrogen bonds are stronger in D2O than in H2O, suggesting that the rate-determining steps of these thermal processes involve breaking hydrogen bonds.

However, isotope effects are typically in the range of 2–3, so the value of 7.7 ± 3.2 for SB hydrolysis in dark state rhodopsin is extremely high. It is known that intrinsic primary isotope effects, involving the rate-limiting step of breaking a bond between oxygen and hydrogen, have a theoretical maximum of $k_{\text{iso}}^{b}/k_{\text{hyd}}^{b} = 6.3$ at 59 °C (31). Our measured value of 7.7 ± 3.2 falls into the range of this theoretical maximum, implying that our observed high isotope effect ($k_{\text{iso}}^{b}/k_{\text{hyd}}^{b}$) is likely due to a primary isotope effect as opposed to a combination of secondary isotope effects caused by, for instance, changes in protein conformation. The hydrolysis of protonated SB species generally proceeds by the nucleophilic attack of water or hydroxide on the SB carbon, forming a carbinolamine intermediate and relieving the positive charge on nitrogen, followed by the subsequent collapse of the tetrahedral intermediate. Previous work by Cooper et al. (32) suggests that, in the rhodopsin-binding pocket, the mechanism is in fact a base-catalyzed attack by hydroxide mediated by a nearby proton acceptor. We speculate that the initial deprotonation of the solvent H2O or D2O before nucleophilic attack on the imine carbon may give rise to the observed primary isotope effect. Without our kinetic model, we could not detect this effect using single-exponential fitting of the thermal isomerization and hydrolysis of SB data (22).

Temperature Dependence of Decay Kinetics—Furthermore, comparing the kinetics of thermal processes at two different temperatures yields information about the energetics of the reactions. As shown in Table 1, both rate constants $k_{\text{iso}}$ and $k_{\text{hyd}}^{C}$ are greater for WT rhodopsin at 59 °C than at 55 °C, whereas similar $k_{\text{iso}}$ was observed at 55 and 59 °C. Because $k_{\text{iso}}$, the rate of opsin-catalyzed cis-trans thermal isomerization, shows little temperature dependence, the activation energy of this process should be small, as expected for a catalytic reaction. In contrast, an increase in temperature of only 4 °C had a 10-fold effect on both $k_{\text{iso}}^{b}$ and $k_{\text{hyd}}^{C}$, suggesting an extremely steep temperature dependence on the rates of thermal isomerization and SB hydrolysis. Applying the Arrhenius model, the apparent activation energy is estimated to be 120 kcal/mol, which greatly exceeds 57 kcal/mol, the photon energy at 500 nm. This finding, although puzzling, is nevertheless implicit in the work of Hubbard (17). Farrens and co-workers (19, 20) also reported an activation energy of 103 kcal/mol for thermal decay by measuring the decrease in absorbance at 500 nm. Because they assayed $A_{\text{iso}}$ as a function of temperature, this value is apparently the activation energy of thermal decay, which they ascribed to hydrolysis of SB. On the other hand, our kinetic model allows determination of individual rates for thermal isomerization and SB hydrolysis, which explicitly shows that the rates of both processes have extremely steep temperature dependence. We are currently in the process of investigating this very unusual kinetic property in an attempt to relate it to the extremely high thermal stability of rhodopsin.
In addition, it is also important to consider the role of lipid bilayers (33–36). It has been especially well established that lipid bilayers can stabilize rhodopsin and other membrane proteins (37–39). With respect to this, we are in the process of applying the quantitative method developed here to study the effect of lipid bilayer on the kinetics of the thermal processes by incorporating rhodopsin into nanodiscs (39–41). We expect that our ongoing and future studies of this kind will provide a more complete picture to relate the unusual kinetic property and thermal stability of rhodopsin to its extremely high photosensitivity.

Implications in Eye Diseases and Molecular Evolution of Vision—Thus, our kinetic model along with the four kinetic measurements can be used as a full functional assay that gives unique, quantitative insight into the chemistry and molecular mechanism of the thermal processes of rhodopsin. The method can be readily extended to other mutations and/or other visual pigments.

Our model can be used to reveal the possible cause of eye diseases such as retinitis pigmentosa (RP), a family of inherited visual disorders characterized by progressive degeneration of the retina and retinal pigment epithelium (42–47). Approximately 25% of autosomal dominant RP cases are known to connect to one of over 100 point mutations in the rhodopsin gene, whereas additional rhodopsin mutations account for some cases of autosomal recessive RP and congenital stationary night blindness (48, 49). By using our kinetic model, we can quantitatively determine which thermal process dominates the thermal decay of a disease-related mutant. A mutation that primarily accelerates thermal isomerization may increase discrete dark noise by mimicking photoactivation of rhodopsin, which could cause the progressive desensitization and degeneration of rod cells seen in RP. On the other hand, a mutation that mostly speeds the hydrolysis of SB in dark state rhodopsin could lead to higher basal activation by constitutively active opsin protein that can stimulate downstream visual signaling in the absence of retinal, which is the proposed mechanism for congenital night blindness (16, 48). A systematic, quantitative analysis of pathogenic mutations in visual pigments could therefore guide the exploration of new ways to predict the course of and/or treat these visual diseases.

Finally, a longstanding question is how rod pigments acquired photosensitivity because they diverged evolutionarily from cone pigments (50–53). Because dark noise is known to be the limiting factor for photosensitivity, an important evolutionary pressure is to suppress dark noise. However, current investigations of the molecular evolution of visual pigments largely rely on detecting color absorption, which is not directly related to dark noise and is thus ineffective for studying the cone-to-rat divergence (50–53). Our kinetic model will enable a complete characterization of the thermal properties of visual pigments. This will provide quantitative results that can be directly related to the photosensitivity of various rod and cone visual pigments and potentially add a new dimension in the investigation of the molecular evolution of vertebrate vision.

Acknowledgment—We thank Ying Guo (Yale University) for discussion.

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