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Published on Web 03/31/2010

Protein Fluctuations as the Possible Origin of the Thermal Activation of Rod Photoreceptors in the Dark

Víctor A. Lórenz-Fonfría,[†,‡] Yuji Furutani,[†,#] Toru Ota,[†] Kazutomo Ido,[†] and Hideki Kandori*[,†]

Department of Frontier Materials, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan, and Unitat de Biofísica, Departament de Bioquímica i de Biologia Molecular, and Centre d'Estudis en Biofísica, Universitat Autònoma de Barcelona, Barcelona 08193, Spain

Received September 12, 2009; E-mail: kandori@nitech.ac.jp

Abstract: Efficient retinal photoisomerization, signal transduction, and amplification contribute to single-photon electrical responses in vertebrates visual cells. However, spontaneous discrete electrical signals arising in the dark, with identical intensity and time profiles as those generated by genuine single photons (dark events), limit the potential capability of the rod visual system to discern single photons from thermal noise. It is accepted that the light and the thermal activation of the rod photoreceptor rhodopsin (Rho) triggers the light and the dark events, respectively. However the activation barrier for the dark events (80−110 kJ/mol) appears to be only half of the barrier for light-dependent activation of Rho (≥180 kJ/mol). On the basis of these observations, it has been postulated that both processes should follow different pathways, but the molecular mechanism for the thermal activation process still remains an open question and subject of debate. Here, performing infrared difference spectroscopy measurements, we found that the -OH group of Thr118 from bovine Rho exhibits a slow but measurable hydrogen/deuterium exchange (HDX) under native conditions. Given the location of Thr118 in the X-ray structures, isolated from the aqueous phase and in steric contact with the buried retinal chromophore, we assume that a protein structural fluctuation must drive the retinal binding pocket (RBP) transiently open. We characterized the kinetics (rate and activation enthalpy) and thermodynamics (equilibrium constant and enthalpy) of this fluctuation from the global analysis of the HDX of Thr118-OH as a function of the temperature and pH. In parallel, using HPLC chromatography, we determined the kinetics of the thermal isomerization of the protonated 11-*cis* retinal in solution, as a model for retinal thermal isomerization in an open RBP. Finally, we propose a quantitative two-step model in which the dark activation of Rho is triggered by thermal isomerization of the retinal in a transiently opened RBP, which accurately reproduced both the experimental activation barrier and the rate of the dark events. We conclude that the absolute sensitivity threshold of our visual system is limited by structural fluctuations of the chromophore binding pocket rather than in the chromophore itself.

Introduction

Vision is an extremely complex process, but it fundamentally relies on the conversion of the external light into internal electrical signals. In vertebrates, this phototransduction process takes place in the outer segments of the visual cells, containing rod and cone cells, each with different photoreceptors. Cone cells are specialized in color vision under high photon fluxes, while rod cells provide monochromatic vision under dim light conditions.[1,2]

The rod photoreceptor, rhodopsin (Rho), is a membrane protein belonging to the large family of G protein-coupled receptors, but presenting some specific features:[3] (i) it contains an apoprotein (opsin) and a retinal chromophore covalently bound to a lysine via a protonated Schiff base, PSB (Figure 1a); (ii) the 11-*cis* retinal acts both as a chromophore and a very effective inverse agonist, locking the opsin in an inactive state;[4] (iii) light-induced *cis-trans* isomerization converts the retinal into a powerful agonist, leading to a photoinduced activation of Rho in milliseconds through several intermediate states (Figure 1d).

As the result of effective molecular transduction/amplification processes, the activation of a single Rho by a photon generates a macroscopic and reproducible physiological electrical response.[1] This remarkable feat is rendered futile by spontaneous electrical signals arising in the dark (dark events), indistinguishable in intensity and time-profile from the genuine single-photon responses.[1,5] The dark events in rod cells arise randomly, at a

[†] Nagoya Institute of Technology.
[‡] Universitat Autònoma de Barcelona.
[#] Present address: Department of Life and Coordination-Complex Molecular Science, Institute for Molecular Science, 38 Nishigo-Naka, Myodaiji, Okazaki 444-8585, Japan.

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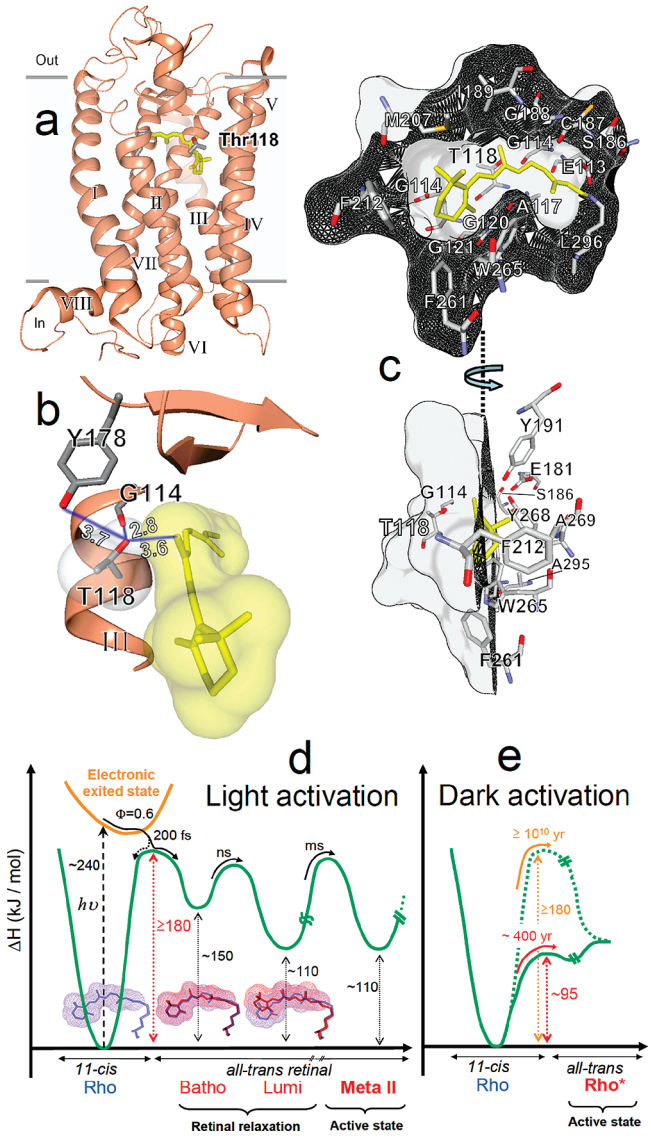

Figure 1. Structure of bovine rhodopsin, with details of the Thr118 environment and the retinal binding pocket, together with Rho activation reaction coordinate. (a) Structure of bovine Rho, PDB 1U19.[70] 11-*cis* Retinal (in yellow) and Thr118 are highlighted. (b) Environment of Thr118 hydroxyl and closer neighbors (distances in Å). (c) Frontal (top) and lateral (bottom) close-up view of the RBP, formed by the amino acids within 4 Å from the retinal. In the frontal view the residues masking the retinal have been removed. The surface of the RBP corresponds to the solvent excluded surface, clipped in half to show the 11-*cis* retinal in the RBP internal cavity. (d) Enthalpy reaction coordinate of light-dependent Rho activation.[2] Note that photoisomerization occurs with negligible changes in the retinal shape (Batho, PDB 2G87),[71] and later with changes mostly restricted to the β-ionone ring in Lumi (PDB 2HPY),[72] formed from Batho via the BS intermediate (not shown). The active state Meta II is formed via Meta I in milliseconds (omitted in the plot). (e) Apparent enthalpy reaction coordinate of Rho dark activation. Note the differences between the apparent activation enthalpy of Rho activation in the dark deduced from the dark events (continuous green line), and the expected value for a dark activation involving the formation of a Batho-like intermediate (dashed green line). Molecular drawings were performed with Ballview.[73]

rate of ~0.006 s^{-1} at 36 °C,[6] with an apparent activation enthalpy of 80−110 kJ/mol.[7−9] The rate of the dark events is similar to the rate of spontaneous perception of light in total dark deduced from psychophysical experiments in humans[1] and behavioral studies in frogs.[10] This similarity suggests that the dark events set the ultimate threshold to trustfully detect dim lights signals in a dark background, which accounts in humans to the simultaneous absorption of near half a dozen of photons in a small retina area.[11]

The physiological similarity of the dark and the single-photon electrical responses, among other compelling evidence,[12,13] points to the thermal *cis-trans* isomerization of the retinal as the most conceivable source of the dark events. If so, it will occur at a rate of ~10^{-11} s^{-1} per Rho at 36 °C, corresponding to a spontaneous 11-*cis* to all-*trans* retinal isomerization once every ~400 years,[6] with an activation enthalpy of 80−110 kJ/mol. Though otherwise solid, the assignment of the thermal *cis-trans* isomerization of the retinal as source for the dark events has been in serious discrepancy with the activation enthalpy for the retinal photoisomerization in Rho, which involves at least 180 kJ/mol.[12,14] To reconcile this notable discrepancy it has been postulated that the thermal and the light isomerization of the retinal in Rho follow different mechanisms,[8] with an energy barrier 2 times lower for the former. Still, the molecular nature of the postulated low-energy retinal isomerization path, or even its real existence, remains controversial.[1,12,15,16]

Recently available atomic structures of Rho[17,18] increased our perplexity about the extended barrier difference between the light and the thermal isomerization of the retinal in Rho. The crystal structures show the retinal binding pocket (RBP) tightly encapsulating the retinal (Figure 1c) and inhibiting by steric constraints changes of the retinal polyene chain even after photoisomerization to all-*trans* in bathorhodopsin (Figure 1d, Batho). This particular observation supports that for thermal isomerization the retinal should be likewise forced to adopt a highly distorted all-*trans* retinal fitting into the ground state RBP, requiring an activation enthalpy of ≥180 kJ/mol as Batho formation,[2,12] and in painful disagreement with the barrier of the dark events (Figure 1e). Additionally, applying transition state theory, we should expect that the thermal formation of a Batho-like all-*trans* retinal at 36 °C would occur only every ~10^{10} years. This is 7 orders of magnitude too infrequent to account for the actual rate of the dark events (Figure 1e).

Thirty years since the first description of the dark events[5] but despite some valuable contributions reviewed later in the Discussion, the origin of the dark events and the thermal activation of Rho remains still open. Here, we introduce a new molecular model to explain the origin and properties of the dark

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events, taking into account previously neglected protein dynamics. We provide experimental evidence that rhodopsin's structure fluctuates under native conditions. More importantly, one of these fluctuations has a magnitude large enough to allow the hydrogen/deuterium exchange (HDX) of the Thr118 hydroxyl, a group located in the RBP, and not accessible to the aqueous phase (Figure 1a). Given the location of Thr118 and its interactions with the retinal and other residues of the RBP (Figure 1a−c), we hypothesize that the same fluctuation will be able to transiently release the 11-*cis* retinal from some of the tight conformational constraints imposed by the RBP, i.e., to open the RBP. We propose a quantitative model in which the thermal activation of Rho originates from the thermal *cis-trans* isomerization of the retinal in this transiently open RBP (schematically shown in Figure 6). Once rhodopsin's all-*trans* agonist is formed thermally, the remaining parts of the activation pathway of Rho will proceed in the dark and cause the dark events in rod cells and the false perception of light in total darkness.

Methods

Thermal Isomerization of *n*-Butylamine 11-*cis* Protonated Schiff Base in Solution. UV irradiation of all-*trans* retinal in acetonitrile, followed by HPLC purification, provided pure 11-*cis* retinal.[19] All subsequent manipulations were performed in darkness. Excess of *n*-butylamine was added to the 11-*cis* retinal to form the *n*-butylamine retinal Schiff base.[20] Full and stable protonation of the 11-*cis* retinal, PRSB, confirmed by UV−vis spectroscopy (Figure S1 in Supporting Information), was accomplished by adding 125 μL of 11-*cis* RSB at 0.5 mM (in ethanol) and 100 μL of 5 M HCl (in water) to 2.775 mL of ethanol. Newly formed 11-*cis* PRSB in aqueous ethanol (3.2%) was rapidly incubated at 40, 50, 60, or 70 °C. Absence of hydrolysis of the PRSB during incubation in the dark was confirmed with UV−vis spectroscopy (Figure S1). Aliquots of 100 μL were periodically taken, and the PRSB was hydrolyzed to form retinal oximes. HPLC analysis was performed as described previously,[19] injecting 10 μL of the retinal oximes in hexane. The chromatograms were normalized by the total amount of retinal, using the extinction coefficient of retinal oxime isomers at 360 nm.[19] Fractions (in %) of retinal isomers were calculated from the relative areas of the peaks in the normalized chromatograms. Three independent experiments were performed for each incubation temperature. The fractions of all-*trans*, 13-*cis*, and 9-*cis* retinal isomers at different temperatures and times were globally modeled as

$$f(t,T) = (A_0 - A_\infty) \times \exp(-t \times k_{iso}(T)) + A_\infty \qquad (1)$$

considering an Eyring temperature dependence for k_{iso}. The fraction of 11-*cis* retinal was fitted as 100% minus the summed fractions of all-*trans*, 13-*cis*, and 9-*cis* retinal.

Hydrogen/Deuterium Exchange (HDX) of Bovine Rhodopsin and Infrared Difference Spectra Collection. Crude rod outer segments (ROS) were isolated and purified from the retinas as described previously[21] and resuspended in 2 mM phosphate buffer (pH 7.5) or in 2 mM borate buffer (pH 8.5 and 9.5). Approximately 40 μL of ROS, containing Rho at ∼2 mg/mL, was dried on a BaF_2 window, hydrated with the vapor phase of few D_2O drops placed next to the film, and closed with another window separated by a rubber O-ring. The final attained hydration level for each sample after 20−60 min of incubation time was ∼1.2 g water/g protein (estimated from the IR absorbance spectrum as described),[22] well above the experimentally evaluated critical level of hydration to obtain solution-like HDX rates (0.15 g water/g of protein).[23] In our experiments, the hydration threshold for solution-like HDX rates was achieved fast enough (<1−2 min) for reliable measurement of the level of H/D exchange of Thr118 after 20−60 min of incubation time. Even for groups with very fast HDX (≤1 min), the estimated relative error in the observed level of HDX than may arise from this hydration delay is ≤10% (for 20 min incubation) and ≤4% (for 60 min incubation). After incubation for a given temperature and time, the sample was rapidly placed into an Oxford OptistatDN cryostat holder, mounted in a DIGILAB FTS7000 FT-IR spectrometer, and cooled down to 77 K. The photoinduced Rho to Batho conversion was archived by illumination with 501 nm light for 2 min, while reconversion of Batho to Rho was done by >610 nm illumination for 1 min. Between one and three independent HDX experiments were performed for each incubation temperature, time, and pH condition. Batho-Rho difference spectra were scaled using the chromophore bands at 1193 and 920 cm^{-1} and offset around 1900−1800 cm^{-1}. The HDX of Thr118 hydroxyl was monitored by the bands at 3487 (+) and 3464 (−) cm^{-1} and 2579 (+) and 2562 (−) cm^{-1}, respectively. As a reporter of the level of exchange we tested (i) the intensity difference between the positive/negative bands, (ii) the absolutes areas of the positive/negative bands, and (iii) the intensity differences of the positive/negative bands in second derivative spectra. All methods gave very similar HDX curves, but the last method gave lower data dispersion (probably related to the ability of the second derivative to minimize baseline differences and enhance narrow bands), and it was the one used in the subsequent quantitative analysis.

Scheme 1

$$\text{close(H)} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \text{open(H)} \xrightarrow{k_{ch}} \text{open(D)}$$

Analysis of the HDX of Thr118 Hydroxyl. The fraction of HDX of Thr118-OH as a function of time will be monoexponential at a given temperature and pH:

$$f(t,T,\text{pH}) = (A_0 - A_\infty) \times \exp(-k_{ex}(T,\text{pH}) \times t) + A_\infty \qquad (2)$$

where A_0 and A_∞ represent here the intensities of the Thr118 hydroxyl bands at zero and at total exchange. A microscopic two-state model was used to model the HDX of Thr118 hydroxyl (see Scheme 1). Given this model, the rate constant of HDX, k_{ex}, will depend on temperature and pH as:[24−26]

$$k_{ex}(T,\text{pH}) = \frac{k_{op}(T) \times k_{ch}(T,\text{pH})}{k_{op}(T) + k_{cl}(T) + k_{ch}(T,\text{pH})} \qquad (3)$$

where k_{op} and k_{cl} are the rate constants of a protein fluctuation making the hydrogen competent for HDX, from where HDX occurs with a chemical rate constant k_{ch}. In contrast to the habitual practice of equaling k_{ch} to the rate constant of HDX found in random coil polypeptides, k_{rc}, we allowed k_{ch} to take any value between k_{rc} and $k_{rc}/20$, i.e., $k_{ch} = k_{rc} \times q$, where q is the HDX attenuation factor in

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the open conformation. All rate constants were considered to be temperature-dependent (following an Eyring behavior), whereas k_{rc} was also considered to be pH-dependent (see below). The experimental HDX data as a function of the temperature, pH, and incubation time were globally analyzed combining eqs 2 and 3 (see below). Once A_0 and A_∞ were estimated, semiexperimental values for k_{ex} were directly obtained solving eq 2 using the experimental data as f.

HDX of Threonine Hydroxyl in Random Coil Polypeptides. The pH dependence of HDX in random-coil polypeptides, k_{rc}, is known to follow the expression[24,27]

$$k_{rc}(\mathrm{pH}) = k_0 + k_a[\mathrm{H^+}] + k_b[\mathrm{OH^-}] = k_0 + k_a 10^{-\mathrm{pH}} + k_b K_w 10^{\mathrm{pH}} \quad (4)$$

where k_a and k_b are rate constants for specific acid and base catalysis for hydrogen exchange (HX), k_0 is the rate constant of direct HX with water, and K_w is the ionization constant of water. The values of k_0, k_a, and k_b depend on temperature, on the chemical nature of the considered group, and to a lesser extent also on steric and inductive effects from neighbor groups,[28] or on isotope effects.[29] Herein we assumed that after substitution of H_2O to D_2O, the pD remains equal to the initial pH ($[H^+] \approx [D^+]$), but we did take care of the K_w differences between H_2O and D_2O, i.e., $[OH^-] \neq [OD^-]$.[29] We made the reasonable assumption that the pH in a hydrated film is equivalent to the pH of the corresponding solution.[30] For Thr-OH, the pH dependence of k_{rc} is characterized at 4 °C by $\log(k_a/s) \approx 7.8$ and $\log(k_b/s) \approx 8.1$ (direct water catalysis is ineffective), obtained by fitting data from Liepinsh et al.[31] to eq 4. At pH > 7 the temperature dependence of k_{rc} for Thr hydroxyl will be given by the sum of the activation enthalpy of k_b and the enthalpic component of K_w (~60 kJ/mol).[29] For acetyl-Thr-OCH_3 their sum is ~70 kJ/mol at pH ≥ 7,[32] similar to the activation enthalpy found for the base catalyzed HX of the amide hydrogen,[28] and the Trp indole hydrogen.[33] $\Delta H_{ch}^{\ddagger}$ was considered to be 60−75 kJ/mol in the analysis performed in this work, allowing for some variation from the value found in the model compound.

Bayesian Inference. An account of Bayesian inference can be found elsewhere.[34] Briefly, the probability of the parameters vector, **a**, conditioned to the data vector, **d**, and some prior information, I, is given by

$$\Pr\langle \mathbf{a}|\mathbf{d}, I\rangle \propto \Pr\langle \mathbf{d}|\mathbf{a}, I\rangle \prod_{i=1}^{M} \Pr\langle a_i|I\rangle \quad (5)$$

where M is the number of parameters. The probability of the data given the model parameters was described by a normal distribution:

$$\Pr\langle \mathbf{d}|\mathbf{a}, I\rangle \propto \exp\left(-\frac{1}{2}\sum_{j=1}^{N}\left(\frac{d_j - y_j(\mathbf{a})}{\sigma_d}\right)^2\right) = \exp\left(-\frac{\chi^2(\mathbf{a})}{2}\right) \quad (6)$$

where N is the total number of experimental data points, **y** is the predicted data vector given parameters **a**, and σ_d is the standard deviation of the errors in the data. For the analysis of the HDX of Thr118 hydroxyl the number of data points was 88. The prior probability of the parameters in eq 5 was set to uniform for A_0 and A_∞; uniform but positive constrained for $\Delta H_{op}^{\ddagger}$ and $\Delta H_{cl}^{\ddagger}$; uniform in logarithmic scale for k_{op} and k_{cl}; uniformly distributed between 60−75 kJ/mol and zero elsewhere for $\Delta H_{ch}^{\ddagger}$; and uniformly distributed in logarithmic scale between 1 and 1/20 and zero elesewhere for q. The value of σ_d was taken as constant and estimated from the residuals of the best fit taking into account the approximate number of degrees of freedom lost. For the analysis of the thermal isomerization of the retinal in solution the number of data points was 308, although only 231 were truly independent due to the normalization. In the fit, the total fraction of all-*trans*, 13-*cis*, and 9-*cis* retinal was restricted, so their sum was always equal or lower than 100%. The prior probability of the parameters was set to uniform between 0 and 100% for A_0 and A_∞ (zero outside); uniform but positive constrained for $\Delta H_{iso}^{\ddagger}$; and uniform in logarithmic scale for k_{iso}. The value of σ_d was considered to be different for each incubation time and temperature, and it was estimated from the variance of the replicated experiments. In both cases, we sampled from eq 5 using the Metropolis−Hastings method, a well-known Markov Chain Monte Carlo sampling algorithm.[34] From the obtained samples we computed histograms approximating the probability distribution of each parameter conditioned to the data and the prior information used. Briefly, a next vector of parameters, $\mathbf{a}_{next}$, was randomly generated from the current ones, $\mathbf{a}_{curr}$, as $\mathbf{a}_{next} = \mathbf{a}_{curr} + \boldsymbol{\varepsilon}$, where $\boldsymbol{\varepsilon}$ is **a** vector of size M with normally distributed random values. When $\Pr\langle \mathbf{a}_{next}|\mathbf{d},I\rangle / \Pr\langle \mathbf{a}_{next}|\mathbf{d},I\rangle \geq r$, where r was a randomly generated number between 0 and 1, $\mathbf{a}_{next}$ was accepted and added to the list of sampled values. Otherwise, $\mathbf{a}_{next}$ was rejected, and $\mathbf{a}_{curr}$ was added to the list without update. The standard deviations used to generate $\boldsymbol{\varepsilon}$ elements were adjusted to approximately one-third of the probability standard deviation expected for each parameter (rejection rate of ~95%), and 10^6 samples were drawn. No significant variations in the obtained probability distributions were observed when the sampling process was repeated for three different times, confirming ergodic sampling. All calculations were implemented in homemade routines for Matlab version 7 (The Math Works, Natick, MA).

Results and Discussion

Thermal Isomerization of the Protonated 11-*cis* Retinal in Solution. Comparing the differences and similarities of the thermal isomerization of the retinal in solution and in Rho could provide a key to understand the nature of the low-energy path for Rho dark activation. Thermal isomerization of free 11-*cis* retinal in different solvents was previously reported using UV−vis spectroscopy,[35] but so far to the best of our knowledge there are no reports on the thermal isomerization of the authentic chromophore present in Rho: a 11-*cis* retinal forming a protonated Schiff Base with a lysine, the 11-*cis* PRSB.[2]

Therefore, we first characterized the thermal isomerization of a protonated *n*-butylamine 11-*cis* retinal Schiff base dissolved in aqueous ethanol, the simplest chemical model of a free 11-*cis* PRSB. The isomeric state of the retinal in the dark was monitored at different temperatures over the time. This was accomplished by HPLC chromatography (Figure 2a), allowing more accurate quantitative evaluation of all isomeric fractions of the retinal than by using UV−vis spectroscopy (Figure S1). At time zero, the fraction of 11-*cis* retinal was higher than 99% (Figure 2b, left). As the incubation time in the dark increased, the fraction of 11-*cis* decreased concomitantly with the formation of all-*trans* retinal (Figure 2b) and to a lesser extent with

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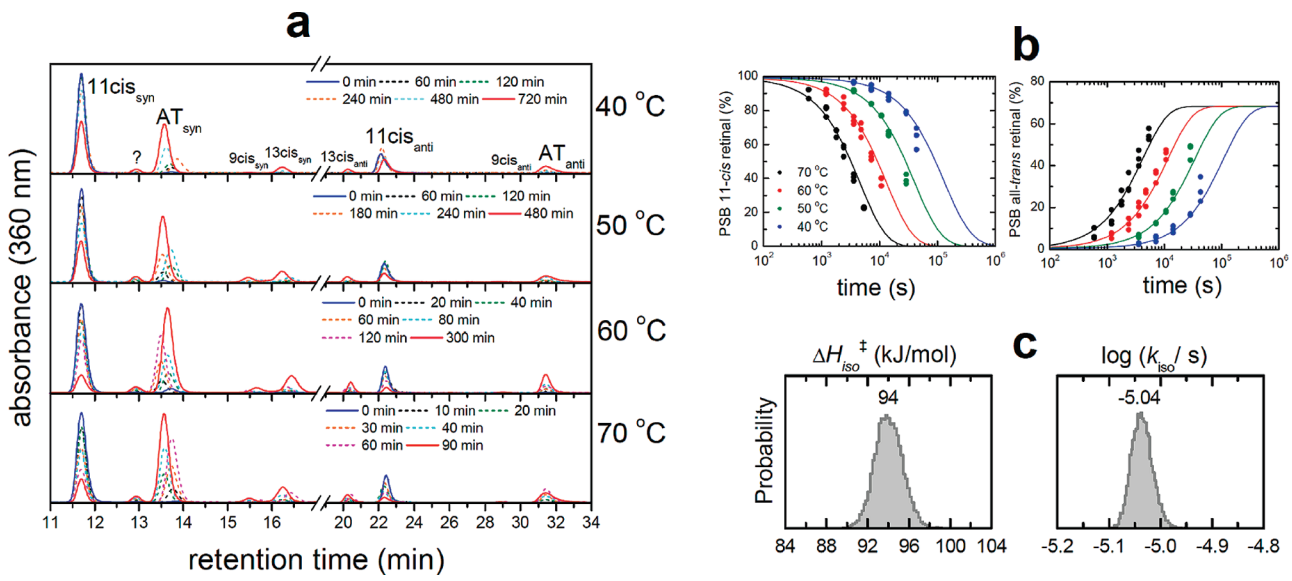

Figure 2. Thermal isomerization of the protonated *n*-butylamine 11-*cis* retinal Schiff base in aqueous ethanol. (a) HPLC chromatograms of the retinal oximes, resolving the population of PRSB isomers and how 11-*cis* retinal thermally isomerizes with the incubation time in the dark at several temperatures (for clarity only one replicate experiment is shown). (b) Experimental fractions (in %) of the 11-*cis* and all-*trans* retinal isomers as a function of time and temperature (filled circles, all three replicate experiments are shown), and best global fits (continuous lines). (c) Probability histograms of the kinetic parameters characterizing the thermal isomerization of the protonated 11-*cis* retinal in solution to all-*trans* at 40 °C, obtained from the global analysis of the data in panel b.

the formation of 13-*cis* and 9-*cis* retinal (see Supporting Information, Figure S2). Global Bayesian analysis of the fractions of retinal isomers (Figure 2b, continuous lines) provided probability distributions for the activation enthalpies and the rate constant of 11-*cis* to all-*trans* thermal isomerization of the PRSB (Figure 2c). The activation enthalpy was estimated to be 94 ± 3 kJ/mol, with a rate constant at 40 °C of $\sim 10^{-5}$ s^{-1} (Figure 2c). Although not directly relevant to the present study, the estimated kinetic values for the 11-*cis* to 13-*cis* and 9-*cis* thermal isomerization were also estimated and are shown in Supporting Information, Figure S2.

Unexpectedly, the estimated energy barrier for the isomerization of free 11-*cis* to all-*trans* PRSB was ~94 kJ/mol, which is well in the range of the energy barrier for 11-*cis* to all-*trans* retinal thermal isomerization in Rho deduced from the dark events, 80−110 kJ/mol.[7−9] However, the estimated rate of all-*trans* thermal isomerization for the free retinal, $\sim 10^{-5}$ s^{-1}, appeared one million-fold accelerated in solution in respect to Rho, $\sim 10^{-11}$ s^{-1}.[6] Therefore, the intrinsic propensity of the 11-*cis* retinal to isomerize to all-*trans* is $\sim 10^6$ times reduced in Rho, although the temperature dependence of the thermal isomerization (i.e., activation enthalpy) seems mostly unaffected by the opsin. These observations suggest that the thermal isomerization of the 11-*cis* PRSB in Rho may occur in a solvent-like environment, where the 11-*cis* PRSB is transiently free of most steric constraints imposed by the opsin.

Hydrogen/Deuterium Exchange of Rhodopsin Thr118 Hydroxyl. To address experimentally whether a protein fluctuation could transiently open the RBP and release thereby steric constraints to the retinal, we performed hydrogen/deuterium exchange (HDX) experiments on bovine Rho in the native rods outer segments. Fluctuations in soluble proteins have been successfully detected and characterized under native conditions by HDX. In proteins, the HDX of a labile hydrogen (mostly those bonded to N and O atoms) occurs through transient high-energy structural fluctuations, separating H-bonded groups and allowing water penetration.[26,36] The potential power of HDX in characterizing protein fluctuations has been successfully explored for soluble proteins, where the HDX of individual amino acids can be customarily resolved by NMR spectroscopy.[26,37] Although much less explored, due to numerous experimental difficulties, similar principles seem applicable for the HDX in membrane polypeptides[38] and proteins.[39] Advances in NMR have made it possible to extend (HDX) experiments to larger macromolecules in solution.[40] Recently, NMR was used to measure semiquantitatively the level of HDX of individual amide hydrogens in some β-barrel membrane proteins,[41,42] although these experiments were performed in detergent micelles. For membrane proteins reconstituted in lipids, HDX has been resolved only to the level of secondary structure motives[43] and to amino acid segments[39,44] by infrared (IR) spectroscopy and mass spectrometry, respectively. Here, using IR difference spectroscopy we report a novel HDX approach, to the best of our knowledge making it possible for the first time to characterize quantitatively the HDX for a single amino acid of a membrane protein in its native lipid environment.

In the Batho minus Rho IR difference spectrum obtained by illumination at 77 K an intense positive/negative peak pair appears at 3487 (Batho) and 3462 (Rho) cm^{-1} (Figure 3a),

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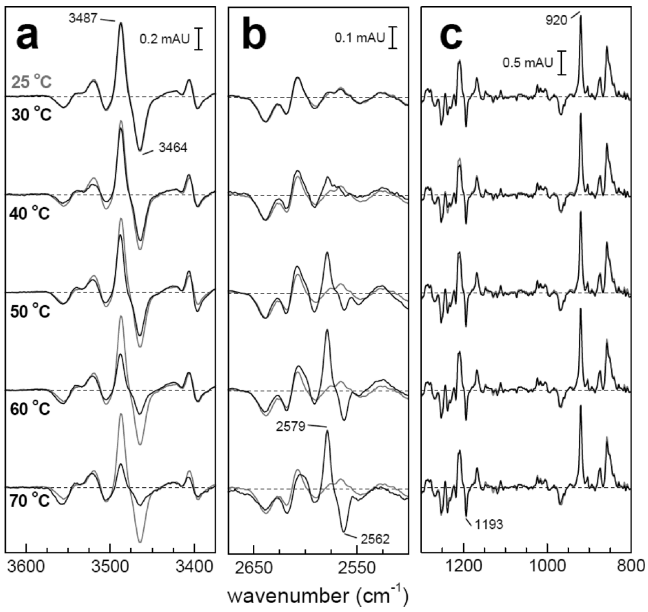

Figure 3. Hydrogen/deuterium exchange (HDX) of bovine rhodopsin. HDX was monitored from the Batho-Rho IR difference spectra of ROS films incubated in D_2O for 20 min at different temperatures at pH 7.5. (a) The O−H stretch difference band of Thr118 at 3487/3464 cm^{-1} in the Batho/Rho spectra decreases in intensity with the incubation temperature, with (b) a concomitant increase of the Thr118 O−D stretch at 2579/2562 cm^{-1}. (c) Batho-Rho difference spectra at different temperatures were scaled using the chromophore bands at 1193 and 920 cm^{-1}, to correct for any intensity differences between the samples. The gray spectrum corresponds to a difference spectrum taken at 25 °C, given as a visual reference.

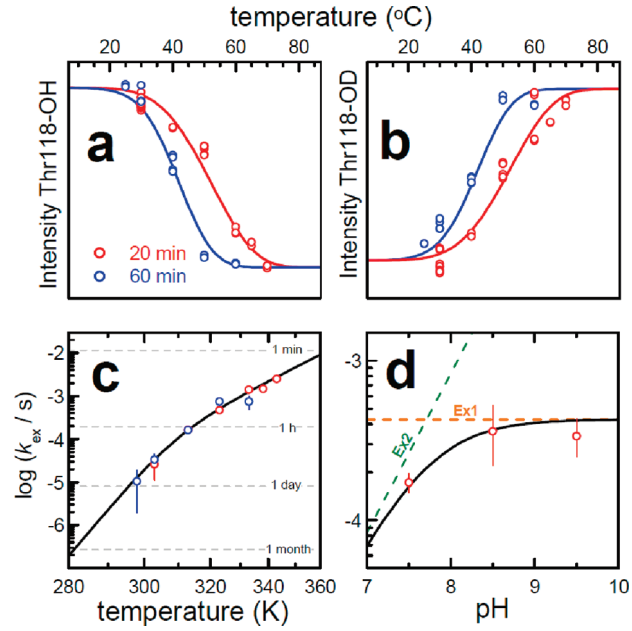

Figure 4. Analysis of the HDX of the Thr118 hydroxyl. (a, b) Experimental peak-to-peak intensities for (a) the Thr118 O−H stretching and (b) the Thr118 O−D stretching bands versus temperature for two different incubation times at pH 7.5 (open circles). (c, d) Rate constants of HDX, k_{ex}, as a function of (c) incubation temperature (at pH 7.5) and (d) pH (at 313 K) (open circles). The error bars correspond to the standard error of the mean. The HDX data were globally modeled with the HDX mechanism for labile hydrogens in proteins (see Scheme 1 and eqs 2 and 3). The best global fit is presented with continuous lines; the probability distributions estimated for relevant parameters are given in Figure 5 and for nuisance parameters in Figure S3 in Supporting Information.

unequivocally assigned to the O−H stretching vibration of Thr118.[45] This vibrational signature allows probing selectively the level of HDX of Thr118 after different incubation conditions, as shown below. Thr118 is a conserved amino acid in all visual rhodopsins of the RH1 family, including bovine rhodopsin.[46] Thr118 is located in helix III of Rho (Figure 1a,b), and constitutes part of the RBP (Figure 1c). Its side chain hydroxyl is at H-bonding distance to the backbone oxygen of Gly114 and is in steric contact with the C_9-methyl group of the retinal (Figure 1b), an essential element for Rho activation.[47] Moreover, Thr118 is deeply embedded within the protein (Figure 1a) and according to the X-ray crystal structures is inaccessible to internal or bulk water molecules.[17,18]

Although apparently not fulfilling any of the well documented requirements for HDX,[36] we found that Thr118 becomes deuterated when Rho was incubated in D_2O. Its deuteration level increases with the incubation temperature (Figure 3a,b and Figure 4a,b) and incubation time (Figure 4a,b). While the peak pair at 3487/3464 cm^{-1} decreases in intensity with increasing incubation temperature (Figure 3a), a new peak pair appears at 2579/2562 cm^{-1}, corresponding to the Thr118 O−D vibration (Figure 3b). The persistence of Thr118 O−H peak pair at higher incubation temperatures (or long incubation times) is due to the presence of contaminating H_2O along with D_2O during the HDX experiments, estimated to represent ∼30% of the total amount of water in the sample from the IR absorbance spectrum (not shown). This uncertainly was accounted for in the posterior quantitative analysis by treating the 100% level of HDX as an unknown. At this point, we should mention that any potential contribution of thermally denatured Rho to the measured HDX can be completely excluded by the functional Batho-Rho difference spectrum by which HDX was probed. Accordingly, the measured HDX corresponds selectively to the native and photoactive ensemble of Rho molecules. The well reproduced spectral changes in the protein and retinal fingerprint region for all incubation temperatures further confirms that functional Rho is probed in the Batho difference spectra (Figure 3c).

The substantial HDX of the Thr118 hydroxyl group implies that under native conditions a protein fluctuation breaks the H-bond of Thr118 with Gly114, separates Thr118 from the retinal C_9-methyl group and Try178 (Figure 1b), and allows a transient penetration of water molecules into the Thr118 vicinity. Unfortunately, the method used to probe selectively the HDX of Thr118 is not readily transferable to other amino acid side chain involved in the RBP of Rho, which do not have the same clear-cut signature in the Batho difference spectra. However, given the above-mentioned location of Thr118 (Figure 1a−c) and the requirements of HDX, we argue that the fluctuation making Thr118 competent for HDX will release at least some of the structural constraints of the RBP on the 11-*cis* PRSB, i.e., it will correspond to a loosening/opening of the RBP as sensed by the retinal. This view is in line with recent MD simulations reporting that fluctuations in the retinal conformation correlate with fluctuations in Thr118.[48] Therefore, we further conducted a more detailed characterization of the HDX of Thr118 hydroxyl.

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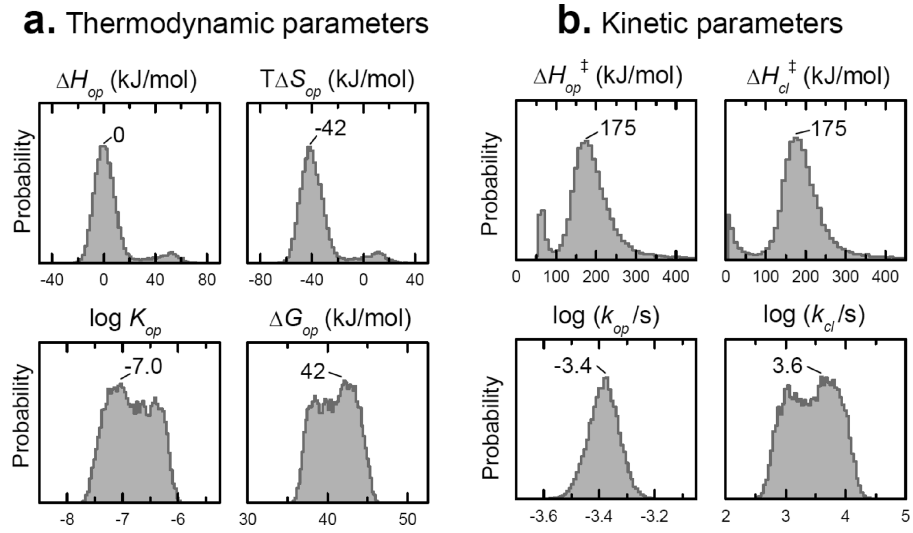

Figure 5. Probability histograms for the (a) thermodynamic and (b) kinetic parameters of the fluctuation process making Thr118 competent for HDX, obtained from the global Bayesian analysis of the experimental data in Figure 4. Note that from the eight parameters shown, only four of them are independent (e.g., ΔH_{op}, K_{op}, $\Delta H_{op}^{\ddagger}$, k_{op}), and the other four parameters (e.g., ΔS_{op}, ΔG_{op}, $\Delta H_{cl}^{\ddagger}$, k_{cl}) are obtained from known relations with the formers.

Quantitative Analysis of the HDX of Thr118 Hydroxyl. Figure 4a,b shows the peak-to-peak intensities of the Thr118-OH and Thr118-OD bands as a function of temperature for two incubation times, and Figure 4c shows the corresponding semiexperimental rate constants of HDX, k_{ex} (open circles), obtained as described in Methods. Similar data were obtained as function of the pH at fixed temperature (not shown), and the corresponding k_{ex} are plotted in Figure 4d (open circles).

A thermodynamic and kinetic characterization of the fluctuation making Thr118 competent for HDX requires a quantitative analysis of the HDX data. Despite its conceptual simplicity, a microscopic two-state model has been used successfully to describe and interpret the HDX of individual hydrogens in proteins under native conditions (see Scheme 1).[24−26,49,50]

In this model, HDX-incompetent closed (Closed) and HDX-competent open (Open) conformations interconvert with rate constants k_{op} and k_{cl}. HDX occurs only from the HDX-competent open conformation, with a chemical rate constant k_{ch}. The value of k_{ch} is essential for the analysis of the HDX data, but its value is generally unknown and not easily measurable. To cope with this problem, in the literature k_{ch} is systematically equaled to the rate of HDX of polypeptides adopting a random coil conformation, k_{rc}. This is done because the dependence of this rate on the chemical group, temperature, and pH is not only straightforward to measure but also fairly well understood and predictable.[24−26]

Studying k_{ex} versus temperature at sufficiently high pH allows the estimation of the kinetic parameters of the opening process (k_{op} and $\Delta H_{op}^{\ddagger}$), a condition known as the Ex1 limit.[25] At low pH, the estimation of thermodynamic parameters (K_{op} and ΔH_{op}) becomes possible, a condition known as the Ex2 limit.[51] Hence, global analysis of k_{ex} as a function of temperature and pH allows, in principle, a full thermodynamic and dynamic characterization of the fluctuation,[25] although conditioned to the values assigned to k_{ch} and its activation enthalpy, $\Delta H_{ch}^{\ddagger}$. However, this approach is sustained in the practice of equaling k_{ch} and k_{rc}, a practice that has been appropriately questioned.[52] Indeed, it has been reported that a hydrophobic environment and/or a partial solvent accessibility in the open conformation can make k_{ch} 20−25 times slower than k_{rc}.[53,54]

Our approach tries to avoid potential artifacts arising from the practice of blindly equaling k_{ch} and k_{rc} by acknowledging the possibility that the HDX of Thr118 in the open conformation could be slower than observed in random coil polypeptides. Therefore, we allowed k_{ch} to take any value from k_{rc} to k_{rc}/20 and $\Delta H_{ch}^{\ddagger}$ to take any value between 60 and 75 kJ/mol (see Methods). Then, we performed a global Bayesian analysis of the HDX data, obtaining probability distributions for the thermodynamic and kinetic parameters involved in the fluctuation process making Thr118 hydroxyl HDX-competent (Figure 5). It should be stressed that the obtained probability distributions presented here take account of our lack of accurate knowledge on the values of k_{ch} and $\Delta H_{ch}^{\ddagger}$, as well as on the level of 0% and 100% HDX (Supporting Information, Figure S3). They also take into account and provide correlations between the estimated parameters, as shown in Supporting Information (Figure S4). The best global fits, derived from the parameters with higher probability given the data, are displayed in Figure 4 (continuous lines), which reproduce the HDX data within the experimental error. We should finally note that, except from k_{cl}, the estimated values were mostly independent of the actual value assigned to k_{ch} and $\Delta H_{ch}^{\ddagger}$ within a physically reasonable range (see Figure S5 in Supporting Information).

Considering that the fluctuations that make Thr118 competent for HDX correspond to an opening of the RBP, our analysis shows that the transient opening of the RBP takes place every ∼30 min in average, closing back in less than 2 ms at 40 °C (Figure 5b). This transient opening is characterized by a free energy cost of ∼40 kJ/mol (equilibrium constant of $\sim 10^{-7}$), with a nearly zero enthalpic contribution (Figure 5a). The activation enthalpies for the opening and closing processes are

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around 175 kJ/mol, although with a large uncertainly due to their broad probability distributions (Figure 5b).

The estimated nearly zero enthalpic contribution for a fluctuation presumably opening the RBP may be unexpected. Given that water molecules are required to reach the vicinity of Thr118 to catalyze its HDX (or more precisely hydroxyl ions), it seems reasonable that some water molecules may enter in contact with other residues of the RBP or even with the retinal. These contacts could restore or effectively compensate the lost opsin-retinal interactions in the open conformation, explaining the low enthalpy change. The large negative entropy change (Figure 5a) would be in line with the loss of freedom of water molecules transferred from the bulk to the RBP vicinity. Although it may seem that these fluctuations are too short-lived (<2 ms) for bulk waters to penetrate to Thr118 (and to interact with the open RBP), we should recall that in the structurally homologous bacteriorhodopsin most internal waters exchange with the bulk on the time-scale of microseconds or less,[55] i.e., water molecules seem to be able to diffuse very quickly within proteins whenever cavities are formed.

General Discussion

Model for the Dark Activation of Rhodopsin Based on Protein Fluctuations. Figure 6 presents our model for the dark activation process of Rho. According to this model, the effective rate and activation enthalpy for retinal isomerization leading to thermal Rho activation, and hence to the dark events, will depend mainly on (i) the thermodynamic parameters of the fluctuation opening the RBP (K_{op} and ΔH_{op}) and (ii) the kinetic parameters of the thermal isomerization of the 11-*cis* PRSB in the open RBP (k_{iso} and $\Delta H_{iso}^{\ddagger}$), as

$$k_{eff} = \frac{k_{op}k_{iso}}{k_{op} + k_{cl} + k_{iso}} \approx K_{op}k_{iso} \quad (7)$$

$$\Delta H_{eff}^{\ddagger} \approx \Delta H_{op} + \Delta H_{iso}^{\ddagger} \quad (8)$$

where the approximations in eqs 7 and 8 consider that closing the open RBP will be much faster than the retinal isomerization in the open RBP: $k_{cl} \gg k_{iso}$ (see Figure 2c and Figure 5b).

To estimate the parameters in the proposed model we applied two experimental approaches: (i) characterization of the thermal isomerization of the 11-*cis* PRSB to all-*trans* in aqueous ethanol as a model for retinal isomerization in the open RBP and (ii) characterization of a fluctuation affecting Thr118 environment by means of HDX, which we take as the experimental indication of a transient opening of the RBP. The apparent enthalpic barrier for retinal isomerization and Rho activation in the dark predicted by our model is 95 ± 16 kJ/mol using eq 7 and the experimentally derived parameters (see Figure 6b and Table 1). This predicted barrier quantitatively agrees with the experimental apparent enthalpic barrier of the dark events found in vertebrates (toads), 82 ± 17 and 98 ± 14 kJ/mol,[7,9] and in invertebrates (limulus), 108 ± 33 kJ/mol.[8] Moreover, the effective rate constant for the thermal *cis-trans* retinal isomerization predicted by eq 7 is $\sim 10^{-12}$ s^{-1} at 36 °C (Table 1), in reasonable agreement with the estimated frequency of $\sim 10^{-11}$ s^{-1} of the dark events per Rho.[6] Note that these estimates for the activation enthalpy and for the rate constant of the dark activation of Rho have been derived completely from the analysis of experimental data, without any *ad hoc* adjustments.

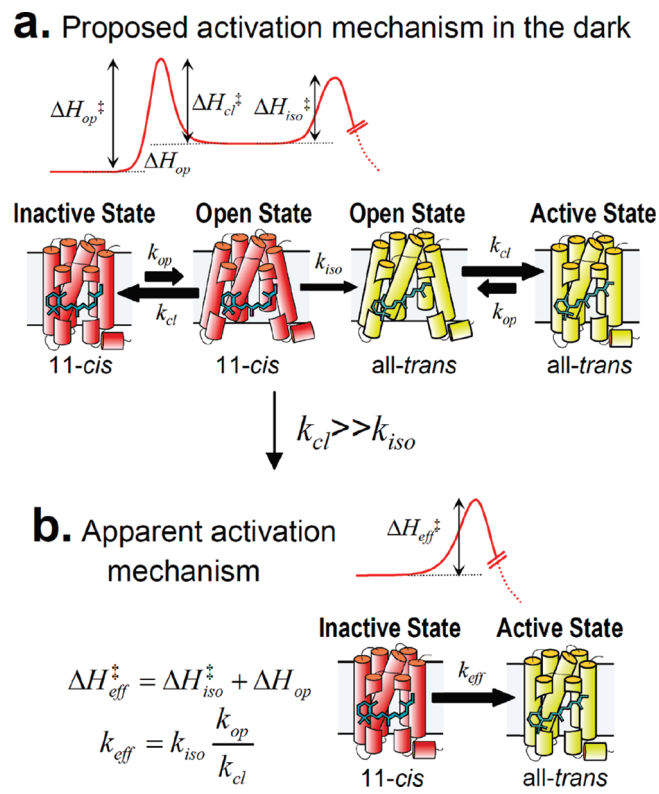

Figure 6. Proposed two-step model for the thermal isomerization of the 11-*cis* retinal in rhodopsin (Rho), leading to light-independent activation of Rho. (a) A protein fluctuation transiently "opens" the retinal binding pocket (RBP) allowing for retinal isomerization free from opsin constraints (solvent-like environment). Upon closure of the RBP, the isomerized all-*trans* chromophore acts as an agonist, leading to activation of the receptor. (b) Since the RBP closing process would be much faster than the retinal isomerization in the solvent-like environment ($k_{cl} \gg k_{iso}$), the model reduces to an apparent single step, and the effective activation enthalpy and rate constant for Rho dark activation will depend only on ΔH_{op}, K_{op}, $\Delta H_{iso}^{\ddagger}$, and k_{iso} values.

We explored to which extent the inherent ambiguities in the HDX data analysis to characterize fluctuations in proteins may question our results. As shown in Figure 7, our model can satisfactorily reproduce the values of the dark events for a quite reasonable interval of parameters used in the analysis of the HDX data. Namely, our estimation of $\Delta H_{eff}^{\ddagger}$ is in agreement with the activation enthalpy of the dark events (Figure 7a−b), independent of the values assumed for the chemical HDX of Thr118 hydroxyl in the open conformation (k_{ch} and $\Delta H_{ch}^{\ddagger}$). While our estimation of k_{eff} is fairly independent of the value used for $\Delta H_{ch}^{\ddagger}$ (Figure 7d), it is inversely related to the assumed value for k_{ch} (Figure 7c). Actually, if k_{ch} was 100 times slower than the rate constant of HDX in random coil poplypeptides, k_{rc} (we assumed instead $k_{rc}/20 \leq k_{ch} \leq k_{rc}$), then our predicted rate constant for retinal isomerization in Rho would be $\sim 10^{-11}$ s^{-1}, in full agreement with the experimental rate constant of the dark events. This observation suggests in turn that the activity of water in the vicinity of Thr118 in the open HDX-competent conformation could be 100 smaller than in solution.

Model of a Stepwise Mechanism for Rhodopsin Dark Activation. According to the proposed model and the collected experimental data, the thermal activation mechanism of Rho is suggested to proceed as follows. (i) Once every ~30 min on average a thermal fluctuation in Rho structure will transiently

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Table 1. Predicted versus Experimental Values for the Thermal Activation of Rhodopsin[a]

retinal isomerization in the open RBP[b]		transient opening of the RBP[c]			thermal activation of Rho (predicted)[d]		thermal activation of Rho (experimental)[e]	
$\Delta H_{iso}^{\ddagger}$	τ_{iso}, h	ΔH_{op}	K_{op}	τ_{op}, min	$\Delta H_{eff}^{\ddagger}$	k_{eff}, s^{-1}	$\Delta H_{eff}^{\ddagger}$	k_{eff}, s^{-1}
94 ± 3	30 ± 3	1 ± 16	$\sim 10^{-7}$	39 ± 6	95 ± 16	$\sim 10^{-12}$	80−110	$\sim 10^{-11}$

[a] Enthalpies are given in kJ/mol. The equilibrium constant and the time constants are given at 40 °C, whereas rate constants are given at 37 °C. Where reported, the confidence interval corresponds to ± 2 standard deviations. [b] Taken from the thermal isomerization of the protonated *n*-butylamine 11-*cis* retinal Schiff base in aqueous ethanol (Figure 2c). [c] Taken from the opening fluctuation making Thr118 hydroxyl competent for HDX (Figure 5). [d] Computed from the model in Figure 6. [e] Taken from the literature.[6−9]

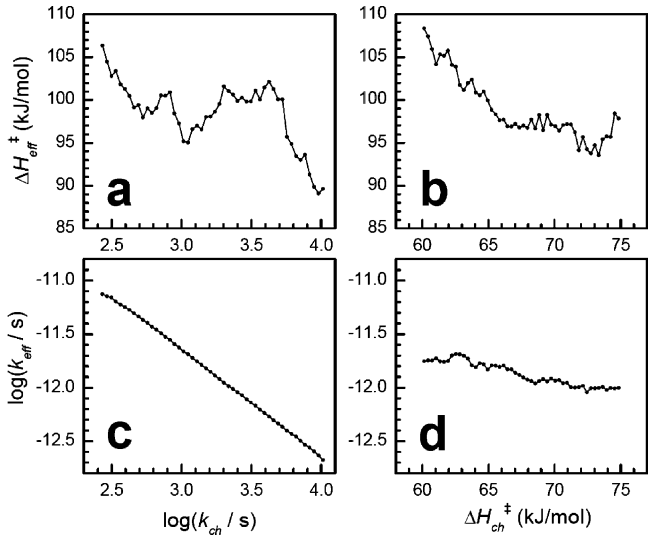

Figure 7. Predicted kinetic values for the thermal isomerization of the retinal in Rho at 36 °C, ($\Delta H_{eff}^{\ddagger}$ and k_{eff}) as a function of the parameters assigned to the chemical HDX of Thr118 hydroxyl in the open RBP ($\Delta H_{ch}^{\ddagger}$ and k_{ch}). The mean of the probability distributions for ΔH_{op} and K_{op} as a function of $\Delta H_{ch}^{\ddagger}$ and k_{ch} values were computed (Supporting Information, Figure S5) and used to calculate the predicted parameters for the thermal isomerization of the retinal in Rho from eqs 7 and 8.

open the RBP, releasing the tight constraints on the retinal conformation and possibly allowing for the temporal presence of water in the retinal vicinity. Only 1 out of $\sim 10^7$ Rho molecules will have an "open RBP" at a given instant. (ii) While the RBP is transiently open, retinal *cis-trans* thermal isomerization will be possible at the rate observed for the free retinal in solution. (iii) In less than 2 ms the "open RBP" will return to the most stable "closed RBP", where retinal *cis-trans* isomerization is effectively inhibited by a ≥180 kJ/mol barrier. (iv) Retinal thermal isomerization will take place once every $\sim 10^{8.5}$ openings of the RBP. In the case that such an opening of the RBP leads to the thermal retinal isomerization, Rho will sense the binding of all-*trans* retinal (an agonist) when returning back to the more stable closed conformation (in less than 2 ms). As a consequence, the reaction path leading to Rho activation is triggered, and a dark event will be generated.

Dark Events in Cone Receptors. Sharing the same chromophore molecule rod and cone receptors should display a similar rate of dark events if, as generally assumed, the thermal activation of photoreceptors is either controlled by the intrinsic propensity of the chromophore for thermal isomerization,[1,10,56] or by the barrier for light-activation.[15] However, it is well established that cone photoreceptors display 10^3-10^4 higher rates of dark events than rod photoreceptors,[13,57,58] while their apparent activation enthalpies for dark events are nearly coincident.[59]

Our model provides a plausible rationale for these observations. Given a fixed chromophore, the frequency of the dark events will be determined by the equilibrium constant for opening the RBP, and hence it will depend on the local stability of the chromophore binding site to fluctuations (eq 7). The higher rate of dark events in cones could be explained if the stability of the RBP was 10^3-10^4 lower in cone than in rod photoreceptors (K_{op} of $10^{-3}-10^{-4}$ instead of 10^{-7}). Although not experimentally proven to our knowledge, a less tight and stable RBP in cones than in rods is explicitly accepted by many authors.[59,60] On the other hand, our model predicts that the apparent activation enthalpy of the dark events should be primarily controlled by the barrier for the thermal isomerization of the chromophore in "solution" (eq 8). Therefore, allowing for some variability due to possible differences in ΔH_{op} among photoreceptors, the activation barrier of the dark events should be roughly similar in both cones and rods, in agreement with experimental observation.[59]

Purkinje Shift and the Dark Events. The Purkinje shift corresponds to the blue-shift of the maximum sensitivity of the eye at low levels of ambient illumination. It occurs as a result of the transition between the primary use of the photopic (cone-based) to the scotopic (rod-based) vision, the latter being systematically blue-shifted from the wavelength that would maximize photon catch. It has been postulated that this blue-shift would be adaptive by reducing the probability of Rho thermal activation.[61] This is indeed supported by the correlation ($R^2 = 0.59$) between rod photoreceptors absorbance maxima, λ_{max}, and their rate of dark events: a 50 nm red-shift in λ_{max} generating in average a ~10-fold increment in the rate of dark events at ~20 °C.[15] A similar correlation is also observed for cone photoreceptors.[15]

Although our model does not address directly the way that blue-shifted photoreceptors could display lower noise levels, it provides some means to explain it. Accordingly to our model, the ratio between the rates of dark events for two photoreceptors with λ_{max} given by λ_2 and λ_2 will be given by

$$\frac{k_{eff}^{\lambda_2}}{k_{eff}^{\lambda_1}} = \exp\left(-\frac{\Delta H_{op}^{\lambda_2} - \Delta H_{op}^{\lambda_1}}{RT}\right) \qquad (9)$$

where we considered other terms besides ΔH_{op} to remain constant. Accordingly to eq 9, we could explain a 10-fold increase in the dark events at 20 °C from a 50 nm red-shift in the chromophore λ_{max} if this shift correlates in turn with a ~5.6

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kJ/mol lower enthalpy for the RBP opening, i.e., with a loss of interactions between the retinal and the opsin. A correlation between the spectral tuning of the retinal by the opsin and the energetics of their interactions could be possible, given the calculation that a 11-*cis* PRSB devoid of all opsin interactions, i.e., *in vacuo*, red-shifts to 720 nm.[62]

Critical Assessment of Previous Models for the Thermal Activation of Rhodopsin. Fourteen years after the first description of the dark noise in rod photoreceptors,[5] Barlow et al. proposed the first plausible explanation for its molecular origin.[8] They hypothesized a two-step mechanism in which a transiently deprotonated SB could reduce, according to their calculations, the effective energy barrier for the *cis-trans* thermal isomerization of the retinal in Rho. We must stress that these calculations were performed before an atomic-resolution structure for Rho was available.[17] Therefore, Barlow et al. were forced to assume a specific retinal geometry for the 11-*cis* and all-*trans* retinal in Rho, notably ignoring the surrounding opsin residues that may contribute to the energy barriers for retinal isomerization and SB deprotonation by steric clashes and electrostatic effects. Nevertheless, this model was meritorious in successfully reproducing for the first time both the activation energy[8] and the rate of the dark events.[12] It also provided a simple rationale, although never confirmed, of why cone are noisier than rod photoreceptors: the pK_a of the SB should be 3−4 units lower in the former.[63] As a demerit, this model did not address the biological significance of the Purkinje shift. However, as a more important failure of this model we should mention the prediction of a strong pH dependence of the rate of the dark events,[12,63] recently experimentally disproved in rods[64] and in cones.[59] Actually, these recent findings make the mechanism proposed by Barlow et al. highly implausible, at least for vertebrate photoreceptors.

Soon after the validity of the Barlow model became seriously questioned, Ala-Laurila et al. presented a new hypothesis.[15] They postulated that the previously reported 2-fold difference between the thermal and light-activation barriers of Rho, suggesting that both represent difference processes, was an analytical artifact originating from the use of Bolzmann statistics to deduce energy barriers from the temperature dependence of the rate constants. Using Hinshelwood instead of Bolzmann statistics and assuming ∼40 modes involved in the activation state for retinal thermal isomerization in Rho, they showed that the thermal barrier could be brought into agreement with the light-activation barrier.[15] Although somehow contrived by the *ad hoc* selection of the number of modes involved in the active state, this hypothesis had the unquestionable merit of giving a quantitative explanation for the inversely correlation of the photoreceptors λ_{max} with their rate of dark events. As important demerits, we should mention that the Ala-Laurila hypothesis did not address the rate of dark events in Rho, which should be many orders of magnitudes lower than observed if thermal and light isomerization of the retinal follows the same path (see Introduction). Moreover, the Ala-Laurila hypothesis did not account by itself for the observation that cone photoreceptors are 10^3-10^4 times noisier than rod photoreceptors, despite displaying rather similar barriers for light activation. Finally, a recent attempt to test experimentally the Ala-Laurila hypothesis for the dark events provided contradictory results.[65] The increase in the rate of the dark events observed by shifting the λ_{max} of a photoreceptor by changing its chromophore was better accounted by Bolzmann than by Hinshelwood statistics, which contradicts their initial assumptions.[65]

Recently, Bokkom et al. proposed that the dark events could be the response of rhodopsin to the light emitted in the cells by various redox chemical processes (biophotons).[16] However, even if biophotons had enough energy to activate rhodopsin, there is not any experimental evidence that this activation could occur with the frequency and temperature dependence observed for the dark events.

Khrenova et al. using QM/MM calculations computed an energy barrier for thermal isomerization of the retinal in rhodopsin (from Rho to Batho) to be ∼92 kJ/mol.[66] Is this estimate was correct, it would mean that the dark events could originate from the thermal formation of Batho, similarly as the light formation of Batho causes the light events. However, the same work estimated the energy difference between Rho and Batho to be 67 kJ/mol,[66] while current experimental estimates all converge to values higher than 135 kJ/mol.[67−69] This discrepancy suggests that in their calculations Khrenova et al. could be underestimating energy differences by a factor of 2, which would return us back to an energy barrier for the thermal formation of Batho of ∼180 kJ/mol.

Outlook

In the present work we have successfully characterized both thermodynamically and kinetically a structural fluctuation in a membrane protein under native conditions. This has been accomplished for the first time to our knowledge, and it has been possible by combining hydrogen/deuterium exchange, difference IR spectroscopy, and global Bayesian analysis. Moreover, this fluctuation has the relevance of affecting the environment of a conserved residue in the rod photoreceptor rhodopsin, which is located nearby its active center. Although not strictly proven, we have provided reasonable evidence that this fluctuation may play a central role in the thermal activation of rhodopsin and be responsible for the spontaneous perception of light in total darkness. The present results illustrate how high-energy conformations in proteins, invisible for most structural methods due to their transient nature and marginal steady-state populations, may control key aspects of protein function and misfunction.

Acknowledgment. We are grateful to Tzvetana Lazarova and Reiner Vogel for critically reading the manuscript and for their suggestions and to Janos K. Lanyi for helpful comments. This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology to H.K. (20050015,

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20108014) and Y.F. (21023014, 21026016) and a UAB postdoctoral fellowship (40607) and a Marie Curie reintegration grant (PIRG03-6A-2008-231063) to V.A.L.F.

Supporting Information Available: UV−vis absorbance spectra of the dark incubated protonated retinal Schiff Base; experimental fractions of 13-*cis* and 9-*cis* retinal isomers as a function of time and temperature determined by HPLC, and their corresponding analysis; probability histograms for the nuisance parameters involved in the analysis of the HDX data; joint probability 2D histograms for pairs of kinetic/thermodynamic parameters in the analysis of the HDX data; joint probability 2D histograms of ΔH_{op} and K_{op} with $\Delta H_{ch}^{\ddagger}$ and k_{ch}. This material is available free of charge via the Internet at http://pubs.acs.org.

JA907756E