Chromophore Structure in Bacteriorhodopsin's N Intermediate: Implications for the Proton-Pumping Mechanism†

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ABSTRACT: By elevating the pH to 9.5 in 3 M KCl, the concentration of the N intermediate in the bacteriorhodopsin photocycle has been enhanced, and time-resolved resonance Raman spectra of this intermediate have been obtained. Kinetic Raman measurements show that N appears with a half-time of 4 ± 2 ms, which agrees satisfactorily with our measured decay time of the M_{412} intermediate (2 ± 1 ms). This argues that M_{412} decays directly to N in the light-adapted photocycle. The configuration of the chromophore about the C_{13}=C_{14} bond was examined by regenerating the protein with [12,14-^{2}H]retinal. The coupled C_{12}-^{2}H + C_{14}-^{2}H rock at 946 cm^{-1} demonstrates that the chromophore in N is 13-cis. The shift of the 1642-cm^{-1} Schiff base stretching mode to 1618 cm^{-1} in D_2O indicates that the Schiff base linkage to the protein is protonated. The insensitivity of the 1168-cm^{-1} C_{14}–C_{15} stretching mode to N-deuteriation establishes a C=N anti (trans) Schiff base configuration. The high frequency of the C_{14}–C_{15} stretching mode as well as the frequency of the 966-cm^{-1} C_{14}-^{2}H – C_{15}-^{2}H rocking mode shows that the chromophore is 14-s-trans. Thus, N contains a 13-cis, 14-s-trans, 15-anti protonated retinal Schiff base. These results, together with the recent confirmation that L_{550} contains a 14-s-trans chromophore [Fodor, S. P. A., Pollard, W. T., Gebhard, R., van den Berg, E. M. M., Lugtenburg, J., & Mathies, R. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2156–2160], argue against models for the bacteriorhodopsin photocycle that employ C_{14}–C_{15} bond rotation or inversion of the Schiff base nitrogen as a "reprotonation switch". An alternative "C–T model" for the photocycle is proposed that invokes isomerization-driven protein conformational changes as the reprotonation switch.

Bacteriorhodopsin (BR)[1] is one of several photoactive pigments contained in the cytoplasmic membrane of the halophilic bacterium *Halobacterium halobium* (Stoeckenius & Bogomolni, 1982). The retinal chromophore is attached to Lys_{216} of bacterioopsin via a protonated Schiff base linkage. Light absorption drives a rapid 13-trans → 13-cis photoisomerization which results in proton transport across the bacterial membrane. The J → K, K → L_{550}, and L_{550} → M_{412} steps in the photocycle (Figure 1) involve the relaxation of the 13-cis chromophore structure and deprotonation of the Schiff base. The chromophore must then be reprotonated and reisomerized to complete the photocycle. The molecular mechanism for this reisomerization is poorly defined. Reprotonation of the Schiff base seems necessary in order to lower the barrier for 13-cis to all-trans isomerization. This suggests that an intermediate containing a 13-cis protonated Schiff base should appear after M_{412}.

Recently, an additional intermediate has been identified in the later part of the BR photocycle which has been called P by Drachev et al. (1986, 1987) or R_{350} by Dancshazy et al. (1986). Kouyama et al. (1988) have shown that this intermediate lies between M_{412} and O_{640} and pointed out its functional significance. Illumination of an alkaline suspension of purple membrane produces a difference spectrum with a narrow depletion band centered at 580 nm and a small absorbance increase in the near-UV with a maximum at 350 nm. This result was attributed to photoconversion of BR_{568} to a long-lived intermediate, called N, which had an absorption maximum near 560 nm and a lower extinction than BR_{568}. Increasing the ionic strength and pH, or lowering the temperature, slowed the thermal decay of N, thereby increasing its concentration. This intermediate was termed N because of its position in the photocycle, consistent with the transient absorption experiments that first suggested its existence (Lozier et al., 1975).

By taking advantage of the slow decay kinetics of N at pH 9.5 and high salt, it is possible to obtain its resonance Raman spectrum. Our time-resolved resonance Raman spectra correlate the decay of M_{412} with the appearance of N. This argues that the N intermediate is formed directly from M_{412}. Specific isotopic substitutions were performed to show that N contains a 13-cis, 14-s-trans, C=N anti protonated Schiff base chromophore. Thus the M_{412} → N step involves Schiff base protonation, and the N → O_{640} step involves 13-cis → 13-trans isomerization. By use of previous knowledge of the photocycle intermediate structures and the structural information presented here, a new photocycle model is presented that uses

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[1] Abbreviations: BR, bacteriorhodopsin; HOOP, hydrogen out of plane; FTIR, Fourier transform infrared; MNDO, modified neglect of differential overlap.

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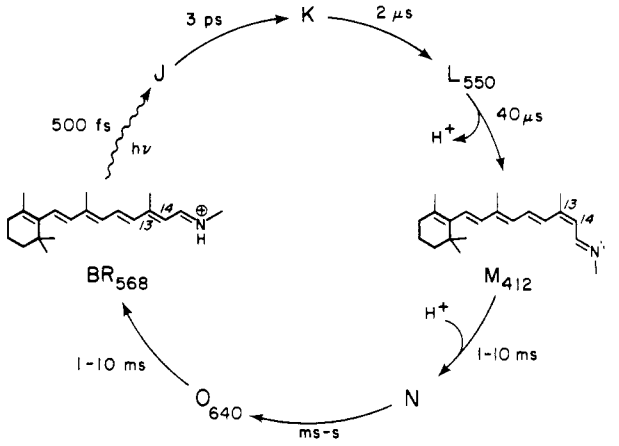

FIGURE 1: Proton-pumping photocycle of bacteriorhodopsin. The decay times in the later part of the photocycle are very sensitive to pH (Kouyama et al., 1988). At high pH the concentration of N builds up to significant levels because of its slow decay (~1 s at pH 9.5).

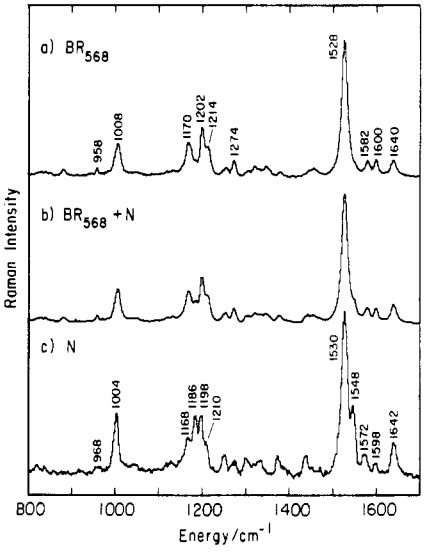

FIGURE 2: Resonance Raman spectra of bacteriorhodopsin's N intermediate in 3 M KCl and 10 mM borate, pH 9.5 at 22 °C. (a) BR_{568} spectrum excited at 514.5 nm. (b) Pump–probe spectrum of BR_{568} + N with a time delay of 100 ms. (c) Spectrum of pure N obtained by subtracting (a) from (b). On the basis of the ethylenic intensities, N contributes ~25% of the scattering in the pump–probe spectrum.

isomerization-induced protein conformational changes as a "reprotonation switch".

Experimental Procedures

The 12,14-^{2}H, 14,15-^{2}H, and 14,15-di-^{13}C derivatives of retinal were synthesized according to previously published procedures (Pardoen et al., 1984, 1986). BR was isolated from an overproducing strain of *H. halobium* (ET1001). The bleaching and regeneration procedures have been described (Fodor et al., 1988).

The BR samples were suspended in 10 mM borate buffer and 3 M KCl at pH 9.5. An ~2 OD/cm suspension was light-adapted and then placed in a recirculating flow system consisting of an unilluminated 50-mL reservoir (at ~22 °C) connected to a 1.5-mm diameter capillary which served as the optical cell. The sample was pumped through the closed loop system by a peristaltic pump at 300 cm/s. The Raman data were obtained by using a two-beam, pump–probe configuration. The amount of photointermediate produced by the laser depends upon the photoalteration parameter, $F = (3.824 \times 10^{-21})P\epsilon\phi/\nu d$ (Mathies et al., 1976). In this equation, P is the laser power (photons/s), ϵ is the extinction coefficient (M^{-1} cm^{-1}), ϕ is the quantum yield for the photochemical reaction, ν is the flow velocity (cm/s), and d is the focused beam diameter (cm). The photocycle was initiated with ~200 mW of 514.5-nm light cylindrically focused onto the 1.5-mm diameter capillary to give a photoalteration parameter of ~1 (ϵ = 35000, ϕ = 0.6). A 20-mW cylindrically focused 514.5-nm beam, displaced downstream from the pump, was used as the probe laser for the N spectra ($F < 0.1$). M_{412} spectra were obtained by using 10 mW of cylindrically focused 406.7-nm krypton laser light as the probe beam ($F < 0.1$).

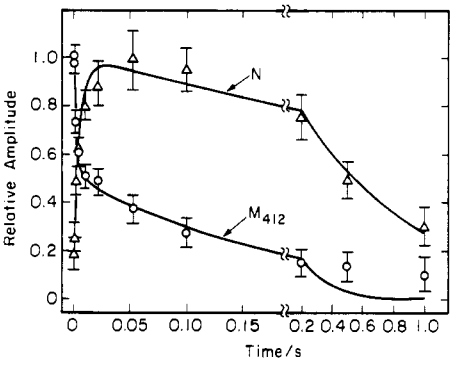

FIGURE 3: M_{412} and N concentration versus time. The concentrations were determined from the respective ethylenic intensities ratioed to a nitrate internal standard. The amplitudes were normalized to 1 at the maximum concentrations observed. Two exponential rate constants were required to adequately model the N and M_{412} data. The decay of M_{412} gave $t_{1/2}$ values of 1.6 ± 1 and 122 ± 40 ms. The rise and decay of N were fit with $t_{1/2}$'s of 4.3 ± 2 and 500 ± 200 ms, respectively.

The time-resolved kinetic Raman data for N and M_{412} were obtained by varying the time delay between pump and probe lasers from 0.5 ms to 1 s. The relative concentrations of M_{412} and N were determined by measuring the ethylenic band height, which was referenced to an internal nitrate standard (1049 cm^{-1}, 0.3 M KNO_3).

Raman data were acquired with a Spex 1401 double monochromator equipped with photon-counting detection and interfaced to a PDP 11/23 computer. The monochromator step size was 2 cm^{-1} with a 2-s dwell time, and the spectral band-pass was ~5 cm^{-1}. The spectra are the average of 5–20 scans.

Results

Our method for obtaining resonance Raman spectra of N is presented in Figure 2. The spectrum of BR_{568} is obtained with just the probe beam. Addition of a pump beam ~100 ms upstream produces the composite spectrum of BR_{568} and N in Figure 2b. Subtraction of spectrum a from spectrum b generates the N resonance Raman spectrum in Figure 2c. The subtraction parameter was selected to minimize any negative or positive BR_{568} peaks in the N spectrum that would result from over- or undersubtraction.

To ensure that the species we are examining is identical with the green-absorbing intermediate identified as N by Kouyama et al. (1988), we measured the 1530-cm^{-1} ethylenic band intensity in the difference spectrum as a function of time. These measurements are plotted along with the amplitude of the M_{412} ethylenic band in Figure 3. The decay of M_{412} and the rise and decay of N were fit to biexponential kinetics. The $t_{1/2}$ values for the decay of M_{412} were 1.6 ± 1 and 122 ± 40 ms, in good agreement with 1.6 and 110 ms from kinetic absorption studies (Kouyama et al., 1988).[2] The amplitude of N rose

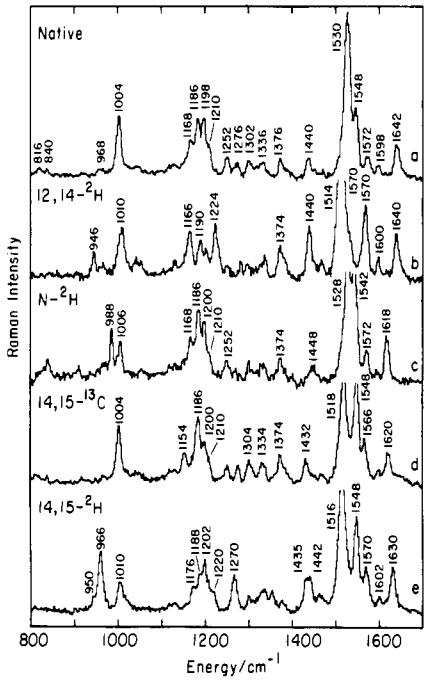

FIGURE 4: Resonance Raman spectra of native N (a) and its 12,14-^{2}H (b), *N*-^{2}H (c), 14,15-^{13}C (d), and 14,15-^{2}H (e) derivatives with a time delay of 100 ms.

with a $t_{1/2}$ of 4.3 ± 2 ms and decayed in 500 ± 200 ms, consistent with the ~800-ms recovery time for BR_{568} absorption under similar conditions (Kouyama et al., 1988). Kinetic absorption experiments were also performed on the samples used here, and the decay times were very similar (S. Sundberg and W. Stoeckenius, unpublished results). The close correspondence of the M_{412} decay and the rise of N in Figure 3 indicates that the species we are identifying as N is produced by the decay of M_{412}. The agreement between the N decay rates obtained from the resonance Raman intensities and the transient absorption spectra also suggests that we are studying the same species. The fact that the N signal rises from near zero amplitude at t = 500 μs where there is no residual L Raman scattering argues that we are not detecting a long-lived L species.

The most intense feature in the N spectrum in Figure 4a is the ethylenic doublet at 1530 and 1548 cm^{-1}. The doubled ethylenic line could arise from two C=C stretching bands or from two different intermediate structures. We have examined the relative intensity of these two ethylenic bands using a variety of time delays, pH, and salt conditions and have observed no changes in their relative amplitudes. Although these observations do not rule out multiple species, they do suggest that we are observing only one. An approximate linear correlation exists between the ethylenic band frequency and the absorption maximum of retinal pigments (Aton et al., 1977). The 1530-cm^{-1} band corresponds to a λ_{max} value of ~560 nm. This value compares favorably to the λ_{max} range of 550–560 nm calculated from the transient absorption data (Kouyama et al., 1988) and further suggests that we are studying the same intermediate.

The resonance Raman spectrum of N contains a great deal of information about the structure of its chromophore. We have performed extensive vibrational analyses of retinal pigments (Smith et al., 1987a,b) which have permitted the development of a variety of methods for determining chromophore structure (Smith et al., 1984, 1985, 1986; Mathies et al., 1987). The configuration about the C_{13}=C_{14} bond can be established by examining the C_{12}-^{2}H + C_{14}-^{2}H rocking vibration in pigments regenerated with [12,14-^{2}H]retinal (Smith et al., 1985). In all-trans chromophores there is strong coupling between the deuterium rocks, and the C_{12}-^{2}H + C_{14}-^{2}H rocking mode appears near 910 cm^{-1}. In 13-cis chromophores, the coupling is weaker and the frequency of the C_{12}-^{2}H + C_{14}-^{2}H rocking mode is characteristically found at ~940 cm^{-1}. The C_{12}-^{2}H + C_{14}-^{2}H rocking mode in N is found at 946 cm^{-1}, demonstrating that N contains a 13-cis chromophore (Figure 4b).

N exhibits a protonated Schiff base stretching mode at 1642 cm^{-1}. The Schiff base stretch shifts down to 1618 cm^{-1} in ^2H_2O, and a new band appears at 988 cm^{-1} which we assign as the N-^{2}H rock (Figure 4c). Both the downshift in the C=N stretch and the appearance of the N-^{2}H rocking vibration show that the chromophore in N is a protonated Schiff base.

The configuration of the C=N bond in protonated Schiff bases can be determined from the sensitivity of the C_{14}-C_{15} stretch to N-deuteriation (Smith et al., 1984). In C=N syn-(cis-) protonated retinal Schiff bases, the C_{14}–C_{15} stretch and the N–H rocking motions are strongly coupled so that N-deuteriation causes a large upshift in the C_{14}–C_{15} stretching frequency. In C=N anti (trans) compounds, the coupling is weak and little or no frequency changes are observed in the C_{14}–C_{15} stretch upon N-deuteriation. The first step in this analysis is to assign the C_{14}–C_{15} stretch in N. The only mode in the 1100–1300-cm^{-1} "fingerprint region" that shifts upon 14,15-^{13}C substitution is the line at 1168 cm^{-1}, which moves to 1154 cm^{-1} (Figure 4d). Since no other bands move in this derivative, C_{14}–C_{15} stretch character must be highly localized in the 1168-cm^{-1} mode. Suspension in D_2O results in no shift of the 1168-cm^{-1} line (Figure 4c), demonstrating that N contains a C=N anti Schiff base.

The conformation about the C_{14}–C_{15} bond in N can also be determined because the vibrational properties of the HC_{14}–$C_{15}H$ group are sensitive to structure. Smith et al. (1986) first pointed out that the C_{14}–C_{15} stretching frequency is sensitive to C_{14}–C_{15} conformation. The high frequency of the C_{14}–C_{15} mode in N (1168 cm^{-1}) is consistent with a 14-s-trans conformation. Recently, we have shown that the C_{14}–C_{15} conformation can also be ascertained from the frequency of the symmetric deuterium rock combination in 14,15-^{2}H-substituted retinals (Fodor et al., 1988). The symmetric rock combination is observed at ~970 cm^{-1} in 14-s-trans chromophores, and it is predicted at ~850 cm^{-1} in 14-s-cis chromophores. For example, L_{550} exhibits a symmetric C_{14}-^{2}H – C_{15}-^{2}H rock combination at 968 cm^{-1} which is within 5 cm^{-1} of the BR_{568} value (963 cm^{-1}; Fodor et al., 1988). MNDO calculations show that the C_{14}-^{2}H – C_{15}-^{2}H rock patterns are not significantly altered by chromophore distortions or by increased π-electron delocalization brought about by changes in the charge environment of the Schiff base (Fodor et al., 1988). The C_{14}-^{2}H – C_{15}-^{2}H rock combination in N is located at 966 cm^{-1} (Figure 4e), which shows that N contains a C_{14}–C_{15} s-trans retinal chromophore.

[2] We believe that the 122-ms component in our M_{412} decay arises from an intrinsic decay process in the light-adapted photocycle and cannot be due to a photolysis product of N (Kouyama et al., 1988). First, the transit time of our sample through the photolysis beam is less than 20 μs. This interaction time is too short to both produce and photolyze N. Second, the recirculation time of the sample (5–8 s) is long compared to the lifetime of N (0.5 s). Third, difference spectra of unphotolyzed BR_{568} between pH 9.5 and 7.0 show no evidence for an increase in the thermal population of N at pH 9.5. Finally, resonance Raman spectra of the fast and slow decay components of M_{412} are identical.

Discussion

To determine the mechanism of proton pumping in BR, it is necessary to know the chromophore structure in each intermediate. The sequence of events that leads to M_{412} formation, 13-trans to 13-cis photoisomerization, and Schiff base deprotonation is well-defined. These structural changes must be reversed to reset the proton pump. Therefore, the identification of an intermediate(s) between M_{412} (13-cis, unprotonated) and O_{640} (all-trans, protonated) is of primary importance. Recent transient absorption studies (Kouyama et al., 1988; Drachev et al., 1986, 1987; Dancshazy et al., 1986) have identified conditions under which such an intermediate (N) can be studied. The kinetic resonance Raman data presented here strongly suggest that N is a product of M_{412} decay and is therefore part of the light-adapted photocycle. The analysis of the isotopic shifts in N demonstrates that it contains a 13-cis, 14-s-trans, C=N anti protonated retinal Schiff base chromophore. Thus the sequence of events in the later half of the photocycle is now determined. First, the Schiff base in M_{412} is reprotonated, generating N. Second, a thermal 13-cis to 13-trans isomerization occurs producing the red-shifted O_{640} chromophore, which relaxes to BR_{568}.

Two other groups using resonance Raman spectroscopy have detected a variety of "N-like" species at high pH (Alshuth & Stockburger, 1986; Maeda et al., 1986; Ogura et al., 1987) but interpreted them differently. Alshuth and Stockburger (1986) reported a species that they called "L" from their kinetic Raman data acquired in a 50-Hz spinning sample chamber. Ogura et al. (1987) and Maeda et al. (1986) suggested that the "L" species observed by Alshuth and Stockburger was a long-lived component or photoproduct in the spinning cell since they could not observe its spectrum at early times ($\sim$100–200 μs) in a single-pass flowing sample system. In light of the present data, these previous experiments can be explained. Since the decay time of N exceeds the $\sim$20-ms revolution time of the sample chamber used by Alshuth and Stockburger (1986), the contribution of N builds up in the cell. This will give the appearance that N (alias "L") is present at all times. The single-pass flow experiments of Ogura et al. (1987) employed delay times of 60–260 μs, which is too fast to observe M decay and the rise of the N intermediate.

Now that the structure of the chromophore in N has been determined, it is possible to incorporate N in the proton-pumping mechanism. An important concept is the idea of a "reprotonation switch" to control the directionality of proton flow. After the Schiff base proton is given up in the $L_{550} \rightarrow M_{412}$ transition to a residue that can transmit the proton to the cell exterior, the reprotonation switch assures that the Schiff base will be reprotonated by a group connected to the cytoplasmic surface. Models have been proposed where C_{14}–C_{15} bond rotation (Schulten & Tavan, 1978; Liu et al., 1985) or Schiff base anti $\rightarrow$ syn inversion is used as the reprotonation switch (Smith et al., 1986). Resonance Raman spectroscopy has provided strong evidence against the C_{14}–C_{15} s-cis model (Smith et al., 1986; Fodor et al., 1988). The C=N inversion model predicts that N would contain a 13-cis, C=N syn protonated retinal Schiff base in disagreement with the results presented here. *It is now clear that M_{412} undergoes reprotonation to form N with no change in Schiff base orientation. This suggests that the conductivity of the proton pump is not controlled by changes in the retinal geometry but instead by a rearrangement of the protein.*

A "C–T model" for a protein-based reprotonation switch is presented in Figure 5. We begin by assuming that the light-adapted protein can exist in two basic forms, T and C,

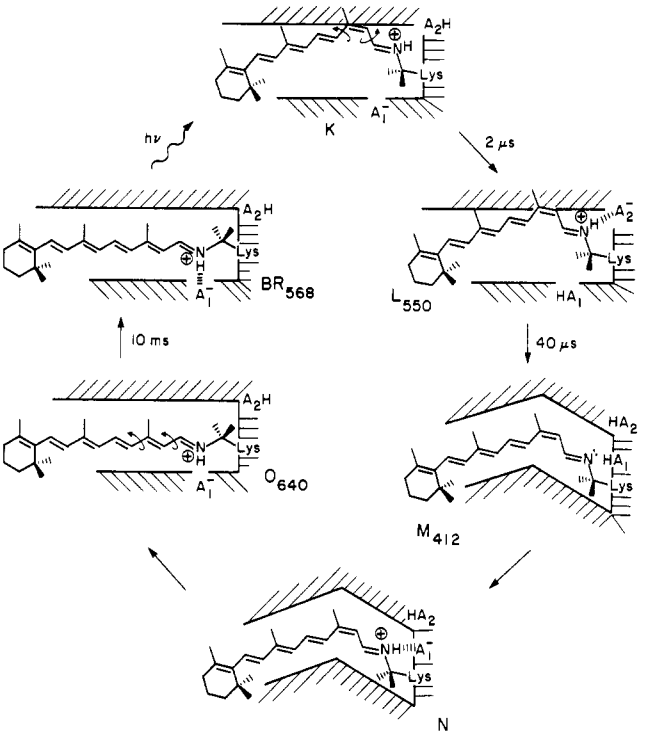

FIGURE 5: "C–T model" for the BR photocycle which utilizes isomerization-driven protein conformational changes as the "reprotonation switch". The protein can have two tertiary structures called T and C. This denotes whether the protein conformation has relaxed to accommodate the 13-trans or 13-cis configuration of the chromophore. Photon absorption by BR_{568} converts the chromophore to 13-cis and translates the Schiff base hydrogen from A_1^- to a new environment near HA_2. During or after the deprotonation of the Schiff base in the L_{550} to M_{412} transition, the protein conformation changes from T to C. This protein conformational change is driven by the affinity of the C-form of the protein for the 13-cis chromophore geometry. The T $\rightarrow$ C change also serves as the reprotonation switch by disconnecting HA_2 from the Schiff base and connecting HA_1.

corresponding to protein conformations accommodating the *all-trans*- or 13-*cis*-retinal geometries, respectively. In analogy with ligand-binding models for allosteric proteins, the T-form is the most stable conformation of the *protein*. Absorption of a photon by BR_{568} isomerizes retinal to the 13-cis configuration in K, and the protein is forced into a strained T state. The isomerization switches the connectivity of the Schiff base moiety from a hydrogen-bonded chain connected to the cytoplasm (HA_1) to another chain connected to the exterior of the cell (HA_2) (Stoeckenius et al., 1978). The steric distortion of the 13-cis K chromophore in the T binding pocket (indicated by arrows in Figure 5) is supported by the observation of strong hydrogen out-of-plane (HOOP) intensity in the resonance Raman spectrum (Braiman & Mathies, 1982).

The K $\rightarrow$ L_{550} step involves proton movements within the protein and relaxation of the chromophore distortions which are accompanied by an increase in Schiff base hydrogen-bond strength and a decrease in HOOP intensity (Eisenstein et al., 1987; Engelhard et al., 1985; Smith et al., 1985). This transition must also involve some local rearrangements of the protein residues in the chromophore binding pocket which can be thought of as "functionally important motions" as discussed by Frauenfelder et al. (1988) and Ansari et al. (1985).

In the $L_{550} \rightarrow M_{412}$ transition we introduce the concept of a protein-based reprotonation switch. The deprotonation of the Schiff base during the $L_{550} \rightarrow M_{412}$ transition shuttles a proton to the membrane exterior along the hydrogen-bonded chain connected to A_2. The protein also undergoes a major conformational change from the T-form to the C-form, which

is more compatible with the 13-cis chromophore configuration. This large conformational change of the protein can be thought of as a "proteinquake" (Ansari et al., 1985; Frauenfelder et al., 1988). Even though the C-form is not the most stable conformation of the *protein*, the binding energy of the 13-cis chromophore for the C-form of the active site makes this transition downhill. This conformational change may also be facilitated by changes in the protonation state of the HA_2 chain. It is reasonable for this structural transition to occur on a microsecond time scale. For example, the allosteric changes from the R → T state in hemoglobins occur in ~20 μs (Hofrichter et al., 1985). Consistent with this model, FTIR measurements show large changes in the amide I and amide II region in the $BR_{568} \rightarrow M_{412}$ difference spectra (Braiman et al., 1987; Bagley et al., 1982; Rothschild & Marrero, 1982; Engelhard et al., 1985). The observation that reduced hydration decreases the magnitude of the amide changes is also consistent with the presence of large protein structural changes (T → C) at this stage in the photocycle (Braiman et al., 1987). We further suggest that the T → C transition shifts residue HA_2 away from the Schiff base and reconnects HA_1 to the Schiff base. This conformational change allows the reprotonation of the Schiff base by the A_1 chain to produce N.

The protonated Schiff base in N has a lower $C_{13}=C_{14}$ isomerization barrier which facilitates the reisomerization. To stabilize the 13-trans product in the $N \rightarrow O_{640}$ transition, the protein also switches from its high free energy C-form to the low free energy T-form. However, conversion to a fully relaxed T-form must not be complete in O_{640} since the chromophore exhibits intense HOOP modes and altered Schiff base properties, indicating structural distortion (Smith et al., 1983). The protein–chromophore complex finally relaxes in the $O_{640} \rightarrow BR_{568}$ transition to a minimally strained all-trans chromophore in a T protein state. It will be interesting to examine the $M_{412} \rightarrow N \rightarrow O_{640}$ portion of the photocycle with FTIR spectroscopy to determine when the amide structural changes, associated with the T → C transition, are reversed.

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