Correlation between absorption maxima and thermal isomerization rates in bacteriorhodopsin

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ABSTRACT The reported rates of thermal 13-*cis* to all-*trans* isomerization of the protonated Schiff base of retinal (PSBR) in solution and in bacteriorhodopsin (BR) are shown to be correlated with the red shift in the absorption maximum of the chromophore, though the linear fit is different for BR and for a model PSBR in solution. Because the red shift in the absorption has been previously shown to be correlated with π-electron delocalization in the chromophore, this suggests that the thermal isomerization rate is largely regulated by the amount of double bond character in the chromophore. Because the linear fit of isomerization rates with absorption maxima is different for BR and the model PSBR, specific interactions of the protein with the chromophore must also be a factor in determining thermal isomerization rates in BR. A model of the later steps in the photocycle of BR is presented in which the 13-*cis* to all-*trans* thermal isomerization occurs during the O intermediate.

INTRODUCTION

Bacteriorhodopsin (BR) is the primary protein in the cell membrane of *Halobacterium halobium.* Within picoseconds of absorption of visible light by light adapted BR (laBR), its chromophore, a protonated Schiff base of retinal (PSBR), isomerizes from all-*trans* to 13-*cis* (Sharkov et al., 1985; Mathies et al., 1989; Ottolenghi and Sheves, 1989). This event initiates a process called the BR photocycle, in which the protein goes through a series of intermediates whose ultimate function is to pump a proton across the cell membrane (Stoeckenius and Bogomolni, 1982; Ottolenghi and Sheves, 1989). The photoisomerization event acts as the energy transforming switch that initiates the BR photocycle, as in those laBR that absorb light but do not isomerize, no photocycle ensues and no protons are pumped. The photocycle is completed in <10 ms at room temperature, and when it is finished, all-*trans*-BR is obtained in its initial state (Lozier et al., 1975; Hofrichter et al., 1989), implying that thermal 13-*cis* to all-*trans* isomerization occurs in <10 ms. Slower thermal isomerization of the PSBR during the photocycle would slow down the regeneration of laBR and retard the rate at which BR could absorb light and pump protons. The regeneration of laBR is slowed in certain single point mutants of the protein (Mogi et al., 1988; Holz et al., 1989; Butt et al., 1989), and in these cases the yield of pumped protons, upon irradiation of the protein with high light fluxes, is limited by the rate of return of laBR. Thus, the rapid thermal isomerization of the PSBR that occurs during the photocycle of BR is critical for the efficient function of the protein.

Thermal isomerization also occurs in BR in the dark, with the nearly 100% all-*trans* PSBR in laBR equilibrating in ~ 20 min in the dark at neutral pH and room temperature to give approximately 33% all-*trans* and 67% 13-*cis* PSBR in dark adapted BR (daBR) (Scherrer et al., 1989). It is known that the 13-*cis*-BR does not pump protons upon absorption of light and that it reverts to the photoactive all-*trans*-BR (laBR) with a quantum yield of less than 0.05 (Ohno et al., 1977; Hofrichter et al., 1989). Thus, thermal isomerization from all-*trans* to 13-*cis* BR decreases the proton pumping photoactivity of BR when the ambient light level is low. At higher light fluxes the 13-*cis*-BR is converted to all-*trans*-BR at a rate that is greater than the thermal back isomerization, and the effective quantum efficiency of proton pumping is greater.

Efficient thermal isomerization of the PSBR in BR appears to be essential for its appropriate function as a photocatalyst within the bacterium. Whereas the nature of the intermediates important in the proton pumping of BR have been the subject of extensive investigation, the details of the thermal isomerization in BR have been studied much less. There are two basic views of how rapid isomerization occurs in BR. One is that the isomerase activity of the protein is due to enhanced π-electron delocalization in the PSBR that is induced by the electrostatic interactions of the protein with the chromophore (Orlandi and Schulten, 1979; Sheves and Baasov, 1984; Tavan et al., 1985). The other is that catalysis of the isomerization is induced by transient nucleophilic addition of an aspartate to C_{13} of the PSBR (Seltzer, 1990). Intermediate to these views is a model in which a negatively charged group is close to the C_{13} of

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the PSBR and contributes to inducing a greater π-electron delocalization in the chromophore. In the current work, previously reported results on the thermal isomerization rates of BR and of model PSBR in solution are used to show that there is a correlation between the thermal isomerization rate and the position of the absorption maximum of the chromophore. Whereas the current work cannot rule out the possibility that a transient nucleophilic addition to C_{13} of the PSBR occurs, it is concluded, in agreement with previous theoretical calculations (Tavan et al., 1985; Seltzer, 1987), that it is possible to ascribe the isomerase activity of BR, in large part, to π-electron delocalization that decreases the strength of the double bonds in the PSBR. A tentative model, based on results reported on single point mutants of BR and on kinetic results on the photocycle kinetics of BR, is presented that shows a picture of the photocycle of BR that is consistent with rapid thermal isomerization.

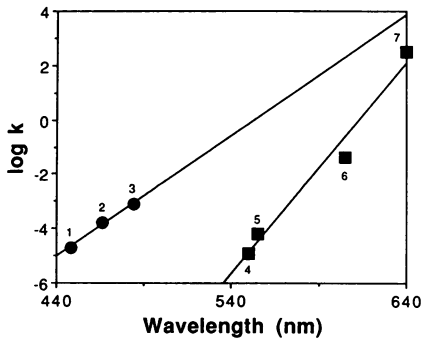

FIGURE 1 log k(k = rate of 13-*cis* to all-*trans* isomerization) versus absorption maximum of: (●) *n*-butyl PSBR in $CDCl_3$ solution (1 = Cl^- salt, 2 = ClO_4^- salt, 3 = 0.35 M CF_3CO_2H) slope = 4.4 × 10 nm^{-1}, y-intercept = −47.4, r = 0.997; (■) BR (4 = pH 10, 5 = pH 7, 6 = pH 3, 7 = photocycle) slope = 7.7 × 10 nm^{-1}, y-intercept = −47.3, r = 0.986. Data are from references cited in text.

ISOMERIZATION RATES

The literature values of the rates of 13-*cis* to all-*trans* isomerization will be plotted versus the absorption maxima for model PSBR. Sheves and Baasov (1984) have reported the isomerization rates for model PSBR in $CDCl_3$. These model compounds differed in the primary amine used to form the Schiff base and/or the acid and its concentration used to protonate the Schiff base. The authors used NMR to obtain the isomerization rates of 13-*cis* to all-*trans* PSBR and reported the half-life for isomerization to the 20:80 13-*cis*:all-*trans* equilibrium mixture they obtained. From these half-lives one can calculate a first-order rate for the 13-*cis* to all-*trans* process, as the observed rate is the sum of the forward and reverse rates and the ratio of these rates is determined by the mixture at equilibrium. On Fig. 1 are plotted the 13-*cis* to all-*trans* isomerization rate for the *n*-butyl PSBR/Cl^-, PSBR/ClO_4^- and the *n*-butyl PSBR protonated with 0.35 M trifluoroacetic acid (TFA), all in $CDCl_3$. For each of these solvent conditions the log of the rate is plotted against the absorption maximum of the chromophore in $CHCl_3$ that was obtained experimentally under similar protonation conditions. For the case of protonation by HCl and $HClO_4$, the absorption maximum of the 13-*cis*,*n*-butyl PSBR is 448 and 466 nm, respectively, whereas for protonation by 0.35 M TFA the absorption maximum is 484 nm. As seen in Fig. 1, a plot of log k versus the absorption maximum for the model PSBR in the three protonation conditions defines a straight line, with r = 0.997, slope = 4.4 × 10^{-2} nm^{-1}, and y-intercept = −47.4. Similarly, if a plot is made of the log of the 13-*cis* to all-*trans* isomerization rate versus the energy at the absorption maximum (in $centimeter^{-1}$) a highly correlated plot is obtained, with r = 0.998, slope = -9.6×10^{-4} cm, and y-intercept = 16.9.

In the case of BR in its native lipid environment of the purple membrane (PM) in different external solvation environments, a correlation can be obtained between the log of the rate of thermal 13-*cis* to all-*trans* isomerization and the absorption maximum. In the following, the estimates used to obtain the rates and absorption maxima may not give the exact values; however, the high correlation obtained would still be found for other reasonable estimated values. Because the plot for the model compounds was of the 13-*cis* to all-*trans* thermal rate at 25°C, the rates of the BR in PM will be estimated for these condition. A number of years ago Ohno et al. (1977) reported the observed rate of dark adaptation for BR in PM at pH between 3.5 and 10 at 30°C. At neutral and basic pH, dark adaptation was seen to follow first order kinetics, whereas at acidic pH deviations from first order were observed. The reported rates were analyzed by Warshel and Ottoenghi (1979) with a model that assumes there are ionizable groups within the protein whose protonation/deprotonation define the forms of BR that have the different isomerization rates. Dark adaptation rates of 0.12, 5 × 10^{-4}, and $<5 \times 10^{-5}$ s^{-1} were determined for low pH, blue BR, neutral pH BR, and high pH BR. To convert these values to 13-*cis* to all-*trans* rates at 25°C, one needs to know the isomer ratio when the thermal equilibrium between the isomers is obtained and the activation energy of the isomerization process. A number of papers have reported on the determination of the isomer ratio in equilibrated, dark adapted BR (daBR) (Ohno et al., 1977; Fischer and Oesterhelt, 1979; Sperling et al., 1979; Scherrer et al., 1989). Even at neutral pH there has been disagreement,

but the most recent report is that there is ~2:1 13-*cis*:all-*trans* PSBR at thermal equilibrium (Scherrer et al., 1989). For low pH BR, which is also known as blue-BR due to its red-shifted absorption and blue color, a few values have been reported: 2:3 and 3:7 for the relative amount of 13-*cis* and all-*trans* PSBR at thermal equilibrium (Fischer and Oesterhelt, 1979; Ohtani et al., 1986); the average of these gives ~1:2 13-*cis*:all-*trans*. The isomer ratio has not been reported for high pH BR, but it is reasonable to expect that it would be in the range of BR in the other conditions, and for the sake of calculation of the isomerization rate it is taken to be 1:1 13-*cis*:all-*trans*. Only a limit of the rate for the observed isomerization is reported for the high pH BR, and as this is the most reliable number available, this will be used. Also, to allow for comparison of the rates with those of the model compounds, the rates estimated for 30°C have been divided by 2.0, a factor that represents the decrease in rate expected at 25°C for a process that has an activation energy of 24 kcal/mol. This is the value reported for the activation energy of the thermal isomerization for BR at neutral pHs (Ohno et al., 1977; Tavan et al., 1985). One expects the activation energy to be somewhat different for the BR at high and low pH, but the differences should not significantly affect the value of log k because of the small change in the temperature in the calculation. From the above, the rate at 25°C for thermal isomerization is estimated to be 4.0×10^{-2}, 6.67×10^{-5}, and 1.25×10^{-5} for low, neutral, and high pH BR in PM.

To plot points for BR similar to those plotted on Fig. 1 for the model PSBR, it is necessary to know not only the rates of thermal isomerization, it is also necessary to know the absorption maximum of the species. The absorption spectrum of 13-*cis* and all-*trans* PSBR are known to be different in BR at neutral pH. There has been some disagreement in the literature as to the absorption maximum of 13-*cis* BR at neutral pH, with values between 548 and 555 nm having been reported (Stoeckenius and Bogomolni, 1982; Scherrer et al., 1989). The value of 555 nm is from a recent and complete study of both the isomer ratio in daBR and the absorption maximum of the 13-*cis* species that makes up ⅔ of it (Scherrer et al., 1989). Thus, the absorption maximum of the 13-*cis* BR is 5 nm to the blue of that of daBR, whose absorption maximum is at 560 nm. The absorption maximum of dark adapted low pH, blue BR is 605 nm (Fischer and Oesterhelt, 1979; Ohtani et al., 1986) and that of high pH BR is ~550 nm (Muccio and Cassim, 1979). These values are used in the plot shown on Fig. 1, as there are no reports on the relative position of the absorption of the 13-*cis* and all-*trans* BR under these conditions. Though small differences in the position of the 13-*cis* BR absorption relative to that of the equilibrated BR for high and low pH BR are likely, such a difference between the actual and plotted value would not significantly affect the correlation observed. In fact, if the 13-*cis* absorption maximum is assumed to be a few nanometers to the blue of the spectrum of the equilibrated species in both high and low pH BR, as it is in neutral pH BR, the correlation between log k and the absorption maximum is higher.

As mentioned in the Introduction, not only does thermal isomerization occur in the dark, it occurs during the photocycle. The rate of isomerization must be very rapid in the photocycle, as the photocycle is completed in ~10 ms at room temperature in neutral solution. Resonance Raman and FTIR have identified the chromophore in the K through N intermediates as 13-*cis* (Rothschild and Marrero, 1982; Fodor et al., 1988; Ames et al., 1989) and during the O intermediate as all-*trans* (Smith et al., 1983). In the work on the resonance Raman spectrum of O, which was done at 40°C, the rise of O is observed to take place in 1.6 ms, whereas the spectrum is obtained between 3 and 6 ms after the photolysis. Thus, what is known is that the isomerization occurs at least some time after the N intermediate decays and within a few milliseconds after the O intermediate has formed. Whereas in models of the photocycle it is common to show that the isomerization from 13-*cis* to all-*trans* occurs concomitant with the N to O transition (Ames and Mathies, 1990), the current work suggests that a different model should be considered. In both the model compound and BR there is a more rapid thermal isomerization for the PSBR when the absorption maximum of the chromophore is further to the red. As will be explained in greater detail below, this can be attributed to the π-electrons being more delocalized in the PSBR when its absorption is red shifted. Thus, it follows that the 13-*cis* to all-*trans* isomerization would be expected to be significantly more rapid in O, which has an absorption maximum at 640 nm, than in N, which has an absorption maximum at 550 nm. When the isomerization might occur during the O intermediate is not clear, but from the reported resonance Raman results (Smith et al., 1983; Ames and Mathies, 1990) it should take place within the initial few milliseconds of the formation of this intermediate. Thus, for the sake of plotting a log k versus absorption maximum value for the 13-*cis* to all-*trans* isomerization an arbitrary value of log k must be chosen, and the recently reported N to O rate obtained at 25°C is used (Hofrichter et al., 1989). If the model proposed here is correct, this value is probably near the lower limit of the actual value, considering the speed at which an apparently constant O Raman spectrum is seen. But even if the actual isomerization rate is faster by as much as two orders of magnitude from this estimated value, the

correlation including it and the other isomerization rates in BR would still exist (for the plot of log k versus absorption maximum, the correlation coefficient would change from 0.986 to 0.966). Fig. 1 shows the correlation including the estimated value for the isomerization rate within the O intermediate and the values obtained for the isomerizations during dark adaptation for BR in PM in its different pH forms, with $r = 0.986$, slop = 7.7×10^{-2} nm^{-1}, y-intercept = -47.4. Similarly, if a plot is made of the log of the 13-*cis* to all-*trans* isomerization rate versus the energy at the absorption maximum (in centimeter^{-1}), a highly correlated plot is obtained, with $r = 0.981$, slope = -2.7×10^{-3} cm, and y-intercept = 44.0.

The above argument that the rapid thermal isomerization of the PSBR in BR during the photocycle occurs during the initial stages of the O intermediate is predicated on the correlation between the log of the isomerization rate and the absorption maximum of the chromophore for the model PSBR and for dark adaptation of BR. If one took the current view in the literature, that isomerization is the event that defines the difference between N and O and that isomerization occurs during N decay, then this would define the absorption maximum of the isomerizing species as 550 nm and the rate of isomerization as the rate of N–O. Using these values for the thermal isomerization step, rather than the one discussed above, gives a line that is essentially not correlated ($r = 0.079$) for the plot of the log of the isomerization rates versus the absorption maximum for BR. As will be discussed below, previous work suggests that it is reasonable to suppose that there would be a correlation between the red shift in the absorption and increases in the thermal isomerization rate.

EXPLANATION FOR CORRELATION BETWEEN ABSORPTION MAXIMUM AND ISOMERIZATION RATES

Previous work strongly suggests that thermal double bond isomerization should occur more rapidly in PSBR whose absorption is red shifted. It has been shown that the energy of the $\nu_{c=c}$ stretch decreases for linear polyenes as the absorption maximum shifts to the red (Rimai et al., 1973), and that this type of correlation is also followed by PSBR model compounds (Baasov and Sheves, 1985), and by both rhodopsin and BR in various states (Doukas et al., 1978; Marcus and Lewis, 1978). Thus, for both the model compounds and BR, as the PSBR red shifts it is expected that π-electron delocalization will increase and double bond strength will decrease, leading to more facile double bond isomerization.

A number of reported theoretical calculations on PSBR or their models have shown that upon moving the counter ion of the protonated nitrogen of the PSBR infinitely far away that the thermal barrier to isomerization of double bonds decreases dramatically (Warshel, 1978; Orlandi and Schulten, 1979; Taven et al., 1985; Seltzer, 1987). As an example, MNDO calculations have shown that moving a negative charge from 2.5 Å to infinitely far from the Schiff base nitrogen changes the C_{13}–C_{14} bond order from 1.712 to 1.457 and the C_{15}–N bond order form 1.610 to 1.396 (Seltzer, 1987). These calculations also show that the barrier for concerted *cis-trans* isomerization about C_{13}–C_{14} and C_{15}–N by a bicycle pedal motion decreases from 62.2 to 24.6 kcal/mol upon moving the counterion from 2.5 Å to infinitely far from the Schiff base nitrogen. There are a number of interactions that regulate the position of the absorption maximum of the chromophore in BR in its various states. From theoretical calculations and studies using BR reconstituted with synthetic analogues of retinal, it has been shown that the position of the counterion with respect to the nitrogen is critical in regulating the position of the absorption, with the farther the counterion is from the nitrogen, the greater the chromophore red shift (Ottolenghi and Sheves, 1989). Thus, moving the counterion away from the Schiff base nitrogen both red shifts the absorption and lowers the thermal isomerization barrier. This is as expected, as both an absorption red shift and a lower isomerization barrier should occur upon the greater π-electron delocalization that results from moving the counterion away from the Schiff base nitrogen.

MODEL FOR THERMAL ISOMERIZATION DURING THE PHOTOCYCLE

If, as the analysis above suggests, isomerization occurs after the O intermediate has formed, this implies that there are actually two states of the O intermediate, with the two differing in the *cis-trans* isomer state of the chromophore. Two O intermediates, named here cO and tO for their 13-*cis* or all-*trans* chromophore, have not been detected in the large number of transient absorption experiments reported on the photocycle of BR (Lozier et al., 1975; Hofrichter et al., 1989; Varo and Lanyi, 1990), indicating that either of three possibilities occurs: the isomerization from cO to tO is so rapid that it cannot be readily observed; the isomerization is rapid and gives an equilibrium between cO and tO, and it is this equilibrium mixture that is observed spectroscopically and that decays to BR through tO; cO and tO are spectrscopically similar, and because their concentrations are never high, they are not distinguishable experimentally.

The shift in absorption of the PSBR between N and O

is large, with the maximum changing from 550 to 640 nm. As stated above, the position of the counterion is a critical factor in regulating the absorption in BR. Both solid-state NMR (de Groot et al., 1989) and single site mutagenesis experiments have shown that the counterion of the protonated nitrogen of the Schiff base is complex and that it probably includes a number of residues, with the mutagenesis work indicating that Asp-85, Asp-212, and Arg-82 are involved (Stern et al., 1989; Subramanium et al., 1990; Otto et al., 1990). In the case of replacement of Asp-85 by Asn, the pigment absorbs at 605 nm, the same position as for low pH, blue BR. Neither the Asp-85 → Asn mutant or blue-BR exhibit an M intermediate upon photolysis, suggesting that Asp-85 accepts the proton from the PSBR during the M intermediate of the photocycle, that it is close to the Schiff base proton, and that neutralization of its negative charge affects the chromophore's absorption maximum in a manner similar to moving the negative counterion a large distance away. The importance of protonation of Asp-85 during M is further supported by low temperature FTIR studies of the photocycle intermediates in the Asp-85 → Asn mutant (Braiman et al., 1988) and by time-resolved FTIR studies of BR (Gerwert et al., 1990). The red shift in the spectrum from N to O is even larger than the red shift that comes with removal of the negative charge at Asp-85. This suggests that one process in the N to O transition is the transfer of a proton to the complex counterion. However, the exact placement of the proton within the complex counterion is not known, and other changes in the interaction of the protein with the chromophore, such as movement of a negative charge near to C_{13}, are probably also important in inducing the very large red shift in the PSBR spectrum that is observed in the O intermediate.

Recently, Ames and Mathies (1990) have obtained rate constants for the pH dependent rate of decay of the N to the O. They showed that in 3.5 M salt that the rate is constant below pH 7 and that it decreases at higher pH. Their data indicates that both an N and an N^+ intermediate occur in the photocycle and that the two N intermediates only differ by the protonation of an internal protein residue. It was inferred that the protonation state of this residue does not affect the spectral properties of BR and that it is only the N^+ intermediate that leads to O. This, along with the current work, suggests that it may be the proton on this residue that protonates the complex counterion during the formation of O. Protonation of the complex counterion during O formation is consistent with recent time-resolved FTIR work that indicates that either Asp-212 or Asp-85 becomes protonated at this point in the photocycle (Gerwert et al., 1990). Ames and Mathies (1990) have postulated that N^+ follows N in the photocycle, as it is only N^+ which will lead to O. However, another kinetic scheme fits with their data. Formation of either N or N^+ could occur from M, depending on the protonation state of this residue in M. Because the M to N rate is not significantly pH dependent, then the protonation state of this residue does not affect this rate. Thus, two essentially spectroscopically identical M intermediates may precede the N and N^+, and they are called here, in analogy, M and M^+. Fig. 2 presents the kinetic scheme

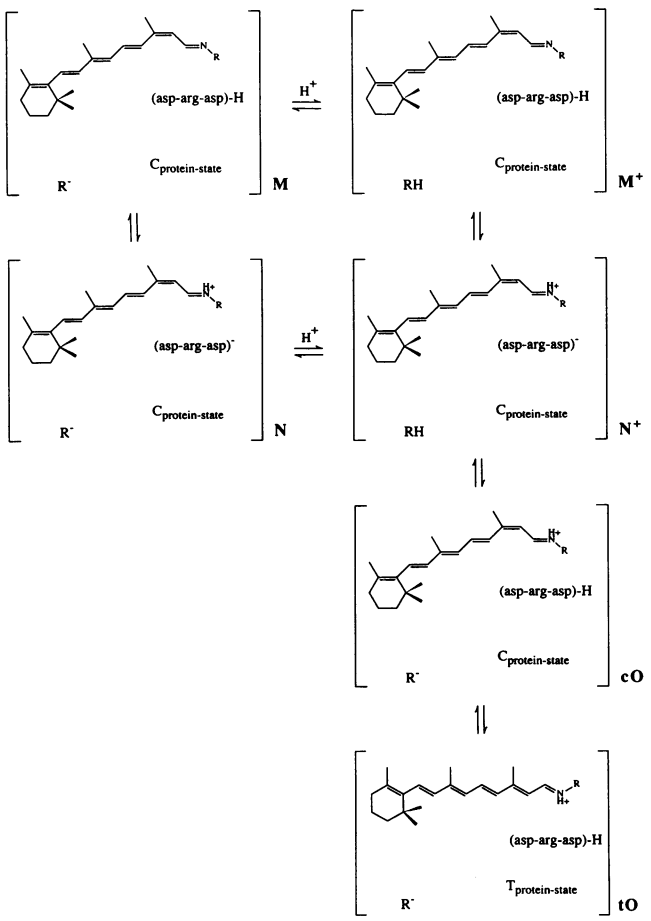

FIGURE 2 Model for the later steps of the photocycle of BR in PM. The 13-*cis* to all-*trans* isomerization step, along with a previously postulated protein conformation change between protein states named C and T (Fodor et al., 1988), is shown to occur during the O intermediate. The protonation state of an as yet unspecified protein residue (R^- or RH) depends on the external pH during the M and N intermediates. Protonation of RH is necessary for the transfer of its proton to the complex-counterion $(asp-asp-arg)^-$ in the N^+ to cO transition. The rate of M^+ to N^+ and M to N are very similar or identical, with the protonation state of RH not significantly affecting the rate of reprotonation of the Schiff base of retinal in the M to N transition. The influence of pH on the M and M^+ and N and N^+ equilibria may be different. The exact position of the proton on the nitrogen of the PSBR is not known, nor is the position of the complex counterion, and their placement is arbitrary.

developed here. Note that the complex counterion is represented by $(asp\text{-}arg\text{-}asp)^-$ or (asp-arg-asp)-H, as there is no consensus in the literature as to the specifics of the protonation state of the three residues during the photocycle (Braiman et al., 1988; Ames and Mathies, 1990; Gerwert et al., 1990). Recent work suggests that a Ca^{2+} may interact with the residues in the complex counterion, and the effect that this ion has on the protonation state of the residues is an open question (Jonas and Ebrey, 1991).

CONCLUSION

Correlations between the maximum in the absorption spectrum and the log of the 13-*cis* to all-*trans* isomerization rate have been shown to exist for a model PSBR and for BR in PM, and the implications of this with regard to the importance of π-electron delocalization in regulating the rate of thermal isomerization have been presented. The results agree with theoretical work that indicates that movement of the counterion of the PSBR away from the nitrogen can lead to red shifts in the spectrum and greater π-electron delocalization. Other changes in the position of charges, such as the relative position of a negative charge near C_{13} of the PSBR, and/or the polarizability of the environment may also make significant contributions to π-electron delocalization in the chromophore. The correlation observed for the model compound and for BR are different, with the isomerization being slower in BR for a chromophore with an equivalent absorption maximum, indicating that other factors are important. Factors that may contribute to the slower isomerization rates in BR might include steric effects of the protein on the chromophore in the binding pocket and the effect that the lipid environment has on the ability of the protein to undergo conformational changes that occur concomitant with the chromophore isomerization. It may be noted that bovine rhodopsin, a retinal pigment with an 11-*cis* PSBR that absorbs near 500 nm, does not thermally isomerize to a significant extent at room temperature. In this pigment the interactions between the protein and the chromophore are very different than those in BR. The bathochromic shift in the PSBR spectrum is not attributed to a movement of the counterion away from the protonated nitrogen (Ottolenghi and Sheves, 1989), but rather to perturbations in the vicinity of C_{13} (Smith et al., 1990). The chromophore binding pocket is considered to be more restricted in rhodopsin than in BR (Gartner et al., 1984; Liu et al., 1984). Thus, the different steric and electronic factors appears to be important in making the thermal isomerization barrier greater in bovine rhodopsin than one would expect solely from the position of its absorption maximum.

Recent work has shown that delipidated BR in different detergent micelles, where the maximum absorption of laBR is between 552 and 558 nm, has dark adaptation rates at room temperature that vary by a factor of 20, depending on the detergent and pH (Milder et al., 1991). This range in the rates has been attributed to the different effect that the different detergents have on protein conformation changes associated with the isomerization and to how the different micelles affect the accessibility of the solvent to the BR. Thus, the correlation seen for BR in PM between isomerization rates and absorption maxima is specific to the solvation condition and is not followed for BR in other solvation conditions. Taken together, the specific environment of the PSBR in BR and the external solvation environment of BR are factors, along with the extent of π-electron delocalization of the chromophore, in regulating the rate of thermal isomerization in BR.

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REFERENCES

Ames, J. B., and R. A. Mathies. 1990. The role of back-reactions and proton uptake during the N → O transition in bacteriorhodopsin's photocycle: a kinetic resonance Raman study. *Biochemistry.* 29:7181–7190.

Ames, J. B., S. P. A. Fodor, R. Gebhard, J. Raap, E. M. M van den Berg, J. Lugtenburg, and R. A. Mathies. 1989. Bacteriorhodopsin's M_{412} intermediate contains a 13-*cis,* 14-s-*trans,* 15-anti-retinal schiff base chromophore. *Biochemistry.* 28:3681–3687.

Baasov, T., and M. Sheves. 1985. Model compounds for the study of spectroscopic properties of visual pigments and bacteriorhodopsin. *J. Am. Chem. Soc.* 107:7524–7533.

Braiman, M. S., T. Mogi, T. Marti, L. J. Stern, H. G. Khorana, and K. J. Rothschild. 1988. Vibrational spectroscopy of bacteriorhodopsin mutants: light-driven proton transport involves protonation changes of aspartic acid residues 85, 96, and 212. *Biochemistry.* 27:8516–8520.

Butt, H. J., K. Fendler, E. Bamberg, J. Tittor, and D. Oesterhelt. 1989. Aspartic acids 96 and 85 play a central role in the function of bacteriorhodopsin as a proton pump. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1657–1663.

de Groot, H. J. M., G. S. Harbison, J. Herzfeld, and R. G. Griffin. 1989. Nuclear magnetic resonance study of the schiff base in bacteriorhodopsin: counterion effects on the ^{15}N shift anisotropy. *Biochemistry.* 28:3346–3353.

Doukas, A. G., B. Aton, R. H. Callender, and T. G. Ebrey. 1978. Resonance Raman studies of bovine metarhodopsin I and metarhodopsin II. *Biochemistry.* 17:2430–2435.

Fischer, U., and D. Oesterhelt. 1979. Chromophore equilibria in bacteriorhodopsin. *Biophys. J.* 28:211–230.

Fodor, S. P. A., J. B. Ames, R. Gebhard, E. M. M. van den Berg, W. Stoeckenius, J. Lugtenburg, and R. A Mathies. 1988. Chromophore structure in bacteriorhodopsin's N intermediate: implications for the proton-pumping mechanism. *Biochemistry.* 27:7097–7101.

Gartner, W., D. Oesterhelt, E. Seifert-Schiller, P. Towner, H. Hopf, and I. Bohm. 1984. Acetylenic retinals form functional bacteriorhodopsins but do not form bovine rhodopsins. *J. Am. Chem. Soc.* 106:5654–5659.

Gerwert, K., G. Souvignier, and B. Hess. 1990. Simultaneous monitoring of light-induced changes in protein side-group protonation, chromophore isomerization, and backbone motion of bacteriorhodopsin by time-resolved Fourier-transform infrared spectroscopy. *Proc. Natl. Acad. Sci. USA* 87:9774–9778.

Hofrichter, J., E. R. Henry, and R. H. Lozier. 1989. Photocycles of bacteriorhodopsin in light- and dark-adapted purple membrane stud-ied by time-resolved absorption spectroscopy. *Biophys. J.* 56:693–706.

Holz, M., L. A. Drachev, T. Mogi, H. Otto, A. D. Kaulen, M. P. Heyn, V. P. Skulachev, and H. G. Khorana. 1989. Replacement of aspartic acid-96 by asparagine in bacteriorhodopsin slows both the decay of the M intermediate and the associated proton movement. *Proc. Natl. Acad. Sci. USA.* 86:2167–2171.

Jonas, R., and T. G. Ebrey. 1991. Binding of a single divalent cation directly correlates with the blue-to-purple transition in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 88:149–153.

Liu, R. S. H., A. E. Asato, M. Denny, and D. Mead. 1984. The nature of restrictions in the binding site of rhodopsin. A model study. *J. Am. Chem. Soc.* 106:8298–8300.

Lozier, R., R. O. Bogomolni, and W. Stoeckenius. 1975. Bacteriorhodopsin: a light-driven proton pump in *Halobacterium halobium. Biophys. J.* 15:955–962.

Marcus, M. A., and A. Lewis. 1978. Resonance Raman spectroscopy of the retinylidene chromophore in bacteriorhodopsin (bR_{570}), bR_{560}, M_{412} and other intermediates: structural conclusions based on kinetics, analogues, models, and isotopically labeled membranes. *Biochemistry.* 17:4722–4735.

Mathies, R. A., J. Lugtenburg, and C. V. Shank. 1989. From femtoseconds to biology: mechanism of light-driven proton pump in bacteriorhodopsin. *SPIE.* 1057:138–145.

Milder, S. J., T. E. Thorgeirsson, L. J. W. Miercke, R. M. Stroud, and D. S. Kliger. 1991. Effects of detergent environments on the photocycle of purified monomeric bacteriorhodopsin. *Biochemistry.* 30:1751–1761.

Mogi, T., L. J. Stern, T. Marti, B. H. Chao, and H. G. Khorana. 1988. Aspartic acid substitutions affect proton translocation by bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 85:4148–4152.

Muccio, D. D., and J. Y. Cassim. 1979. Interpretations of the effects of pH on the spectra of purple membrane. *J. Mol. Biol.* 135:595–609.

Ohno, K., Y. Takeuchi, and M. Yoshida. 1977. Effect of light-adaptation on the photoreaction of bacteriorhodopsin from *Halobacterium halobium. Biochim. Biophys. Acta.* 462:575–582.

Ohtani, H., T. Kobayashi, J. Iwai, and A. Ikegami. 1986. Picosecond and nanosecond spectroscopies of the photochemical cycles of acidified bacteriorhodopsin. *Biochemistry.* 25:3356–3363.

Orlandi, G., and K. Schulten. 1979. Coupling of stereochemistry and proton donor-acceptor properties of a schiff base. A model of a light-driven proton pump. *Chem. Phys. Lett.* 64:370–374.

Otto, H., T. Marti, M. Holz, T. Mogi, L. J. Stern, F. Engel, H. G. Khorana, and M. P. Heyn. 1990. Substitution of amino acids asp-85, asp-212, and arg-82 in bacteriorhodopsin affects the proton release phase of the pump and the pK of the schiff base. *Proc. Natl. Acad. Sci. USA* 87:1018–1022.

Ottolenghi, M., and M. Sheves. 1989. Synthetic retinals as probes for the binding site and photoreactions in rhodopsins. *J. Membr. Biol.* 112:192–212.

Rimai, L., M. E. Heyde, and D. Gill. 1973. Vibrational spectra of some carotenoids and related linear polyenes. A Raman spectroscopic study. *J. Am. Chem. Soc.* 95:4493–4501.

Rothschild, K. J., and H. Marrero. 1982. Infrared evidence that the schiff base of bacteriorhodopsin is protonated: bR570 and K intermediates. *Proc. Natl. Acad. Sci. USA.* 79:4045–4049.

Scherrer, P., M. K. Mathew, W. Sperling, and W. Stoeckenius. 1989. Retinal isomer ratio in dark-adapted purple membrane and bacteriorhodopsin monomers. *Biochemistry.* 28:829–834.

Seltzer, S. 1987. MNDO barrier heights for catalyzed bicycle-pedal, hula twist, and ordinary *cist-trans* isomerizations of protonated retinal schiff base. *J. Am. Chem. Soc.* 109:1627–1631.

Seltzer, S. 1990. Models for bacteriorhodopsin-catalyzed dark *cis-trans* isomerization of bound retinal. *J. Am. Chem. Soc.* 112:4477–4483.

Sharkov, A. V., A. V. Parkulev, S. V. Chekalin, and Y. A. Matveetz. 1985. Primary events in bacteriorhodopsin probed by subpicosecond spectroscopy. *Biochim. Biophys. Acta.* 808:94–102.

Sheves, M., and T. Baasov. 1984. Factors affecting the rate of thermal isomerization of 13-*cis* bacteriorhodopsin to all-*trans. J. Am. Chem. Soc.* 106:6840–6841.

Smith, S. O., J. A. Pardoen, P. P. J. Mulder, B. Curry, J. Lugtenburg, and R. Mathies. 1983. Chromophore structure in bacteriorhodopsin's O_{640} photointermediate. *Biochemistry.* 22:6141–6148.

Smith, S. O., I. Palings, M. E. Miley, J. Courtin, H. de Groot, J. Lugtenburg, R. A. Mathies, and R. G. Griffin. 1990. Solid-state NMR studies of the mechanism of the opsin shift in the visual pigment rhodopsin. *Biochemistry.* 29:8158–8164.

Sperling, W., C. N. Rafferty, K.-D. Kohl, and N. A. Dencher. 1979. Isomeric composition of bacteriorhodopsin under different environmental light conditions. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 97:129–132.

Stoeckenius, W., and R. A. Bogomolni. 1982. Bacteriorhodopsin and related pigments of halobacteria. *Annu. Rev. Biochem.* 52:587–616.

Stern, L. J., P. L. Ahl, T. Marti, T. Mogi, M. Dunach, S. Berkowitz, K. J. Rothschild, and H. G. Khorana. 1989. Substitution of membrane-embedded aspartic acids in bacteriorhodopsin causes specific changes in different steps of the photochemical cycle. *Biochemistry.* 28:10035–10042.

Subramaniam, S., T. Marti, and H. G. Khorana. 1990. Protonation state of asp (glu)-85 regulates the purple-to-blue transition in bacteriorhodopsin mutants arg-82 → ala and asp85 → glu: the blue form is inactive in proton translocation. *Proc. Natl. Acad. Sci. USA.* 87:1013–1017.

Tavan, P., K. Schulten, and D. Oesterhelt. 1985. The effect of protonation and electrical interactions on the stereochemistry of retinal schiff bases. *Biophys. J.* 47:415–430.

Warshel, A. 1978. Charge stabilization mechanism in the visual and purple membrane pigments. *Proc. Natl. Acad. Sci. USA.* 75:2558–2562.

Warshel, A., and M. Ottolenghi. 1979. Kinetic and spectroscopic effects of protein-chromophore electrostatic interactions in bacteriorhodopsin. *Photochem. Photobiol.* 30:291–293.

Varo, G., and J. K. Lanyi. 1990. Pathways of the rise and decay of the M photointermediate(s) of bacteriorhodopsin. *Biochemistry.* 29:2241–2250.