Solvent Isotope Effects on Retinal Cis-Trans Isomerization in the Dark Adaptation of Bacteriorhodopsin

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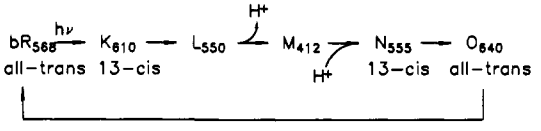
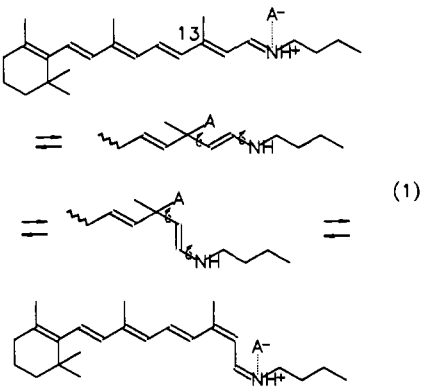
Abstract: The solvent isotope effect on the first-order rate constant for dark adaptation of bacteriorhodopsin, near neutral pH (pD), is inverse; $k_D/k_H = 1.24$. The fit of the variation of isotope effect with the atom fraction of deuterium in the solvent to the Gross-Butler equation leads to the conclusion that the proton(s) in motion is (are) less tightly bound in the reactant than in a reactive intermediate, formed prior to the rate-controlling step of cis-trans isomerization. The near-unity isotope effect on the equilibrium between the bound 13-*cis*- and *all-trans*-retinals in the dark-adapted state mixture indicates that the isotope effects on the forward and reverse rate constants for isomerization are approximately equal to each other and equal to the isotope effect on the observed rate constant for dark adaptation. The results support a previously proposed mechanism of rate-controlling Asp-212 nucleophilic catalysis of retinal cis-trans isomerization in the dark adaptation process.

The purple membrane of *Halobacterium holobium* is a light-driven proton pump.[1] The membrane contains bacteriorhodopsin (bR), a single-chain polypeptide of 248 amino acids, woven into the membrane as seven connected α-helical rods positioned roughly perpendicular to the plane of the membrane. The color derives from the presence of 1 equiv of retinal bound to lysine-216 through a protonated Schiff base. Light initiates a photocycle



the first step of which is a photoisomerization of the bound all-*trans* protonated retinal Schiff base to its 13-cis isomer. The subsequent steps are nonphotolytic. Cis-trans isomerization, in the first step, is obligatory for proton pumping.[2] In order for continued turnover, the bound 13-cis isomer must be reisomerized to its all-trans form, in a later step (N → O), to reset the system.[3] Another dark cis-trans isomerization occurs when the light is extinguished. bR^{LA} (light-adapted bR) transforms to bR^{DA} (dark-adapted bR) where approximately half of the retinal is converted to the 13-cis form. The transformation is really a double isomerization since the all-trans,15-anti isomer is converted to the 13-cis,15-syn isomer[4] and is dynamic while in the dark-adapted form.[5] Apomembrane reconstituted and kept in the dark with a 1:1 mixture of *all-trans*- and 13-*cis*-retinal where only one isomer is radioactively labeled results in both isomers becoming labeled.[5] The isomers of bR^{DA} interconvert with a rate constant equal to that for the bR^{LA} to bR^{DA} reaction. The mechanism of dark cis-trans isomerization is an integral part of the overall mechanism of light-driven proton pumping.

The major part of the protonated retinal Schiff base moiety is believed to be surrounded by protein and is well shielded from solvent. The Schiff base proton, however, exchanges rapidly with solvent water. Since the chromophore appears to be shielded from solvent, we have suggested that cis-trans isomerization is catalyzed by the protein itself.[5,6] The carboxylate group of aspartate-212 could catalyze cis-trans isomerization through nucleophilic attack on retinal's C13 atom of the protonated Schiff base. The carboxyl group of Asp-212, known to be deprotonated,[7] is part of a complex counterion to the positively charged Schiff base. The carboxyl group could, by a microconformational change, move from this position to near retinal's C13 where it could form a bond to that atom.[6] The two double bonds between C13 and the protonated nitrogen would become single bonds about which rotation could occur by a bicycle-pedal mechanism to provide a doubly isomerized intermediate in the dark adaptation process (eq 1, where A^- is



(1)

the carboxylate group of Asp-212) or, alternatively, rotation about the C13-C14 bond to provide a singly isomerized intermediate as in the N → O conversion.[6] Rupture of the oxygen-C13 bond alone and return of the carboxyl group to its original position would provide the isomerized retinal. A similar mechanism is used by a cis-trans isomerizing enzyme in a double isomerization through a bicycle-pedal mechanism.[8]

Semiempirical MNDO calculations support such a mechanism for bR.[6a] Recent studies with zwitterionic model compounds having a secondary amine group for retinal Schiff base formation and a tethered carboxylate group to mimic the presence of as-

(1) For reviews see: (a) Stoeckenius, W.; Bogomolni, R. A. *Annu. Rev.* **1982**, *52*, 587-616. (b) Khorana, H. G. *J. Biol. Chem.* **1988**, *263*, 7439-7442. (c) Stoeckenius, W. *Trends Biochem. Sci.* **1985**, *10*, 483-486. (d) Dencher, N. A. *Photochem. Photobiol.* **1983**, *38*, 753-757. (e) Ovchinnikov, Y. A. *FEBS Lett.* **1982**, *148*, 179-191.

(2) (a) Fang, J.-M.; Carriker, J. D.; Balogh-Nair, V.; Nakanishi, K. *J. Am. Chem. Soc.* **1983**, *105*, 5162-5164. (b) Chang, C. H.; Govindjee, R.; Ebrey, T.; Bagley, K. A.; Dollinger, G.; Eisenstien, L.; Marque, J.; Roder, H.; Vittitow, J.; Fang, J.-M.; Nakanishi, K. *Biophys. J.* **1985**, *47*, 509-512.

(3) Smith, S. O.; Myers, A. B.; Pardoen, A.; Winkel, C.; Mulder, P. P. J.; Lugtenberg, J.; Mathies, R. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2055-2059.

(4) Harbison, G. S.; Smith, S. O.; Pardoen, J. A.; Winkel, C.; Lugtenburg, J.; Herzfeld, J.; Mathies, R.; Griffin, R. G.; *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 1706-1709.

(5) Seltzer, S.; Zuckermann, R. *J. Am. Chem. Soc.* **1985**, *107*, 5523-5525.

(6) (a) Seltzer, S. *J. Am. Chem. Soc.* **1987**, *109*, 1627-1631; (b) *J. Am. Chem. Soc.* **1990**, *112*, 4477-4483. (c) Birnbaum, D.; Seltzer, S. *Bioorg. Chem.* **1991**, *19*, 18-28.

(7) (a) Heyn, M. P.; Khorana, H. G.; et al. *Biol. Chem. Hoppe-Seyler* **1989**, *370*, 623. (b) Otto, H.; Marti, T.; Holz, M.; Mogi, T.; Stern, L. J.; Engel, F.; Khorana, H. G.; Heyn, M. P. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1018-1022.

(8) (a) Feliu, A. L.; Smith, K. J.; Seltzer, S. *J. Am. Chem. Soc.* **1984**, *106*, 3046-3047. (b) Seltzer, S.; Hane, J. *Bioorg. Chem.* **1988**, *16*, 394-407.

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partate-212's carboxylate group indicate that indeed the carboxylate can catalyze cis–trans isomerization of a positively charged retinal Schiff base.[6b] Moreover, the recent observation of an inverse secondary β-deuterium kinetic isotope effect upon dark adaptation of bR, reconstituted with retinal-*20,20,20-d*$_3$, has been shown to be readily rationalized by a mechanism involving addition of an anion to retinal's C13 atom.[6c] Replacement of Asp-212 by Ala or Asn leads to a bR which dark adapts at a rate about 5 times more slowly than native bR, suggesting a direct role for that residue in dark cis–trans isomerization.[9]

A 3.5-Å resolution map of bR has been recently reported.[10] Besides involvement as part of a counterion to the positively charged Schiff base, Aspartate-212 is reported to be hydrogen bonded to the hydroxyl proton of Tyrosine-185. Moreover, the plane of the aromatic ring is located parallel and close to retinal's polyene plane, suggesting the possibility of π overlap between tyrosine and the chromophore. An interesting variant of the nucleophilically catalyzed isomerization mechanism, outlined above, has been suggested by Rothschild and co-workers.[11] They propose that tyrosine's hydroxyl proton moves to aspartate's carboxylate, allowing the tyrosinate anion to function as the nucleophile which attacks retinal's C13.[11] The map shows the tyrosinate oxygen at C4′ in close position to interact with C13. Interestingly, the mutant bR with phenylalanine substituted for Tyr-185 (Y185F) exhibits a very much reduced rate of dark adaptation.[9,11] Tyrosine deprotonation would also render the ortho carbons nucleophilic. An alternative to their mechanism can be suggested here in which the 3′- or 5′-carbon of tyrosinate forms a bond to retinal's C13 to catalyze isomerization in the way that Asp-212's carboxylate might.

In an attempt to gain evidence for or against these mechanisms, rates of dark adaptation were measured in mixed H_2O/D_2O solutions and are reported here. The results indicate that it is unlikely that proton motion is occurring in the rate-controlling step of dark cis–trans isomerization. Moreover, from the direction of the isotope effect in mixtures of H_2O and D_2O it appears that a proton has moved from a weakly bound position to a more tightly held position prior to the rate-controlling step.

Experimental Section

HEPES was obtained from Sigma Chemical Co. D_2O was obtained from the Atomic Energy Commission and was redistilled before use. An aliquot of D_2O was diluted with HPLC grade acetonitrile and subjected to NMR analysis (Bruker AM 300). Several integrations of the two ^1H signals indicated that the D_2O contained 98.90 atom % D. Reagent H_2O was purified with a Milli Q (Millipore Corp.) system. Purple membrane was isolated from *H. halobium* by the method of Oesterhelt and Stoeckenius[12] and purified by the method of Becher and Cassim.[13] The OD_{280}/OD_{568} ratio was 1.906.

Five HEPES buffers (0.01 M), 0.1 M in NaCl, were prepared in H_2O by titrating the buffer with 0.1 M NaOH solution to the desired pH (Metrohm Model 605 pH meter). Similarly, five HEPES buffers (0.01 M) in D_2O, 0.1 M in NaCl, were prepared in similar fashion using NaOD (Aldrich Chemical Co.) to obtain the desired pD. The pD in "100%" D_2O solutions was determined by adding 0.400 to the pH meter reading. For mixed H_2O/D_2O solutions the equation $\delta = 0.076n^2 + 0.3314n$, reported by Schowen and Schowen,[14] was assumed to hold. In these cases, δ was added to the meter reading.

Kinetics. To 1.0 mL of the desired buffer mixture in a cuvette was added 35 μL of a concentrated solution (OD_{568} 20.2) of purple membrane in H_2O. The sample was mixed, irradiated for 1 h, and then allowed to stand in the dark in the thermostated cell holder (34.1 °C) of the spectrometer overnight prior to making any measurements. The kinetics of dark adaptation was determined by measuring the OD_{592} (Varian DMS 80 spectrophotometer) with time after the sample was irradiated with a white fluorescent light for 3 min. Kinetic measurements were carried out at least five times for each solution. Data acquisition and analysis were computer controlled by a program called KNTCS written in our laboratory within the ASYST (Keithly Instruments) framework. The reaction was followed for 6–8 half-lives during which time at least 200 data points were taken. A nonlinear least-squares fit of the data to a first-order decay was carried out. Any point deviating by more than 2.7 times the standard deviation calculated for all points was rejected, and a new rate constant was calculated. The procedure was repeated until the deviation of all points were less than 2.7 times the standard deviation. Fitting of data to various forms of the Gross–Butler equation were carried out with GENPLOT.

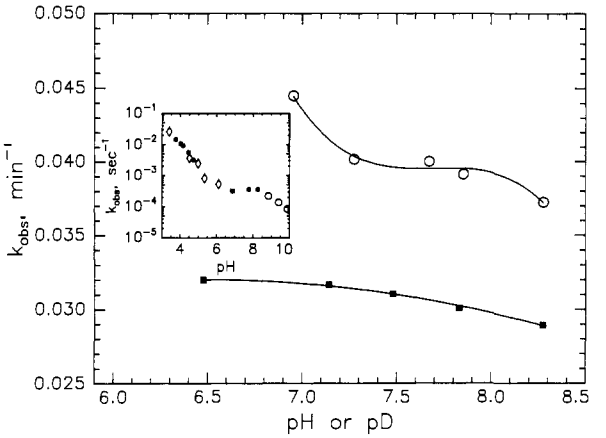

Figure 1. Observed first-order rate constant for dark adaptation of bacteriorhodopsin at 34 °C in 0.01 M HEPES buffer containing 0.1 M NaCl in H_2O (boxes) and in D_2O (circles) plotted *linearly* as a function of pH or pD, depending on the solvent. Inset: Data of Ohno.[16] The observed first-order rate constant (see text) for dark adaptation in H_2O buffers plotted *semilogarithmically* as a function of pH. Buffers: diamonds, 10 mM KCl, 1 mM sodium acetate; solid circles, 10 mM KCl, 1 mM glycine; solid boxes, 10 mM KCl, 1 mM Tris·HCl; circles, 0.2 M KCl, 20 mM glycine.

Equilibrium Isotope Effect. Samples of bR in H_2O and D_2O were prepared at the same concentration in the same way and in the same buffers as used for the kinetics as described above. The contents were irradiated for about 3 min and then allowed to dark-adapt for about 4 h. Retinal extraction was carried out by a modification of the method of Groenendijk et al.[15] Aliquots of the membrane solution (0.5 mL) were added to 0.4 mL of a 2.4 M solution of hydroxylamine in methanol (pH 7.14). (The hydroxylamine solution was prepared as described previously.[6b] The pH of the stock hydroxylamine solution in the present study was determined by diluting an aliquot of the methanol solution with 3–5 volumes of water and measuring the pH with a pH meter.) Reaction with hydroxylamine was allowed to proceed for 5 min after which 0.5 mL of heptane was added; the mixture was then vortexed and centrifuged in an Eppendorf microcentrifuge. The aqueous methanol solution was withdrawn and the heptane solution washed twice with 0.2-mL portions of water. Isomer distribution was determined by HPLC as described previously[6b,15] except that in this study a 5-μm silica 50 guard column (Alltech Associates) was installed at the inlet to trap any contaminating lipids that might be present. With a flow rate of 1.0 mL/min of the 10% ether/hexane, typical elution times of 8.21, 8.97, 12.18, and 20.45 min were obtained for *syn-all-trans-*, *syn-*13*-cis-*, *anti-*13*-cis-*, and *anti-all-trans-*retinal oxime, respectively. The signals were detected at 340 nm by an Altex 153 monitor and integrated by a Hewlett-Packard 3392A integrator.

Results

The rates of dark adaptation of bR^{LA} were first measured in H_2O (pH 6.48–8.27) and in D_2O (pD 6.95–8.28) HEPES buffer solutions by determining the rate of decrease of the 592-nm absorbance. The results are plotted *linearly* as observed first-order rate constant vs pH or pD and shown in Figure 1. In the inset is shown a *semilogarithmic* plot of the pH–rate data in H_2O previously reported by Ohno.[16] Various buffer systems were used

(9) Duñach, M.; Marti, T.; Khorana, H. G.; Rothschild, K. J. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9873–9877.
(10) Henderson, R.; Baldwin, J. M.; Ceska, T. A.; Zemlin, F.; Beckmann, E.; Downing, K. H. *J. Mol. Biol.* **1990**, *213*, 899–929.
(11) Rothschild, K. J.; Braiman, M. S.; He, Y.-W.; Marti, T.; Khorana, H. G. *J. Biol. Chem.* **1990**, *265*, 16985–16991.
(12) Oesterhelt, D.; Stoeckenius, W. *Methods Enzymol.* **1974**, *31*, 667–678.
(13) Becher, B. M.; Cassim, J. Y. *Prep. Biochem.* **1975**, *5*, 161–175.
(14) Schowen, K. B.; Schowen, R. L. *Methods Enzymol.* **1982**, *87*, 551–606.
(15) (a) Groenendijk, G. W. T.; De Grip, W. J.; Daemen, F. J. M.; *Biophys. Biochem. Acta* **1980**, *617*, 430–438; (b) *Anal. Biochem.* **1979**, *99*, 304–310.

Table I. First-Order Rate Constants for Dark Adaptation of Bacteriorhodopsin in H_2O/D_2O Mixtures[a]

atom fraction D	10^2k (min^{-1})	$10^2 \times$ avg dev
0.0	3.25_9	0.02_0
0.1839	3.33_8	0.02_0
0.4768	3.55_6	0.01_9
0.7651	3.79_3	0.01_1
0.9550	4.00_0	0.02_7

[a] Kinetics were carried out at 34.1 °C in 0.1 M NaCl. Mixtures of 0.01 M HEPES (0.0 atom fraction D, pH 7.141; 0.989 atom fraction D, pD 7.671) buffers were used. Purple membrane solution (35 μL) in H_2O containing no buffer was added to each 1.0 mL of the isotopically mixed solvent. Each rate constant is the average of at least five runs.

in the earlier study, and these are indicated in the legend. The pH and pD ranges selected in the present study were designed to provide regions that were relatively insensitive to small changes in pH or pD. This is realized as shown by the data in the figure. Further studies in mixed H_2O (pH 7.141)/D_2O (pD 7.671) solutions of 0.01 HEPES (0.1 M in NaCl) buffers were then carried out in the same way. Examination of the data in the figure suggests that with the choice of these buffers very little to no variation in the rate can be expected due to changes in pH or pD alone when these two buffers are mixed. The first-order rate constants for dark adaptation, measured in mixtures of H_2O/D_2O, are tabulated, together with their errors, in Table I. An *inverse* solvent kinetic isotope effect, $k_D/k_H = 1.24$, is evident.

The observed rate constant for dark adaptation is really the rate constant for the approach to a cis/trans equilibrium mixture and consequently $k_{obs} = k_f + k_r$, where f and r refer to forward and reverse, respectively. The kinetic isotope effect, k_D/k_H, is therefore equal to $(k^D_f + k^D_r)/(k^H_f + k^H_r)$. The isotope effect on the equilibrium constant was measured in order to separate isotope effects for forward and reverse reactions. The ratio of *all-trans*- to 13-*cis*-retinal in bR^{DA} was determined by converting the chromophores to their oximes with hydroxylamine (NH_2OH:bR ~ 10^5:1) in methanol and subsequently analyzing the mixture by HPLC on silica gel. Groenendijk et al.[15] have shown that >95% of the retinals in rhodopsin-containing membranes are converted to their oximes without isomerization if the ratio of hydroxylamine to the rhodopsin is at least 1000:1. Samples of bR in the same HEPES buffers in H_2O and in D_2O used for kinetic measurements were allowed to dark-adapt at 34 °C, and then these were allowed to react with concentrated neutral hydroxylamine in methanol. The ratio of the sums of the integrated signals at 340 nm for 13-*cis*- to *all-trans*-retinal oxime isomers from an H_2O buffer was found to be practically identical with that from our highest atom fraction deuterium D_2O buffer, making it fruitless to measure the equilibrium isotope effect at intermediate levels of solvent deuteration. Assuming equal response factors, an apparent $K_D/K_H = 1.032 \pm 0.006$ (K = [cis]/[trans], three measurements each) was obtained. Since the area ratios differ only slightly, little error in the isotope effect is expected by the assumption of equal response factors.

Since K_D/K_H is close to unity, the isotope effects for forward and reverse reactions are almost the same and approximately equal to the isotope effect on the observed rate constant. Recasting the expression for $(k_H/k_D)_{obs}$ leads to

$$(k_H/k_D)_{obs} = (k^H_f/k^D_f)(1 + 1/K_H)/(1 + 1/K_D)$$

Previous estimates of K are around unity;[17] a recent study suggests that K is closer to 2.[18] If $K = 1$, then $k^H_f/k^D_f = (k_H/k_D)_{obs}/1.015$. If $K = 2$, then $k^H_f/k^D_f = (k_H/k_D)_{obs}/1.010$. Similarly, if $K = 1$, then $k^H_r/k^D_r = (k_H/k_D)_{obs}/0.985$ or $(k_H/k_D)_{obs}/0.980$ if $K = 2$. At intermediate levels of solvent deuteration the correction factors will be even closer to unity than they are in the "100%" deuterated solvent.

Discussion

FTIR studies[19] have suggested that the transformation of all-trans,15-anti to 13-cis,15-syn upon dark adaptation results in the protonation of a single tyrosine. These studies suggest that one of the eleven tyrosines in bR is deprotonated in bR^{LA}_{568} and becomes protonated in bR^{DA}_{548}. Subsequent studies carried out with site-directed mutated bR's where each of the 11 tyrosines was singly replaced with phenylalanine[20] suggest that it is Tyr-185 which undergoes the protonation change. These studies also report that the state of protonation/deprotonation of Asp and Glu carboxyls remains constant in the dark adaptation process.

Recent solid-state ^{13}C NMR measurements,[21] however, appear to contradict this picture. Although in bR^{DA}_{558} about half of one tyrosine is expected to be deprotonated, according to the conclusion from the FTIR studies, the NMR studies indicate that less than one-third of a tyrosinate residue is present. This conclusion is based on the absence of a [^{13}C]tyrosinate resonance in neutral bR^{DA} which otherwise can be seen when bR^{DA} is taken to pH 13. The results of a very recent ultraviolet resonance Raman (UVRR) study also challenge the idea that a tyrosinate becomes protonated in dark adaptation.[22] The failure to observe a prominent 1600-cm^{-1} adsorption due to tyrosinate when bR is excited with ultraviolet light which is otherwise seen at pH 11 with added tyrosine led these workers to report the absence of tyrosinate in bR^{LA}.

The observation of a near-unity equilibrium isotope effect ($K_D/K_H = 1.03$) also argues *against* the idea that Tyr-185 is deprotonated in bR_{LA} and protonated in bR_{548}. If this were the case, a larger isotope effect would have been observed (vide infra).

The data of Henderson et al.[10] place Asp-212 4 Å from the Schiff base. The new structural data also indicate that the hydroxyl oxygen of Tyr-185 is within reacting distance of retinal's C13, making it reasonable to suggest that nucleophilic catalyzed cis–trans isomerization of retinal is brought about by addition of the tyrosinate anion to retinal's C13 during or after proton transfer of tyrosine's hydroxyl proton to Asp-212's carboxylate.[11] The present experiments were initiated to see if evidence could be found for proton movement from a hydrogen bond between the oxygen of Tyr-185 and the carboxyl oxygen of Asp-212, to the carboxyl of Asp-212 alone, during or before the rate-controlling step of dark cis–trans isomerization, which occurs during dark adaptation. The many studies of Schowen,[14,23] Kresge,[24] Albery,[25] and Gold[26] have provided the foundation for study of chemical reactions in mixed-isotopic aqueous solutions. Schowen in particular has applied this method to enzyme systems to characterize the "proton inventory", i.e., the changes in proton bonding, on going from the reactant to the transition state. This can often be determined by measuring rates of reaction in hydroxylic solvents of varying deuterium atom fraction n. Schowen and co-workers have categorized different shapes of curves of k_n/k_0 vs n as supporting

(16) Ohno, K.; Takeuchi, Y.; Yoshida, M. *Biochem. Biophys. Acta* **1977**, *462*, 575–582.
(17) (a) Maeda, A.; Iwasa, T.; Yoshizawa, T. *J. Biochem.* (*Tokyo*) **1977**, *82*, 1599–1604. (b) Pettei, M. J.; Yudd, A. P.; Nakanishi, K.; Henselman, R.; Stoeckenius, W.; *Biochemistry* **1977**, *16*, 1955–1959. (c) Tsuda, M.; Ebrey, T. G. *Biophys. J.* **1980**, *30*, 149–158.
(18) Scherrer, P.; Mathew, M. K.; Sperling, W.; Stoeckenius, W. *Biochemistry* **1989**, *28*, 829–834.
(19) (a) Dollinger, G.; Eisenstein, L.; Lin, S.-L.; Nakanishi, K.; Termini, J. *Biochemistry* **1986**, *25*, 6524–6533. (b) Roepe, P. D.; Ahl, P. L.; Herzfeld, J.; Lugtenburg, J.; Rothschild, K. J. *J. Biol. Chem.* **1988**, *263*, 5110-5117.
(20) Braiman, M. S.; Mogi, T.; Stern, L. J.; Hackett, N. R.; Chao, B. H.; Khorana, H. G.; Rothschild, K. J. *Proteins: Struct. Funct. Genet.* **1988**, *3*, 219–229.
(21) Herzfeld, J.; Das Gupta, S. K.; Farrar, M. R.; Harbison, G. S.; McDermott, A. E.; Pelletier, S. L.; Raleigh, D. P.; Smith, S. O.; Winkel, C.; Lugtenberg, J.; Griffin, R. G. *Biochemistry* **1990**, *29*, 5567–5574.
(22) (a) Ames, J. B.; Bolton, S. R.; Mathies, R. A. *Biophys. J.* **1991**, *59*, 554a. (b) Ames, J. B.; Bolton, S. R.; Netto, M. M.; Mathies, R. A. *J. Am. Chem. Soc.* **1990**, *112*, 9007–9009.
(23) (a) Venkatasubban, K. S.; Schowen, R. L. *CRC Crit. Rev. Biochem.* **1984**, *17*, 1–44. (b) Alvarez, F. J.; Schowen, R. L. In *Isotopes in Organic Chemistry*; Buncel, E., Lee, C. C., Eds.; Elsevier: New York, 1987; Vol. 7, pp 1–60.
(24) Kresge, A. J.; O'Ferrall, R. A. M.; Powell, M. F. In *Isotopes in Organic Chemistry*; Buncel, E., Lee, C. C., Eds.; Elsevier: New York, 1987; Vol. 7, pp 177–273.
(25) Albery, J. In *Proton-Transfer Reactions*; Caldin, E. F., Gold, V., Eds.; Chapman and Hall: London, 1975; pp 263–315.
(26) Gold, V. *Adv. Phys. Org. Chem.* **1969**, *7*, 259–331.

different specific mechanisms. The justification for their analyses stems from the Gross–Butler equation where the ϕ's are the single-site fractionation factors

$$k_n/k_0 = \prod_i(1 - n + n\phi^T_i)/\prod_j(1 - n + n\phi^R_j)$$

with respect to fully deuterated and protonated solvent. For the equilibrium RH + SD = RD + SH, where RH and RD are protonated and deuterated reactant and SH and SD are protium and deuterium solvent, respectively, the fractionation factor ϕ is equal to the ratio of mole fraction ratios; $(x_{RD}/x_{RH})/(x_{SD}/x_{SH})$. The product in the Gross–Butler equation is taken over all isotopically substituted groups in the reactant which undergo bonding changes on its way to the transition state. A similar product is taken for sites in the transition state. If, however, the mathematical product in the numerator were to be taken over all isotopically substituted groups in the chemical product, the ratio of numerator and denominator mathematical products would yield the equilibrium isotope effect K_n/K_0 at deuterium atom fraction n. The kinetics and equilibrium constant were measured in H_2O, D_2O, and mixed H_2O/D_2O solutions to gain some knowledge of how the fractionation factors vary on going from reactant to transition state and from reactant to product for dark adaptation.

The first-order rate constants in the pH 6.5–8.5 region provided by Ohno[16] (semilog plot inset, Figure 1) are shown to be relatively independent of pH. We examined this region further in H_2O and D_2O. Similar HEPES (0.01 M) buffers, 0.1 M in NaCl, were prepared in the two solvents. The results are shown in a *linear* plot of first-order rate constants vs pH or pD. The results in H_2O mirror those of Ohno. The results in D_2O suggest that the group responsible for the acidic wing of the H_2O data increases its pK_a in D_2O, and consequently its effect is seen at a greater pD in D_2O than at the pH observed in H_2O. Otherwise, the D_2O data are also relatively independent of pD. Therefore, it would be expected that rates and the equilibrium constant for dark adaptation, measured in mixtures of H_2O and D_2O buffers in this flat pH region, would not vary because of minor changes in pH or pD. Any rate or equilibrium constant changes observed could reliably be ascribed to isotope effects.

While bR^{LA}_{568} has its chromophore almost completely in the all-trans,15-anti form, dark-adapted bR is a mixture of the all-trans,15-anti and 13-cis,15-syn protonated retinal Schiff base with absorption maxima at 568 and 548 nm, respectively. As noted above, the conclusions from FTIR studies are that Tyr-185 is deprotonated in bR^{LA}_{568} but becomes protonated in bR^{DA}_{548}. If this were the case, then bR^{DA} would have protonated and deprotonated Tyr-185 in equilibrium with each other. K_D/K_H, calculated through use of the Gross–Butler equation and the fractionation factors for phenol and oxonium ion,[14,23,24] would be approximately equal to 3. This process can be ruled out since it is contrary to observation. Like the ^{13}C NMR and the UVRR experiments, the very small isotope effect observed here on the equilibrium constant for bR^{DA} makes it unlikely that there is such a change in the state of protonation of Tyr-185 between bR^{LA} and bR^{DA}.

The first-order rate constants for dark adaptation measured in mixtures of HEPES buffers of pH 7.14 (H_2O) and pD 7.67 (D_2O) are given in Table I and shown plotted in Figure 2. Different functions have been used to fit the data, and these are discussed below. The isotope effect, as measured at $n = 0$ and $n = 1$, is decidedly *inverse*, $k_D/k_H = 1.24$, and the shape of the curve concave.

As already indicated, the expression for the observed kinetic isotope effect contains isotopically differentiated forward and reverse first-order rate constants which can be partitioned into separate isotope effects on the forward and the reverse reactions by substituting the equilibrium isotope effect and its equivalent into the expression. Because K_D/K_H is so close to unity, the isotope effects on the forward (trans to 13-cis) and reverse reactions are approximately equal and equal to the observed isotope effect on the first-order rate constant for dark adaptation (vide supra). For the sake of simplicity, they will be treated as being equal in the following discussion.

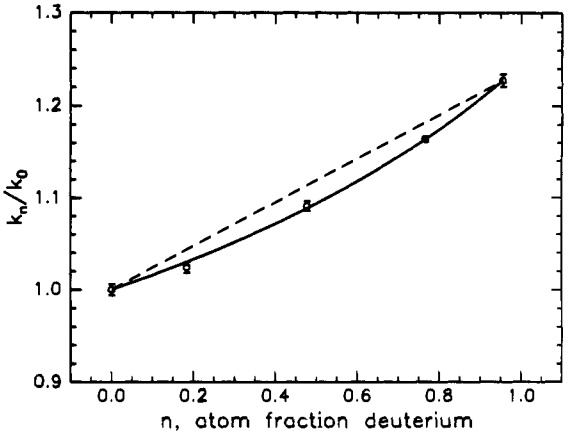

Figure 2. Ratio of the observed first rate constant for dark adaptation of bacteriorhodopsin in mixed H_2O/D_2O buffer to that measured in H_2O in the same buffer as a function of atom fraction n of deuterium in the solvent. See the Experimental Section for details. The solid line is for the equation $k_n/k_0 = (1 - n + n\phi^P)/(1 - n + n\phi^R)$, where $\phi^P = 0.77$ and $\phi^R = 0.62$.

Observation of a solvent isotope effect in such a complex system as bR cannot, by itself, pinpoint the site(s) which generate the effect. The data, however, can be used to eliminate or support mechanisms which have been proposed from other observations.

A mechanism involving a *rate-controlling* proton transfer from the tyrosine hydrogen-bonded OH to the carboxyl group of Asp-212, with concomitant partial bonding of tyrosinate oxygen to retinal's C13, would, like other rate-controlling proton transfers, be expected to generate a sizable normal effect; $k_H/k_D > 2$. A transition state where the proton is somewhat midway between the oxygens of Tyr-185 and Asp-212's carboxyl would be a state where bonds to both donor and acceptor are considerably weakened and would result in a sizable normal isotope effect. Similarly, a mechanism involving a slow proton transfer between the same donor and acceptor with concerted attack of tyrosine's C3′ or C5′ ring atoms on retinal's C13 is also held unlikely for the same reason. A *rate-controlling* proton transfer in the opposite direction from the hydrogen-bonded species to form a non-hydrogen-bonded tyrosine would also be expected to generate a sizable normal kinetic isotope effect. The inverse isotope effect observed here more likely reflects proton motion occurring prior to the rate-controlling step. Referring to the Gross–Butler equation, the inverse nature of the effect and the concave nature of the plot suggest that one or perhaps two protons (vide infra) have moved from a site in the reactant which is less tightly bound to a site in an intermediate which is more tightly bound (ϕ^P's > ϕ^R's). A more quantitative fit of the data to the Gross–Butler equation was sought.

Since the mathematical products in both numerator and denominator in eq 1 are taken over all isotopically substituted sites in the reactant and transition state (or product in a preequilibrium), the Gross–Butler equation will have different forms depending on the number of active protons and on the mechanism of reaction. This has been fully discussed by Schowen.[14,23] Accordingly, we examined the fit of the data for k_n/k_0 vs n to various mechanisms.

Bonding Change of One Proton. As discussed above, the small *inverse* isotope effect, observed for dark isomerization, is most likely due to a preequilibrium prior to the rate-controlling step. If this involves a single proton, changing its bonding on going from a reactant to a reactive intermediate, which in turn undergoes cis–trans isomerization without further bonding change, with respect to other exchangeable protons in the system, then eq 1 reduces to $k_n/k_0 = (1 - n + n\phi^P)/(1 - n + n\phi^R)$. The least-squares fit of the data to this function is shown as the solid line in Figure 2. The fit yields values of $\phi^P = 0.77 \pm 0.05$ and $\phi^R = 0.62 \pm 0.04$ ($\sum$ (residuals)$^2 = 1.4 \times 10^{-5}$). The fit indicates that bonding to the proton in the intermediate is *tighter* than it is in the reactant. Tabulations of single site fractionation factors are available in the review articles by Schowen[14,23] and Kresge.[24] The ϕ's for

phenol and acetic acid, models for tyrosine and aspartic acid, are 1.13 and 0.89, respectively, indicating that the tyrosine proton is held more tightly than the acetate proton. The ϕ for an Asp hydrogen-bonded Tyr is expected to be 1.0.[23a] These are, however, for the species solvated by water. The tyrosine and aspartate residues of interest here are in the binding pocket of bR, which provides a less polar environment, believed to be devoid of water. Fortunately, Kreevoy and Liang[27] have measured fractionation factors for homo- and heteroconjugate hydrogen-bonded species in acetonitrile. In this solvent, fractionation factors for homoconjugate hydrogen bis(phenolate) and hyrogen bis(carboxylate) complexes range from about 0.3 to 0.4. Theoretical analysis by Kreevoy indicates that fractionation factors will be smallest when the lowest stretching vibrational energy level in a double-minimum potential is near the central maximum. As heteroconjugate hydrogen-bonded species become more unsymmetrical, however, their fractionation factors are predicted to rise and approach unity. Fractionation factors for hydrogen-bonded species in water are closer to unity than when in aprotic solvents, which has been rationalized by Kreevoy.[27] The fractionation factors derived from fitting the present data are therefore reasonable for signaling a change in bonding for an Asp-212 hydrogen-bonded Tyr-185 located in an environment lacking solvent water.

Since the heavier isotope prefers the tighter bond, a small *normal* effect ($k_H > k_D$) would have been expected if the proton moved from an aspartate–tyrosine hydrogen bond to the carboxylate group in a preequilibrium prior to cis–trans isomerization as in the mechanism previously suggested by Rothschild et al.[11] If proton motion, however, were in the opposite direction, i.e., from the hydrogen bond between the Asp-212 carboxyl and the phenolate oxygen of Tyr-185, to a non-hydrogen-bonded tyrosine, then one would expect a tighter bond to the proton in the intermediate compared to the reactant as is observed. Proton motion in this direction would free Asp-212's carboxyl to add reversibly to retinal's C13, thereby catalyzing cis–trans isomerization by the mechanism previously proposed.[5,6]

The possibility that the results represent a loosely bound reactant proton ($\phi^R = 0.81 \pm 0.004$) attaining a fraction factor of unity in an intermediate prior to the rate-controlling step appears less likely in view of the considerably poorer fit ($\sum(\text{residuals})^2 = 7 \times 10^{-5}$).

Bonding Changes of Two or More Protons. On the possibility that the Schiff base proton and the tyrosine proton are both hydrogen-bonded to the carboxylate of Asp-212 and these hydrogen bonds are broken in a preequilibrium, it was of interest to see how good the fit might be for two protons in the reactant changing their bonding on going to an intermediate. The fit (not shown, $\phi^R_1 = 0.65 \pm 6.2$, $\phi^R_2 = 0.66 \pm 8.3$, $\phi^P_1 = 0.73 \pm 17.8$, $\phi^P_2 = 0.73 \pm 19.9$) is accompanied by very large σ's, which are presumably due to the fit of four variables to five data points and the strong correlation of the variables with each other and consequently severely limits their usefulness. It is interesting, however, that here too ϕ^P's > ϕ^R's, suggesting that tigher bonds are formed on going from the reactant to an intermediate prior to the rate-controlling step. While the trend in the direction of fractionation factors is in accord with those measured for phenol, amines, and acetic acid in water as models for Tyr-185, the protonated Schiff base, and Asp-212, respectively, in the binding pocket of bR, the magnitudes of the observed ϕ's are smaller than those listed for water as solvent as already discussed for the one-proton model case. The fit for this model is somewhat poorer ($\sum(\text{residuals})^2 = 4.1 \times 10^{-5}$) than for the one-proton case.

Alternatively, if a large number of sites were each to contribute a small isotope effect, the fit would be considerably worse ($\sum(\text{residuals})^2 = 1.2 \times 10^{-4}$). So the best fit and most reasonable fit is for a single proton in the reactant which changes its bonding on forming a reactive intermediate. The two-proton model, however, cannot be ruled out. Moreover, a process involving additional protons where tightness of bonding is conserved from reactant to transition state so that no addition isotope effect is generated cannot be ruled out either.

Conclusions

The results of proton inventory studies described above support the following mechanism for cis–trans isomerization in dark adaptation. Starting with bR^{LA}, a proton on Tyr-185, hydrogen-bonded to the carboxylate of Asp-212, disrupts its hydrogen bond to the carboxylate group to form a reactive intermediate in a preequilibrium step. The now freed carboxylate group of Asp-212 attacks retinal's C13 to provide nucleophilic catalysis in rate-controlling trans → cis isomerization as previously proposed.[5,6] The rate-controlling step would not generate an isotope effect. The carboxylate returns to its original position and reestablishes its hydrogen bond with Tyr-185. The preequilibrium step is the source of the inverse isotope effect on the forward rate constant since the proton moves from a weaker to a stronger binding. The step reestablishing the hydrogen bond *after* the rate-controlling step results in no contribution to the kinetic isotope effect, but the near-unity K_D/K_H suggests that the tightness of proton binding in the reactant (trans isomer) and product (cis isomer, bR_{548}) is about equal.

The reverse reaction is similar. The proton in the hydrogen bond between Tyr-185 and Asp-212 moves to Tyr-185 in a preequilibrium step to allow the carboxyl to catalyze cis–trans isomerization. The carboxyl then returns and reestablishes the hydrogen bond. Again, the movement of the proton in the preequilibrium step generates the inverse kinetic isotope effect on the reverse reaction (cis → trans), but movement after the rate-controlling step does not contribute to the effect.

As stated above, a two-proton mechanism, the second possibly being the Schiff base proton, and also hydrogen bonded to Asp-212, could be accommodated in the same way by the observed proton inventory data. In this case both hydrogen bonds would break in a preequilibrium process before Asp-212's carboxyl would attack retinal's C13 in a rate-controlling cis–trans isomerization.

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Registry No. Asp, 56-84-8; Tyr, 60-18-4; retinal, 116-31-4; deuterium, 7782-39-0.

(27) Kreevoy, M. M.; Liang, T. M. *J. Am. Chem. Soc.* **1980**, *102*, 3315–3322.