BIOORGANIC CHEMISTRY 19, 18–28 (1991)

The Secondary β-Deuterium Isotope Effect in Dark Adaptation of Bacteriorhodopsin Containing Retinal-*20,20,20-d*$_3$

DOV BIRNBAUM[1] AND STANLEY SELTZER[2]

Chemistry Department, Brookhaven National Laboratory, Upton, New York 11973

Received July 9, 1990

The secondary deuterium isotope effect on the rate of dark adaptation of photobleached purple membrane reconstituted with retinal-*20,20,20-d*$_3$ is $k_H/k_D = 0.87 \pm 0.02$. The inverse isotope effect which reflects the change in hyperconjugative stabilization of positive charge at retinal's C13 in going from reactant to the transition state of the rate-controlling step is rationalized, with the aid of MNDO calculations, as being due to the addition of a nucleophile, presumably the carboxylate of aspartate-212, to retinal's C13 to catalyze the double *cis–trans* isomerization which is attendant with the dark-adaptation process.

The purple membrane from *Halobacteria holobium* is a light-driven proton pump (*1*). Bacteriorhodopsin (bR), a single chain polypeptide of 248 amino acids, is the membrane's only protein. bR, which exhibits a high degree of α-helicity, is woven into the membrane as seven columns. The ability to activate the membrane by light results from the presence of 1 eq of retinal, bound to the protein through a protonated Schiff base with lysine-216. Absorption of light initiates a photocycle wherein the first step is the photocatalyzed isomerization of the bound *all-trans*-retinal to its 13-*cis*-isomer. Subsequent nonphotolytic thermal reactions in the cycle result in the ejection of a proton from the extracellular side, the association of a proton from the cytoplasmic side, and reisomerization of the bound 13-*cis*-retinal to its *all-trans*-isomer in order to reset the system for another cycle. It has been shown that trans-cis isomerization in the first step of the cycle is obligatory for proton pumping (*2*). Consequently it is clear that dark reisomerization of the bound 13-*cis*-retinal to the *all-trans*-isomer is required for continued light-driven proton pumping.

Thermal dark *cis–trans* isomerization also occurs upon dark adaptation. Light adapted bacteriorhodopsin (bR^{LA}) where almost all of the retinal is present as the *all-trans*,15-*anti*-isomer, is converted to the dark adapted form (bR^{DA}) where approximately half of the retinal has been doubly isomerized to the 13-*cis*,15-*syn*-isomer, presumably by a bicycle-pedal mechanism (*3*). Moreover, reversible isomerization between 13-*cis* and *all-trans*-retinal is dynamic while in the dark-adapted state (*4*).

[1] On sabbatical leave from A.D.A., Israel.

[2] To whom correspondence should be addressed.

0045-2068/91 $3.00

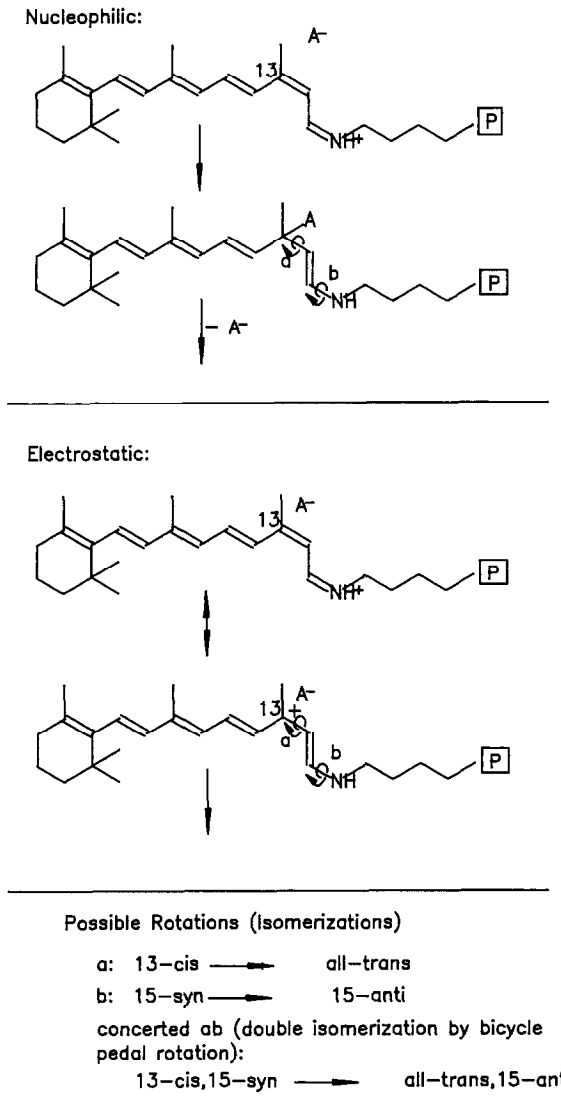
Retinal would appear to be relatively well shielded from the solvent by the protein and lipid bilayer that encapsulates it (*5*). The Schiff base proton, however, has been shown to be readily exchangeable with solvent (*6*) and the iminium carbon succeptible to attack, under illumination, by hydroxylamine (*7*) or sodium borohydride (*8*). It would seem therefore that the agent catalyzing thermal isomerization derives from the protein. Through analogy with the mechanism used by an enzyme to catalyze *cis–trans* isomerization of a very different substrate, we have suggested the possible role of a nucleophile (A^-) to catalyze isomerization of retinal (*4*). Addition of a nucleophile to retinal's C13 would convert double bonds, between C13 and the protonated nitrogen, to single bonds and allow internal rotation about either the C13–C14 or C15–N bonds or both (Scheme 1). This could account for the regiospecificity in retinal isomerization. A similar catalytic effect, initiated by bringing a negative charge above retinal's C13 has also been proposed (*4, 9*). Negative charge, above C13 in the anticipated low dielectric medium of the membrane, would electrostatically stabilize positive charge accumulation at C13 and in so doing would weaken double bonds between C13 and nitrogen and lower the barrier for isomerization. MNDO calculations (*10*) support these mechanisms (*9*).

A likely candidate for the catalytically active nucleophile or negative charge was suggested to be aspartate-212 (*4, 9*). The tertiary structure of the protein puts aspartate-212 and lysine-216 one above the other. We have suggested that the carboxyl group of Asp-212, most probably involved in a salt bridge with the protonated Schiff base nitrogen, could move by a microconformational change, to the region of C13 to catalyze isomerization. Very recently Asp-212 has been identified together with Asp-85 and Asp-96 as being near the Schiff base nitrogen (*11*). Moreover, Asp-212 has recently been reported to be deprotonated in the ground state (*12*), in concert with the idea that its carboxylate anion can act as the catalytically active group. Asp-85, similarly, is deprotonated and could, in principle, fulfill the same role proposed here for Asp-212.

If either the nucleophilic or electrostatic stabilization mechanism operates, alteration of the positive charge density at C13 and the degree of hyperconjugation stabilization by the methyl group at C13 (i.e., C20 methyl) would be anticipated. If these effects are substantial they might be detected in the dark isomerization step as a secondary β-deuterium kinetic isotope effect for a purple membrane preparation containing a retinal analogue deuterated in the C20 methyl group. This paper reports the secondary kinetic isotope effects for dark adaptation for purple membrane reconstituted with retinal-*20,20,20-*d_3. The results are rationalized by considering the changes in the carbon–hydrogen bond orders obtained through MNDO semiempirical quantum calculations using a 30-atom molecule as a model for the protonated retinal Schiff base.

METHODS

General. Gas chromatography was carried out on a Hewlett–Packard 5890A equipped with an HP-1 megabore column (0.53 mm × 30 m), flame-ionization



Possible Rotations (isomerizations)

a: 13–cis ⟶ all–trans

b: 15–syn ⟶ 15–anti

concerted ab (double isomerization by bicycle pedal rotation):

13–cis,15–syn ⟶ all–trans,15–anti

SCHEME 1

detector, and a HP 3396A integrator. NMR spectra were taken with a Bruker AM 300 instrument. Mass spectra were measured on a Finnigan 5100 instrument.

Retinal-20,20,20-d_3 (**1**). 3-Methyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)penta-2(*E*),4(*E*)-dienal (**2**) was prepared from β-ionone and diisopropyl cyanomethylphosphonate (*13*) with subsequent reduction of the nitrile with diisobutylaluminum hydride according to the method of Pardoen *et al.* (*14*). The aldehyde was purified by flash chromatography (2.5% ether/hexane) to a purity of 93–94% as indicated by GC-MS, 218.3 (M^+). Aldehyde **2** was condensed with acetone in

the presence of sodium hydroxide according to the method of Pardoen *et al.* (*15*) to provide 6-methyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)octa-3(*E*),5(*E*),7(*E*)-trien-2-one (**3**). **3** was purified by flash chromatography (15% ether/hexane) to a purity of 93–97% as indicated by GC-MS, 258.2 (M^+). The previously published method for proton exchange of **3** to form 6-methyl-8-(2,6,6-trimethylcyclohexen-1-yl)octa-3(*E*),5(*E*),7(*E*)-trien-2-one-*1,1,1-d*$_3$ (**4**) was modified in that the first aqueous wash was carried out with D_2O. Yield, 86%. GC purity, 92%. GC-MS, 261.0 (M^+); I_{261}/I_{260}, 83.3/16.7. No methyl proton signal was visible at δ 2.29. Ketone **4** was treated with (trimethylsilyl)acetaldehyde *tert*-butylimine (*16*) in THF in the presence of lithium diisopropylamide as described to obtain retinal-*20,20,20-d*$_3$ (*15*). MS, 287.2 (M^+); I_{287}/I_{286}, 94.5/5.5.

Purple membrane was isolated from a single batch of *H. holobium* and purified according to the method of Becher and Cassim (*17*). Membranes were photobleached in the standard way with 0.31 M hydroxylamine, pH 7.3, and light from a 500-W tungsten–halogen projector lamp passed through a Corning 3-69 filter (*18, 19*). One membrane batch was washed twice with water to provide apomembrane A containing retinal oxime. A similar preparation was washed nine times with 2% fatty acid-free bovine serum albumin in 0.1 M Hepes, pH 7.3, and then four times with 0.1 M Hepes to provide apomembrane B.

A portion of each membrane was reconstituted in the dark with either an excess of retinal-*20,20,20-d*$_3$ or retinal-d_0, delivered to the membrane as a concentrated solution in 95% ethanol. After reconstitution of apomembrane A the bacteriorhodopsin preparations were washed four times with 0.01 M Hepes, pH 7.3. After a similar reconstitution of apomembrane B the resulting bacteriorhodopsin was washed four times with the 2% bovine serum albumin solution described above and then four times with 0.1 M Hepes. The membrane was pelleted and taken up in 0.01 M Hepes.

Kinetics. The rates of dark adaptation were measured at 592 nm with a Varian DMS 80 spectrometer whose cell compartment was thermostated by an external circulating bath. Optical density–time measurements were taken under computer control using a program called KNTCS written within the ASYST software framework. Approximately 200 data points, with equal time intervals, were taken for each run over a period of 10 half-lives. The data were subjected to a least-squares fit to a first-order rate law. Any point whose deviation from the calculated value was greater than 2.7 times the standard deviation of all points was rejected automatically and the least-squares fit was recalculated. The process was repeated until the deviations of all points were less than 2.7 times the standard deviation.

Semiempirical quantum calculations. All calculations were carried out as described previously (*9*).

RESULTS

Retinal-*20,20,20-d*$_3$ was synthesized, with minor modification, according to published methods. The d_3 molecule was present in greater than 98% as detected by mass spectrometry. Apomembrane was prepared from purple membrane in

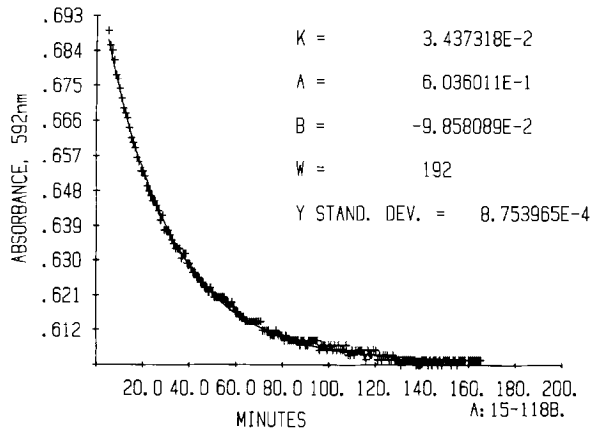


FIG. 1. Typical kinetic data for dark adaptation of reconstituted purple membrane. The curve is the least-squares fit to a first-order rate law.

two different ways and combined with either natural retinal or retinal-*20,20,20-d*$_3$. In one batch the apomembrane (B) was washed before and after with 2% fatty acid-free bovine serum albumin (BSA) to remove retinal oxime formed during photobleaching. Washing was continued until no retinal oxime could be detected spectrophotometrically in the BSA extract. In the other preparation (A) no attempt was made to remove retinal oxime before or after reconstitution. In all cases reconstitution was carried out in the dark with an excess of the retinal used. The kinetics of dark adaptation of each reconstituted retinal was measured spectrophotometrically at 592 nm over 10 half-lives. Measurements were made under computer control and the data fitted to a first-order rate law (see Methods). A sample of the acquired data and the fitted curve is shown in Fig. 1. The observed dark adaptation first order rate constants ($k_{\text{forward}} + k_{\text{reverse}}$) for protio and deuterio purple membranes are given in Table 1. Along with that are the rate constants for the BSA-washed protio purple membrane at different temperatures between 29 and 44°C. A plot of ln k vs $1/T$ yields an activation energy of 26.1 kcal/mol. BSA-

TABLE 1

Observed First-Order Rate Constants for Dark Adaptation of Reconstituted Purple Membrane

t (°C)	BSA washed	Retinal	k_{obs} (min^{-1})[a]	k_H/k_D
35.2	No	d_0	$(4.41 \pm 0.09) \times 10^{-2}$ [5]	
35.2	No	d_3	$(5.05 \pm 0.05) \times 10^{-2}$ [7]	0.87 ± 0.02
34.7	Yes	d_0	$(3.57 \pm 0.06) \times 10^{-2}$ [5]	
34.7	Yes	d_3	$(3.51 \pm 0.13) \times 10^{-2}$ [6]	1.02 ± 0.04
29.7	Yes	d_0	$(1.75 \pm 0.09) \times 10^{-2}$ [5]	
39.3	Yes	d_0	$(6.84 \pm 0.10) \times 10^{-2}$ [4]	
44.1	Yes	d_0	$(1.27 \pm 0.01) \times 10^{-1}$ [4]	

[a] Number of kinetic runs is given in brackets.

washed protio and deuterio purple membrane exhibit nearly the same first-order rate constants for dark adaptation ($k_H/k_D = 1.02 \pm 0.04$) while unwashed purple membranes exhibit a $k_H/k_D = 0.87 \pm 0.02$.

DISCUSSION

Bleached membrane, containing retinal oxime, very slowly undergoes limited autoreconstitution (i.e., Schiff base reformation with the protein). The mechanism probably involves attack of free lysine-216 on the imino carbon of the oxime (*20*). If the amount of autoreconstitution were significant, a bleached preparation, subsequently reconstituted with retinal-*20,20,20-d*$_3$ would be contaminated with bR-d_0. It seemed prudent to subject bleached membrane to repetitive BSA washings to remove retinal oxime. This treatment, however, appears to have deleterious effects (*vide infra*) on the membrane's properties. In other preparations, bleached membrane, containing retinal oxime, was reconstituted with natural or deuterated retinal immediately after removal of excess hydroxylamine. Autoreconstitution is negligible and exchange with retinal oxime is unlikely under these conditions. The protonated retinal Schiff base linkage with lysine-216 is very stable (*vide supra*) and exchange with retinal oxime would require reaction with free amine (*20*).

The secondary β-deuterium isotope effect appears to be dependent on the method of membrane preparation. Thorough washing with BSA leads to a near unity kinetic isotope effect suggesting only minor changes in the degree of β-C–H hyperconjugation in the C20 methyl group of bound retinal in the rate-controlling step of the dark-adaptation reaction. The isotope effect is substantial and *inverse*, however, for reconstituted membrane not previously subjected to BSA washes.

A substantial increase in light scattering is observed in the bleached membrane after washing with BSA. Fatty acid-free BSA, which was used here to remove retinal oxime, has a strong affinity for lipophilic materials and it is probable that some lipid as well is removed from the membrane. The rate of isomerization of BSA-washed membrane is about 15% slower than unwashed membrane after account is taken of the slightly different temperatures of measurement. The activation energy of dark adaptation is slightly larger (26.1 kcal/mol) than that published for native purple membrane (25 kcal/mol) (*21*). Furthermore, the BSA-washed membrane reconstitutes to a smaller degree and at an apparent slower rate than the unwashed bleached membrane. Of the two types of preparations it is our belief that the unwashed one is closer to the native system. If one or more important lipid molecules are removed it is altogether possible that an earlier stage of reaction, conformational change not involving hyperconjugative changes, may become rate controlling in this preparation.

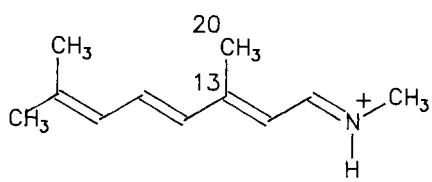
Two mechanisms, one involving nucleophilic addition the other electrostatic stabilization, have been recognized as possible low-energy pathways for thermal *cis–trans* isomerization. In each the carboxylate of aspartate-212 is suggested to move from its position near the positively charged Schiff base nitrogen to C13 of retinal. In the nucleophilic addition mechanism a bond would be formed to that

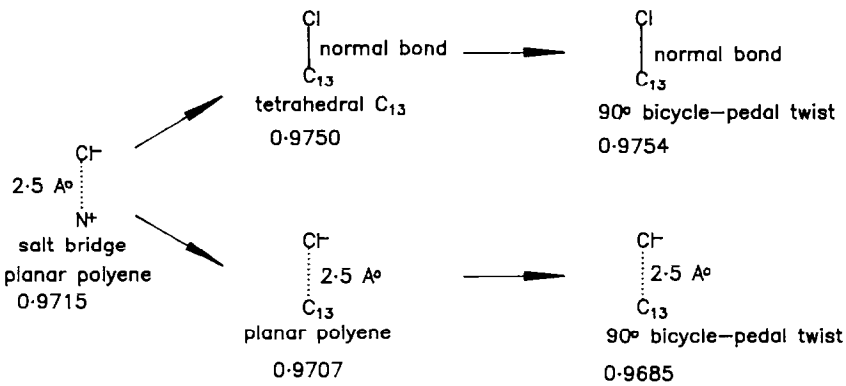
atom while in the electrostatic stabilization mechanism the carboxylate oxygen would be positioned at greater than bonding distance and would induce positive charge development at C13. We turned to semiempirical molecular orbital calculations to see if we could predict the direction of the isotope effect and, by comparison with a well-studied β-deuterium isotope effect system, the solvolysis of *tert*-butyl chloride, we hoped to estimate the magnitude of the effect.

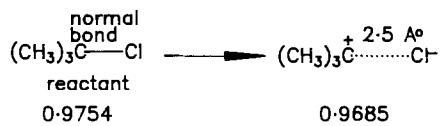
The major contributor to the generation of a secondary deuterium kinetic isotope effect is a difference in C–H force constants between reactant and transition state at the site of deuterium substitution. Secondary β-deuterium isotope effects have been attributed to β-C–H hyperconjugative stabilization of charge or free spin density at Cα. Quantum calculations for the naked *tert*-butyl cation illustrate this effect. The calculations indicate longer (weaker) C–H and shorter (stronger) C–C bonds in the cation than in an uncharged precursor (*22*). An increase in hyperconjugative stabilization in going from neutral reactant to a charged transition state leads to a weakening of β-C–H stretching force constants and a normal (i.e., $k_H/k_D > 1$) isotope effect (*23*). A strengthening of the force constant would be expected to lead to an inverse isotope effect ($k_H/k_D < 1$).

Bond orders correlate with stretching force constants (*24*) and rather than calculate force constant changes to be used in exact calculations of isotope effects by the Wolfsberg–Schachtschneider treatment, it was decided to estimate isotope effects by calculating C20 C–H bond order changes on going from the reactant to a supposed transition state for each mechanism by using the MNDO semiempirical method (*10*).

We recently reported MNDO calculations of C13–C14 and C15–N *cis–trans* isomerization barriers for a model (**I**) of protonated *all-trans*-retinal Schiff base (*9*). In that calculation the effect of placing a point negative charge (i.e., with no bonding electrons) or an anion (acetate or chloride) above the nitrogen or C13 atom, perpendicular to the polyene plane, was determined. In general, a negatively charged species, positioned above nitrogen leads to a higher barrier for isomerization while a negatively charged species above C13 leads to a lower barrier of isomerization as compared to the system where a negatively charged species is absent from the polyeniminium system. It was found that using acetate, a model for Asp-212, as the negatively charged species, yielded very similar results as when chloride was used except that a saving in computational time was realized. Similar calculations were carried out in this study to determine how C20–H bond orders change when the anion moves to different positions with respect to the polyene framework. The geometries about the site of anion–cation interaction, about the site of isomerization, and around the C20 methyl group







SCHEME 2

were all optimized in the calculation except where specific bond lengths or torsional angles were to be maintained. In Scheme 2 are shown schematically the position of the chloride ion with respect to the nitrogen or C13 atom, whether the model represents a planar (reactant-like) or a 90° twisted (transition-state-like) retinal Schiff base, and the average value of C20–H bond orders. The particular C20–H σ-orbital which allows the greatest overlap with the p-orbital of C13 exhibits the largest C20–H bond order change. The average of all of three C20–H bond orders, however, is given. Two paths are traced. Each path is artificially divided into two stages in order to determine the source of the isotope effect. These are not necessarily separate steps; they could act together. In the first path the anion, originally 2.5 Å above the positively charged nitrogen, is moved, in the first stage, to form a normal bond with C13 and then in the second stage, the polyene undergoes concerted internal rotation about the C13–C14 and C15–N bonds via a bicycle-pedal mechanism to the 90° twisted geometry. In the second path, the anion, again above nitrogen, is moved to 2.5 Å above C13 and the polyene is then allowed to undergo bicycle-pedal twisting to the transition state in the second stage. The former is a model for the nucleophilic mechanism while the latter is a model for the electrostatic stabilization mechanism. There is a substantial increase in the C20–H bond order when chloride moves from nitrogen to form a bond with C13. This is due to C20 hyperconjugative stabilization of the positive charge on nitrogen *in the reactant* which is attenuated in the product where the charge is neutralized. The second stage, involving a half bicycle-pedal twist (90°), is accompanied by a small increase in C–H bond order. An increase in bond order

would be accompanied by an increase in C–H stretching force constant and would lead to an *inverse* secondary effect. In the second path there is a small decrease of C–H bond order when chloride moves from 2.5 Å above nitrogen to 2.5 Å above C13 and a larger decrease when the planar polyene is twisted concertedly 90° about the C13–C14 and C15–N bonds. A *normal* secondary β-effect would be anticipated if dark adaptation proceeded along the second path.

It was of interest to calculate the changes for *tert*-butyl chloride when it undergoes solvolysis. It is believed that the first intermediate in solvolysis is the ion pair (*25*). MNDO calculations for *tert*-butyl chloride were carried out where all bond lengths and angles were optimized. The average reactant C–H bond order is found to be 0.9754. A calculation was then carried out for the ion pair where the C–Cl distance was arbitrarily set to 2.5 Å and all other geometric parameters optimized as a model for the transition state. In this case the MNDO calculation yields a geometry (C–C–Cl angle 97.3°) which is a little more than halfway between the reactant (C–C–Cl angle 106.6°) and a planar *tert*-butyl carbocation (C–C–Cl angle 90°). The average C–H bond order decreases to 0.9685. Assuming that the transition-state geometry is chosen correctly, this decrease would be responsible for the observed secondary deuterium isotope effect of $k_H/k_D = 1.34$ per one fully deuterated methyl group.

Judging from the magnitude of the bond order change and the size of the secondary β-isotope effect in *tert*-butyl chloride solvolysis, the calculations for the model protonated retinal Schiff base suggest that a very small normal secondary isotope effect will arise from the movement of an anion from above the positively charged nitrogen to above the C13 atom if the polyene framework remains planar. A somewhat larger normal effect is anticipated in going from that stage to a 90° bicycle-pedal twisted transition-state geometry on its way to a concerted double *cis–trans* isomerization. If one uses the *tert*-butyl chloride case as a calibration point for the relationship of magnitude of isotope effect with the size of C–H bond order change and assumes a linear relationship then the electrostatic stabilization path would be expected to produce a normal isotope effect about half as large as that for the *tert*-butyl chloride case.

An inverse secondary effect is predicted for the nucleophilic addition path. When chloride is above the positively charged nitrogen some of that charge is stabilized through C20 hyperconjugation. Covalent bonding of the anion to C13, resulting in tetrahedral geometry at that site, destroys the positive charge and reduces the degree of 20-methyl hyperconjugation. This is seen as a substantial increase in C20–H bond order. One-half of a bicycle-pedal rotation leads to only a small further increase of bond order. Using the magnitude of the bond order change as a crude guide to the magnitude of the isotope effect, the expected isotope effect for a reaction coordinate involving a reactant with an anion initially above the nitrogen transformed to a transition state involving a half twisted pair of isomerizing double bonds catalyzed by the prior formation of a full covalent bond between anion and C13 would be inverse and about half as large as that realized in the solvolysis of *tert*-butyl chloride. If only partial bond formation between anion and C13 is realized at that transition state then the isotope effect would be proportionally smaller.

The observed first-order rate constant for dark adaptation is really the rate constant for the approach to dark adaptation equilibrium and as such is equal to the sum of forward (k_f) and reverse (k_r) rate constants for thermal isomerization between bR(*all-trans*,15-*anti*) and bR(13-*cis*,15-*syn*). The observed isotope effect then, $(k_H/k_D)_{obs}$ equals $(k_f^H + k_r^H)/(k_f^D + k_r^D)$. We have proposed an isomerization pathway catalyzed by nucleophilic addition for which the initial state is represented by an anion above the positively charged nitrogen of the Schiff base where the two are involved in a salt bridge. Movement of the anion, in the first stage, to form a covalent bond with C13 is calculated to lead to an inverse β-deuterium effect. Subsequent bicycle-pedal rotation is anticipated to lead to only a small additional effect. Whether the reaction proceeds from *cis* to *trans* or *trans* to *cis* the pathway is still suggested to proceed *from* an initial state in which the anion is involved in a salt bridge with the positively charged nitrogen *toward* covalent bond formation between anion and C13 in the transition state. Therefore both k_f^H/k_f^D and k_r^H/k_r^D are anticipated to be inverse. They will be nearly equal to each other if the transition state is symmetrically placed between the two initial states. The observed isotope effect for the membrane preparation not modified by BSA appears to support the proposed mechanism of a nucleophilically catalyzed *cis–trans* isomerization. While we have originally suggested Asp-212 as the species catalyzing dark adaptation, we recognize that Asp-85 may also be a possible candidate for this role.

Nucleophilic additions to catalyze *cis–trans* isomerization chemically (*26*) and enzymatically (*27*) are well documented. While more efficient nucleophiles than a carboxylate ion have previously been recognized as serving in this role, a recent model study demonstrates the ability of a tethered carboxylate to catalyze *cis–trans* isomerization of a positively charged retinal Schiff base (*28*). Carboxylate groups have also been shown to serve as nucleophilic agents in enzyme-catalyzed hydrolysis of glucosides (*29*).

ACKNOWLEDGMENTS

This research was carried out under Contract DE-AC02-76CH00016 with the U.S. Department of Energy and supported by its division of Chemical Sciences, Office of Basic Energy Sciences. The authors thank Dr. Richard L. Schowen, University of Kansas, for suggesting the β-deuterium test. The authors also thank Ms. Elinor Norton for the mass spectral determinations.

REFERENCES

1. For reviews, see: (a) STOECKENIUS, W., AND BOGOMOLNI, R. A. (1982) *Annu. Rev. Biochem.* **52,** 587–616. (b) STOECKENIUS, W. (1985) *Trends Biochem. Sci.* **10,** 483–486. (c) DENCHER, N. A. (1983) *Photochem. Photobiol.* **38,** 753–757. (d) OVCHINNIKOV, Y. A. (1982) *FEBS Lett.* **148,** 179–191.
2. (a) FANG, J.-M., CARRIKER, J. D., BALOGH-NAIR, V., AND NAKANISHI, K. (1983) *J. Amer. Chem. Soc.* **105,** 5162–5164. (b) CHANG, C. H., GOVINDJEE, R., EBREY, T., BAGLEY, K. A., DOLLINGER, G., EISENSTEIN, L., MARQUE, J., RODER, H., VITTITOW, J., FANG, J.-M., AND NAKANISHI, K. (1985) *Biophys. J.* **47,** 509–512.

3. (a) HARBISON, G. S., SMITH, S. O., PARDOEN, J. A., WINKEL, C., LUGTENBURG, J., HERZFELD, J., MATHIES, R., AND GRIFFIN, R. G. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1706–1709. See also: (b) WARSHEL, A. (1976) *Nature (London)* **260,** 679–683. (c) FELIU, A. L., SMITH, K. J., AND SELTZER, S. (1984) *J. Amer. Chem. Soc.* **106,** 3046–3047. (d) SELTZER, S., AND HANE, J. (1988) *Bioorg. Chem.* **16,** 394–407.
4. SELTZER, S., AND ZUCKERMANN, R. J. (1985) *J. Amer. Chem. Soc.* **107,** 5523–5525.
5. (a) HEYN, M. P., WESTERHAUSEN, J., WALLAT, I., AND SEIFF, F. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2146–2150. (b) KING, G. I., MOWERY, P. C., STOECKENIUS, W., CRESPI, H. L., AND SCHOENBORN, B. P. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4726–4730. (c) ENGELMAN, D. M., HENDERSON, R., MCLACHLAN, A. D., AND WALLACE, B. A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2023–2027. (d) HUANG, K. S., RADHAKRISHNAN, R., BAYLEY, H., AND KHORANA, H. G. (1982) *J. Biol. Chem.* **257,** 13,616–13,623.
6. (a) HARBISON, G. S., ROBERTS, J. E., HERZFELD, J., AND GRIFFIN, R. G. (1988) *J. Amer. Chem. Soc.* **110,** 7221–7223. (b) DOUKAS, A. G., PANDE, A., SUZUKI, T., CALLENDER, R. H., HONIG, B., AND OTTOLENGHI, M. (1981) *Biophys. J.* **33,** 275–279. (c) EHRENBERG, B., LEWIS, A., PORTA, T. K., NAGLE, J. F., AND STOECKENIUS, W. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6571–6573.
7. OESTERHELT, D., SCHUHMANN, L., AND GRUBER, H. (1974) *FEBS Lett.* **44,** 257–261.
8. WOLBER, P. K., AND STOECKENIUS, W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2303–2307.
9. SELTZER, S. (1987) *J. Amer. Chem. Soc.* **109,** 1627–1631.
10. DEWAR, M. J. S., AND THIEL, W. (1977) *J. Amer. Chem. Soc.* **99,** 4899–4907. MOPAC, a molecular orbital package, (QCPE-455) was obtained through the Quantum Chemistry Program Exchange.
11. (a) HEYN, M. P., KHORANA, H. G., *et al.* (1989) *Biol. Chem. Hoppe-Seyler* **370,** 623. (b) OTTO, H., MARTI, T., HOLZ, M., MOGI, T., STERN, L. J., ENGEL, F., KHORANA, H. G., AND HEYN, M. P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1018–1022.
12. BRAIMAN, M. S., MOGI, T., MARTI, T., STERN, L. J., KHORANA, H. G., AND ROTHSCHILD, K. J. (1988) *Biochemistry* **27,** 8516–8520.
13. DUGGER, R. W., AND HEATHCOCK, C. A. (1980) *Synth. Commun.* **10,** 509.
14. PARDOEN, J. A., MULDER, E. M. M., VAN DEN BERG, E. M. M., AND LUGTENBERG, J. (1984) *Canad. J. Chem.* **63,** 1431.
15. PARDOEN, J. A., VAN DEN BERG, E. M. M., WINKEL, C., AND LUGTENBERG, J. (1986) *Recl. Trav. Chim. Pays-Bas* **105,** 92–98.
16. COREY, E. J., ENDERS, D., AND BOCK, M. G. (1976) *Tetrahedron Lett.* 7–10.
17. BECHER, B. M., AND CASSIM, J. Y. (1975) *Prep. Biochem.* **5,** 161–175.
18. OESTERHELT, D. (1982) *in* Methods in Enzymology (Packer, L., Ed.), Part I, Vol. 88, p. 10–17, Academic Press, San Diego.
19. Wavelength pass greater than 510 nm.
20. JENCKS, W. P. (1964) *in* Progress in Physical Organic Chemistry (Cohen, S. G., Streitwieser, A., Jr., and Taft, R. W., Eds.), Vol. 2, pp. 63–128, Interscience Publishers, New York.
21. OHNO, K., TAKEUCHI, Y., AND YOSHIDA, M. (1977) *Biochim. Biophys. Acta* **462,** 575–582.
22. HEHRE, W. J. (1975) *Acc. Chem. Res.* **8,** 369–376.
23. SHINER, V. J., JR., *in* Isotope Effects in Chemical Reactions (Collins, C. J., and Bowman, N. B., Eds.), pp. 90–159, Van Nosrand Reinhold Co., New York.
24. PAULING, L. (1960) The Nature of the Chemical Bond, pp. 231–241, Cornell Univ. Press, Ithaca, NY.
25. ABRAHAM, M. H. (1973) *J. Chem. Soc., Perkin Trans. 2* 1893–1899.
26. SELTZER, S. (1972) *in* The Enzymes (Boyer, P., Ed.), 3rd ed., Vol. 3, pp. 361–406, Academic Press, New York.
27. SELTZER, S. (1989) *in* Coenzymes and Cofactors, (Dolphin, D., Poulson, R., and Avramovic, O., Eds.), Vol. 3A, pp. 733–751, Wiley-Interscience, New York.
28. SELTZER, S. (1990) *J. Amer. Chem. Soc.* **112,** 4477–4483.
29. WITHERS, S. G., WARREN, A. J., STREET, I. P., RUPITZ, K., KEMPTON, J. K., AND AEBERSOLD, R. (1990) *J. Amer. Chem. Soc.* **112,** 5887–5889 and references cited therein.