

Interactions between the Protonated Schiff Base and Its Counterion in the Photointermediates of Bacteriorhodopsin

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Received February 24, 1997[Ⓢ]

Abstract: In a previous study, we have shown that halide salts of the protonated Schiff base (pSB) of *all-trans*-retinal with aniline crystallize in the 6-*s-trans* conformation. These compounds are therefore better solid state models for the chromophores of the *all-trans* states of bacteriorhodopsin (bR) than the conventional butyl imine compounds. We have now extended these studies to the halide salts of the pSB of 13-*cis*-retinal with aniline. The 5-¹³C chemical shifts again indicate 6-*s-trans* conformations. These compounds are thus good models for the chromophores of the 13-*cis* photocycle intermediates of bR. As for the *all-trans* compounds, the frequencies of maximum visible absorbance and the ¹⁵N chemical shifts of the 13-*cis* compounds are linearly related to the strength of the pSB–counterion (CI) interaction as measured by $(1/d^2)$, where d is the center-to-center distance between the pSB charge and the CI charge. However, the relationship is steeper for the 13-*cis* compounds. With these calibrations, we estimate that $d = 4.0, 3.9, 3.7, 3.6,$ and $3.8 \text{ \AA} (\pm 0.3 \text{ \AA})$ for the J₆₂₅, K₅₉₀, L₅₅₀, N₅₂₀, and bR₅₅₅ states of bR, respectively. These distances compare with similarly determined values of about 4.16 ± 0.03 and $4.66 \pm 0.04 \text{ \AA}$ for the *all-trans* bR₅₆₈ and O₆₄₀ states, respectively. The results suggest that the pSB–CI interaction is stronger in all the 13-*cis* photocycle intermediates, including the red-shifted J and K intermediates, than in bR₅₆₈. The apparent tightening of the pSB–CI interaction in the J → N sequence is consistent with models of the early photocycle in which electrostatic attractions between the pSB and counterions constitute an important constraint.

Bacteriorhodopsin (bR) is a membrane protein produced in large quantities by *Halobacterium salinarium* (also known as *Halobacterium halobium*).¹ It consists of 248 amino acid residues, forming seven transmembrane α -helices with a retinal chromophore linked to the protein via a Schiff base (SB) at lysine residue 216 in helix G.² bR uses light to pump protons from the cytoplasmic side of the membrane to the extracellular side. The resulting proton gradient is used to drive the synthesis of ATP, an energy carrier needed to power other cellular activities.

The generally accepted bR photocycle is shown in Figure 1. The SB is protonated in all states except M. The counterion (CI) in bR₅₆₈ is generally considered to be a H-bonded complex formed by water and three residues—Arg82, Asp85, and Asp212.^{3,4} (Related abbreviations: bR₅₆₈, the 6-*s-trans*, *all-trans*, 15-*anti* component of dark-adapted bacteriorhodopsin and sole component of light-adapted bacteriorhodopsin; bR₅₅₅, the 6-*s-trans*, 13-*cis*, 15-*syn* component of dark-adapted bacteriorhodopsin; J or J₆₂₅, the primary photoproduct of bR₅₆₈; K or K₅₉₀, the relaxation product of J and the precursor of L; L or L₅₅₀, the photointermediate of bR₅₆₈ preceding M₄₁₂; M or M₄₁₂, the only photointermediates of bR₅₆₈ in which the Schiff base

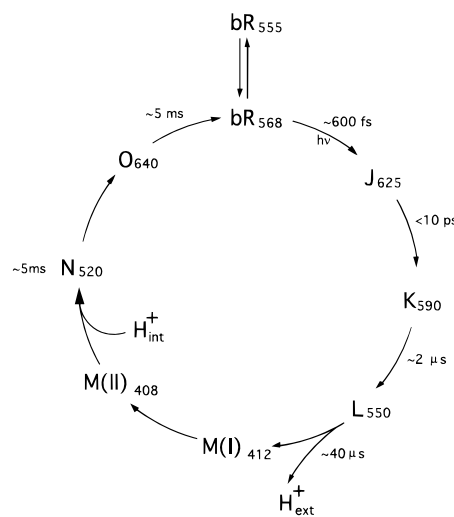


Figure 1. Photocycle of bR₅₆₈ showing the relationships among the functionally important forms. Subscripts represent the wavelengths of maximum visible absorbance. The retinal Schiff base is protonated in all but the M states, and the retinal polyene chain is 13-*cis* in all but the O₆₄₀ and bR₅₆₈ states where it is *all-trans*.

is deprotonated; N or N₅₂₀, the photointermediate of bR₅₆₈ following M₄₁₂.) Spectroscopic data for the photointermediates^{5–10} indicate that, while bR₅₆₈ and O₆₄₀ have an *all-trans* chromophore, the chromophore in all the other species is

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[Ⓢ] Abstract published in *Advance ACS Abstracts*, September 15, 1997.

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13-*cis*. However, the spectroscopic characteristics also clearly show further distinctions among the chromophores of these two classes.

Recently we have found, for halide salts of an *all-trans* protonated Schiff base (pSB) of retinal with aniline, that both the frequency of maximum visible absorption and the ^{15}N chemical shift are linearly related to the energy of electrostatic attraction between the pSB and the halide ion, as estimated from the inverse square of the center-to-center distances between the two.¹¹ On the basis of this correlation, we used the visible spectrum and ^{15}N chemical shift to infer a 4.16 Å center-to-center distance between the pSB and its counterion in bR₅₆₈. This is consistent with the electron-cryomicroscopy-based model of Henderson et al.¹² and its refinement.¹³ For O₆₄₀, using the frequency of maximum visible absorbance alone, the SB–CI distance is similarly estimated to be 4.66 Å.

In the present work, we seek to make a similar calibration of spectroscopic data for the 13-*cis* states of retinal in bR. Again, the aniline pSB is used to stabilize the 6-*s-trans* conformation, and a halide series is used to vary counterion strength. The results suggest that the pSB–CI distances in bR are shorter in the 13-*cis* states than in all the *all-trans* states. Furthermore, relaxation from J₆₂₅ to N₅₂₀ in the 13-*cis* side of the photocycle, and from O₆₄₀ to bR₅₆₈ in the *all-trans* side of the photocycle, both appear to involve strengthening of electrostatic interactions at the Schiff base.

Materials and Methods

Synthesis of 13-*cis* pSBs. The 13-*cis* SBs were prepared in exactly the same manner as the *all-trans* SBs.¹¹ However, the subsequent protonation step used an aqueous solution of hydrogen halide, which proved to be safe in the preparation of retinal pSBs.¹⁴ Commercial concentrated aqueous HCl, HBr, or HI (37, 48, and 56 wt %, respectively) was added very slowly, with vigorous shaking or stirring, to the SB dissolved in ether at –10 °C. The amount of acid added was roughly twice that needed to combine stoichiometrically with the SB, in order to increase the pSB yield. A dark product was generated during the addition of the acids. The solvents (ether + water) were evaporated away with nitrogen. The product was washed with ether several times in order to remove the remaining reactants. The product was dried completely under vacuum for solid-state NMR (SSNMR) and UV–vis spectroscopy.

Measurement of Solid-State UV–Vis Absorption. Solid-state absorption spectra were obtained in the range 300–700 nm on a Perkin-Elmer Lambda3 spectrophotometer, using the approach of Kobayashi et al.¹⁵ The iminium salt was ground very carefully until no particles were large enough to be seen with the naked eye. A very thin layer of the fine powder was placed on the surface of a microscope coverslip. Another coverslip of the same quality was used as a reference.

^{15}N and ^{13}C SSNMR Measurements. Spectra were collected on a custom-designed spectrometer, using custom-designed probes, operating at ^1H , ^{15}N , and ^{13}C frequencies of 400, 40.5, and 100.0 MHz, respectively. Standard cross-polarization/magic angle spinning (CP/MAS) was employed with proton decoupling during acquisition. The isotropic signals are flanked by spinning sidebands spaced at the

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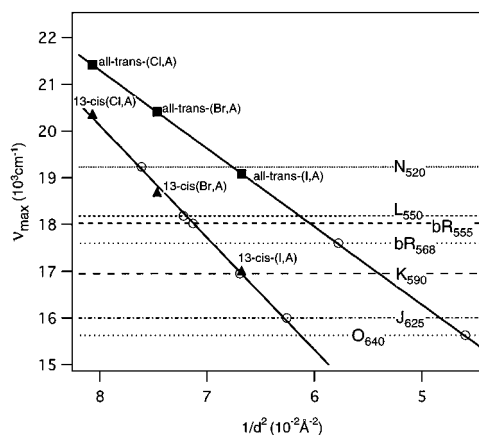


Figure 2. The frequency of maximum solid-state visible absorption of the indicated salts of retinal pSBs with aniline (A) vs $1/d^2$, where d is the sum of the crystallographic radius of N^{3-} and the halide counterion. Squares represent *all-trans* retinal compounds, and triangles represent 13-*cis* retinal compounds. The specific pSBs are further identified by the notation (X, A), where X indicates the halide (Cl, Br, or I) and A identifies aniline. The solid lines show the linear least-squares fit of the experimental points for *all-trans* and 13-*cis* compounds. The broken lines mark the absorptions of various bR states. The open circles mark the intersections of the broken lines with the corresponding solid lines, indicating the estimated values of $1/d^2$ for each of the bR states.

spinning frequency. The typical conditions were as follows: ($\pi/2$) for ^1H 4.0 μs , CP mixing time 2.0 ms, recycling delay 3 s, sample spinning speed ~ 2 kHz for ^{15}N and ~ 4 kHz for ^{13}C , dwell time 20 μs , and digitization size 1024. The chemical shift tensor elements were calculated from the intensities of the spinning sidebands relative to the centerbands.¹⁶ ^{15}N chemical shifts were referenced to external solid $^{15}\text{NH}_4\text{Cl}$ (11.9 ppm downfield of 5.6 M aqueous $^{15}\text{NH}_4\text{Cl}$). ^{13}C chemical shifts were referenced to external solid adamantane (38.56 ppm downfield relative to tetramethylsilane).

Data Analysis. Linear least-squares fits and associated error analysis were performed using the IGOR Pro software package from WaveMetrics, Inc. (Lake Oswego, OR).

Results

The value of the downfield $5\text{-}^{13}\text{C}$ chemical shift tensor element, σ_{33} , is diagnostic for the 6-*s* conformation, with values of 237 and 217 ppm for 6-*s-trans* and 6-*s-cis*-retinoic acid,¹⁷ respectively. For the 13-*cis*-aniline pSBs in the solid state, the experimental values for σ_{33} are 235, 239, and 238 ppm for the counterions Cl^- , Br^- , and I^- , respectively. Thus the 13-*cis*-aniline pSBs are all in the 6-*s-trans* form, like the *all-trans*-aniline pSBs¹¹ and the *N*-methylaniline pSB¹⁸ studied previously.

Figure 2 shows the expected red shift for weaker counterions.¹⁹ As for the *all-trans* compounds,¹¹ the frequency of maximum visible absorbance for the 13-*cis* compounds is linearly related to $1/d^2$, where d is the center-to-center distance between the SB and the CI. For the 13-*cis* compounds the linear least-squares fit for the variation of λ_{max} (in nm) with d (in Å) is

$$(10^4/\lambda_{\text{max}}) = (0.94 \pm 1.31) + (2.40 \pm 0.18)(100/d^2)$$

as compared to

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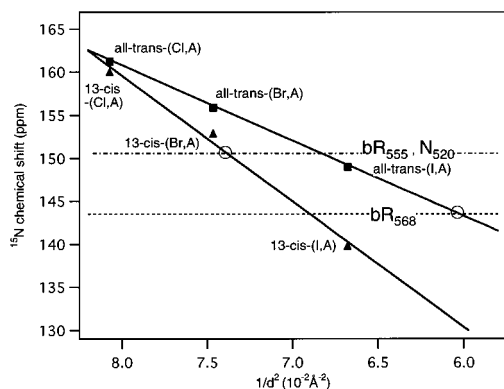


Figure 3. ^{15}N isotropic chemical shift plotted against $1/d^2$. The notation for the pSBs is as in Figure 2. The broken lines mark the ^{15}N chemical shifts for the indicated bR states.

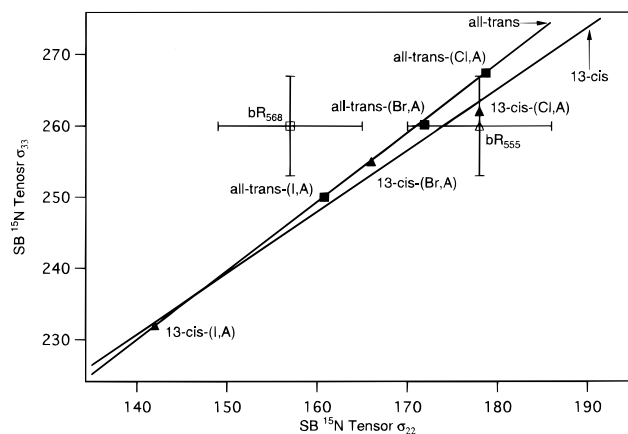


Figure 4. The downfield elements of the ^{15}N shift tensor plotted against each other. The notation for the pSBs is as in Figure 2. The open symbols and associated error bars apply to the indicated states of bR.

$$(10^4/\lambda_{\max}) = (7.93 \pm 0.05) + (1.67 \pm 0.01)(100/d^2)$$

for the *all-trans* compounds.

As reported previously,³ the positive charge at the SB nitrogen is directly related to the counterion strength: the weaker the counterion, the greater the delocalization of the positive charge along the polyene chain and the lower the positive charge at the SB nitrogen. This increase in electron density at the nitrogen leads to increased shielding of the nitrogen and an upfield shift of the SB signal in the ^{15}N NMR spectrum. Figure 3 shows that, as for the *all-trans* compounds,¹¹ the ^{15}N chemical shift for the *13-cis* compounds is linearly related to $1/d^2$. The linear least-squares fit for the variation of the chemical shift δ (in ppm) with d (in Å) is

$$\delta = (42.8 \pm 10.3) + (14.60 \pm 1.38)(100/d^2)$$

for the *13-cis* compounds, as compared to

$$\delta = (90.56 \pm 0.02) + (8.752 \pm 0.003)(100/d^2)$$

for the *all-trans* compounds.

Examining the three principal values of the chemical shift, it is found for the *13-cis* compounds, as for the *all-trans* compounds,¹¹ that the upfield element (σ_{11}) is essentially constant and the other two elements vary proportionally (Figure 4). This suggests that a single effect is responsible, as expected since we have sought only to modulate the electronic interactions of the pSB by simple substitution of counterions. Such behavior

Table 1. Center-to-Center SB–CI Distances in bR Derived from Figure 2

conformation	state	d (Å)
<i>13-cis</i>	J ₆₂₅	4.0 ± 0.3
	K ₅₉₀	3.9 ± 0.3
	L ₅₅₀	3.7 ± 0.3
	N ₅₂₀	3.6 ± 0.3
	bR ₅₅₅	3.8 ± 0.3
<i>all-trans</i>	bR ₅₆₈	4.16 ± 0.03
	O ₆₄₀	4.66 ± 0.04

was first observed in a comprehensive series of salts of *6-s-cis*, *all-trans*-retinal *n*-butylimine.³

Discussion

The linear relationship between the energy of the visible transition and the strength of the pSB–CI interaction has been derived theoretically.²⁰ We have now demonstrated this relationship experimentally in both *all-trans* and *13-cis* pSBs of retinal with aniline. Furthermore, we have shown that the relationship extends to the ^{15}N chemical shifts of these pSBs.

The surprise in the new data is that the *13-cis* compounds are substantially red shifted from the *all-trans* compounds. This is in contrast to very small differences (≤ 8 nm) between *all-trans* and *13-cis* isomers of retinol, 3-dehydroretinol, retinal, 3-dehydroretinal, and retinaldehydeoxime in ethanol or hexane.²¹ Since there can be no conjugation between the polyene and aniline π -systems,¹¹ the effect must be a peculiarity of ion pair formation. Indeed, the red shift depends strongly on the size of the pSB counterion and disappears for a center-to-center distance of 3.22 Å (corresponding roughly to a fluoride CI). Thus, the electronic behavior when the pSB–CI separation is small is similar to that observed long ago for the uncharged polyenes. The fact that divergent behavior is seen as the pSB–CI separation grows is reasonable given that, for more diffuse counterions, the electrostatic effects may go beyond the SB to other elements of the polyene chain, depending on how they are arranged by bond isomerization.

Under the assumption that the mechanisms dominating the spectroscopic properties of bR are similar to those for the model compounds, we can use the model compound data to estimate center-to-center pSB–CI distances in all states of bR with a protonated SB. From the λ_{\max} values of the various bR states (see horizontal lines in Figure 2), and the linear least-squares fits of the visible data for the *13-cis* model compounds

$$(100/d^2) = (-0.35 \pm 0.57) + (0.415 \pm 0.030)(10^4/\lambda_{\max})$$

and the *all-trans* model compounds

$$(100/d^2) = (-4.74 \pm 0.05) + (0.598 \pm 0.002)(10^4/\lambda_{\max})$$

we obtain the center-to-center SB–CI distances for bR shown in Table 1. Similar distances are obtained from the ^{15}N data for the states of bR for which ^{15}N chemical shifts are known (see the horizontal lines in Figure 3).

One interesting result of this analysis is that, although the J₆₂₅ and K₅₉₀ states are red-shifted relative to bR₅₆₈, the pSB–CI interaction seems to be stronger in the former than the latter. Thus, *all-trans* to *13-cis* photoisomerization appears to have the effect of moving the pSB closer to a negatively charged group. This suggests that electrostatic interactions may actually

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influence the photoisomerization process such that, when photoexcitation reduces double-bond character along the polyene chain, rotation around the 13–14 bond allows the pSB to be drawn closer to a negatively charged group. In succeeding steps of the photocycle, electrostatic attraction seems to strengthen further, until the chromophore reisomerizes to *all-trans*, presumably under steric pressure from the protein. The pSB of the resulting O₆₄₀ state has an even weaker counterion interaction than bR₅₆₈. But this is because Asp85 became protonated in the L → M transition. The resting relationship between the *all-trans* pSB and its counterion is restored by deprotonation of Asp85 in the O₆₄₀ → bR₅₆₈ transition.

The above analysis is oversimplified in the sense that other factors in addition to the pSB–CI interaction influence the visible spectrum of the chromophore. In particular, twisting around single and double bonds is expected to cause blue and red shifts, respectively.^{22,23} ¹⁵N chemical shift data for the early photocycle intermediates would be helpful in distinguishing such effects from electrostatic effects. However, because the differences in the visible spectra of 13-*cis* and *all-trans*-retinal SBs

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for a given counterion are quite large (see Figure 2), the basic conclusion that the retinal pSB is closer to its counterion in the 13-*cis* K₅₉₀, L₅₅₀, and N₅₂₀ states of bR than in the *all-trans* bR₅₆₈ state is likely to be robust.

Conclusions

Studies of the spectroscopic properties of retinal Schiff base salts show powerful systematic effects of counterion strength. In particular, for both 13-*cis* and *all-trans* halides, the frequency of maximum visible absorbance and the ¹⁵N chemical shift are found to vary linearly with the energy of the ion pair interactions. However, this variation is steeper for the 13-*cis* compounds than for the *all-trans* compounds. Using these calibrations, we estimate the center-to-center distances between the pSB and its counterion for all the spectroscopically established states of bR. The results support a view of the bR photocycle in which electrostatic interactions of the pSB play an important role.

Acknowledgment. This research was supported by the National Institutes of Health (GM-36810, GM-23289, and RR-00995). The authors thank Sunil DasGupta for helpful discussions regarding the synthesis of pSBs.

JA9706007