that the magnetic properties of the metal sites are not homogenized by the strong electron delocalization demonstrated by the isomer shift and quadrupole splitting parameters.

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Analysis of NMR and Absorption Spectroscopic Data in Bacteriorhodopsin: Models for Protein–Chromophore Interactions

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Abstract: Detailed models for the interaction between the chromophore of bacteriorhodopsin and its protein environment are presented. The models are based on an analysis of the solid-state ^{13}C NMR data and the spectroscopic data derived from modified bacteriorhodopsin pigments in which the chromophore is replaced with a series of dihydroretinals. Semiempirical quantum mechanical methods are used to guide the analysis. The main features of the models include the following: a counterion whose electrostatic interaction with the Schiff base is weaker than that of a chloride counterion in solution; a negative charge near C5; a positive charge near C7; and a ring–chain conformation with a smaller torsional angle around the 6–7 single bond than is found in solution. Models with both s-cis and s-trans ring–chain conformations give excellent agreement with the absorption spectroscopic and chemical shift data. Although a number of recent experiments have led to the suggestion that an s-trans conformation is present in bR, arguments are presented that indicate that an s-cis ring–chain conformation cannot be ruled out.

Bacteriorhodopsin (bR) belongs to a group of biological pigments (retinal proteins) that use a protonated Schiff base of retinal as a chromophore. Although all of the pigments in this group contain retinal as a chromophore, both their absorption maxima (λ_{max}) and their biological function can be quite different. Thus, it is the arrangement of amino acids in each protein that both regulates the spectral properties of the chromophore and determines how the energy of the absorbed photon is to be used. In the absence of high-resolution three-dimensional structures of these pigments, a variety of spectroscopic techniques have been used to study the interactions between the protein and the chromophore.

Models for wavelength-determining interactions in the retinal binding site of bR have been based on both absorption spectra and solid-state NMR data. The essential absorption measurements were carried out on bR and on modified bR's in which the chromophore was replaced with a series of dihydroretinals (see Table I).[1-3] The NMR results were obtained in the series of experiments of Harbison et al., who succeeded in measuring the ^{13}C and ^{15}N chemical shifts of the retinal chromophore of bR.[4-8]

Table I. Calculated and Experimental λ_{max} (nm) of PRSB and bR and Opsin Shifts (OS) (cm^{-1})

chromophore	all-trans	5,6-H_2	7,8-H_2	9,10-H_2
PRSB[a]	445	431	392[b]	322
(calcd)[a]	448	435	392	336[c]
bR[a]	567	478	440	
OS	4830	2340	2780	
(calcd)	571	480	437	
OS	4800	2160	2630	
bR[d]	568	475	445	

[a] Data taken from Spudich et al.[2]. [b] Nakanishi et al.[1] and Lugtenburg et al.[3] reported a value of 385 nm for the λ_{max} of 7,8-dihydro-PRSB. We reproduced this λ_{max} using a C=N syn conformation in our calculations. It is thus possible that the discrepancy in the data is a result of different configurations about the C=N bond. [c] The 322-nm λ_{max} is reproduced when a C=N syn conformation is used in our calculations (see above). [d] Data from Lugtenburg et al.[3]

The major qualitative features of the models proposed to account for the 570-nm absorption maximum of bR include (1) a weak counterion–Schiff base interaction, (2) an s-trans conformation about the C6–C7 single bond (the ring–chain angle) rather than the s-cis conformation that is the energetically favored form in solution,[9] and (3) a negative charge near C5 that forms an ion pair with a positive charge near C7. Quantum mechanical calculations of absorption maxima were used to derive a model for chromophore–protein interactions in which the location of the charged groups on the protein was explicitly defined. However, the interpretation of the NMR data was based entirely on qualitative arguments. In this paper the NMR data are also

(1) Nakanishi, K.; Balogh-Nair, V.; Arnaboldi, M.; Tsujimoto, K.; Honig, B. *J. Am. Chem. Soc.* **1980**, *102*, 7945–7947.
(2) Spudich, J. L.; McCain, D. A.; Nakanishi, K.; Okabe, M.; Shimizu, N.; Rodman, H.; Honig, B.; Bogomolni, R. A. *Biophys. J.* **1986**, *49*, 479–483.
(3) Lugtenburg, J.; Muradin-Szweykowska, M.; Heeremans, C.; Pardoen, J. A.; Harbison, G. S.; Herzfeld, J.; Griffin, R. G.; Smith, S. O.; Mathies, R. A. *J. Am. Chem. Soc.* **1986**, *10*,, 3104–3105.
(4) Harbison, G. S.; Herzfeld, J.; Griffin, R. G. *Biochemistry* **1983**, *22*, 1–5.
(5) 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.
(6) Harbison, G. S.; Smith, S. O.; Pardoen, J. A.; Mulder, P. P. J.; Lugtenburg, J.; Herzfeld, J.; Mathies, R.; Griffin, R. G. *Biochemistry* **1984**, *23*, 2662–2667.
(7) Harbison, G. S.; Mulder, P. P. J.; Herzfeld, J.; Griffin, R. G. *J. Am. Chem. Soc.* **1985**, *107*, 4809–4819.
(8) Harbison, G. S.; Smith, S. O.; Pardoen, J. A.; Courtin, J. M. L.; Lugtenburg, J.; Herzfeld, J.; Mathies, R. A.; Griffin, R. G. *Biochemistry* **1985**, *24*, 6955–6962.
(9) Honig, G.; Warshel, A.; Karplus, M. *Acc. Chem. Res.* **1975**, *8*, 92–100.

0002-7863/88/1510-1943$01.50/0

interpreted on the basis of quantum mechanical calculations. The goal is to derive models that are consistent, in a quantitative sense, with both absorption and NMR measurements.

The reliability of semiempirical methods in the prediction of absorption maxima of retinal analogues has been established in previous work.[1,2] The first goal of this study is to learn whether chemical shift data are also consistent with the results of theoretical calculations and, in doing so, to determine the extent to which qualitative conclusions derived from these data are likely to be correct. This is accomplished through the analysis of NMR measurements on model compounds. The conclusions from this part of the study are then used as a basis for deriving models of retinal–protein interactions in the bR binding site that provide satisfactory quantitative agreement between theory and experiment. The combination of NMR and absorption data is found to provide fairly severe constraints, which appear to reliably define the spectroscopic determinants in the chromophore binding site.

The calculations presented here have no bearing on the question of the ring–chain torsional angle in bR. However, a study with retinal analogues by van der Steen and co-workers[10] and NMR experiments[7,8] tend to support the conclusion that the ring–chain conformation is s-trans. Our own analysis, presented in the Discussion, suggests that the NMR results can also be interpreted in terms of an s-cis conformation in which the 6–7 torsional angle is somewhat more planar than in solution. Since the major emphasis of the work is the electrostatic environment of the chromophore and not the ring–chain angle, models that assume both an s-trans and s-cis conformation are presented.

Methods

The total chemical shift of nucleus A can be considered to be the summation of the electronic contributions (for review see Martin et al.[11])

$$\sigma^{A} = \underbrace{\sigma^{AA}_{dia} + \sigma^{AA}_{para}}_{\text{intraatomic}} + \underbrace{\sum \sigma^{BA}_{B \neq A} + \sigma^{A}_{deloc} + \sigma^{A}_{solv}}_{\text{interatomic}}$$

where the first term (the diamagnetic term) is due to electrons in a spherical s state, the second term (the paramagnetic term) corrects for nonspherical electronic distributions, the third term is the summation of contribution from intratomic currents induced by all atoms B ≠ A and is related to their magnetic anisotropy, the fourth term corrects for ring currents, and the last term represents the contributions from solvent. This expression is a complex function of electron densities, bond orders, geometry, and excitation energies. Therefore, calculation of total chemical shifts is difficult and often inaccurate.

A major determinant of the chemical shift of a nucleus is, however, its electron density. Spiesecke and Schneider[12] observed that the ^{13}C chemical shifts of the carbon atoms in the cyclic aromatic compounds $C_5H_5^-$, C_6H_6, $C_7H_7^+$, and $C_8H_8^{2-}$ change linearly with π-electron density with a slope of −160 ppm/electron. Similar results have been obtained by Lauterbur[13] for aromatic compounds and Tokuhiro and Fraenkel[14] for azines. The carbons in all of these compounds are in very similar environments. Tokuhiro and Fraenkel carried out an extensive study calculating ^{13}C chemical shifts by a number of different semiempirical methods. Their results were dependent on the method of calculation, but the best results were obtained by using the CNDO approximation. Although they did observe a linear correlation between chemical shift and π-electron density, the linearity improved considerably when σ electrons were also included in the charge densities. The slope found in their study was −155 ppm/electron.

In contrast to the compounds mentioned in the previous paragraph, retinal is made up of carbons having very different chemical environments. As a consequence, a comparison of charge density with *absolute* chemical shift will not yield high correlations (see Results for further discussion of this point). However, the molecules of interest in this study are all retinal analogues. Each carbon in one analogue is in a very similar environment to the corresponding atom in another analogue. For example, C9 of retinal (RET) and C9 of the unprotonated Schiff base of retinal (RSB) are both in the conjugated chain and have an attached methyl group. As a consequence, *differences* in chemical shifts between any two corresponding atoms in the retinal analogues are likely to correlate well with their *differences* in charge densities, with a slope of about −160 ppm/electron. Note that, since differences are involved, a graph of changes in chemical shift versus changes in charge density should in principle pass through the origin.

Table II. Comparison of Correlations between Charge Density and Chemical Shift Obtained when Considering Differences between Two Analogues (Columns 1 and 2) and a Single Molecule (Columns 3 and 4) (Standard Deviation (SD) in ppm and Slope in ppm/Electron)

	RSB–RET[a]	PRSB[b]–RSB	RET	RSB
corr	0.98	0.96	0.95	0.85
SD	2.13	2.42	5.64	5.00
slope	−186	−125	−124	−87
y_{int}	−1.6	1.0	−3.6	−2.1

[a] Experimental chemical shifts from Inoue et al.[21] [b] A counterion distance of 3.7 Å was used in the calculation for PRSB. The chemical shifts are from PRSB in CCl_4 with a TFA counterion.[16]

There have a number of previous studies that used this basic approach in analyzing chemical shifts of retinal and Schiff base analogues of retinal.[15-17] Shriver et al.[15] calculated ^{13}C chemical shifts by using the Pople–Karplus relation for planar conjugated alkenes and the π-electron method of Pariser, Parr, and Pople (PPP). They found large errors in their calculated values for C14 and C15, which they attribute to departures in this part of the molecule from the type of structure for which the Pople–Karplus theory was designed. Inoue et al.[16] correlated differences in π-electron density calculated with CNDO/2-MO with differences in chemical shift. They also found a large deviation in C15, but C14 was well correlated. Their calculated line (slope −98 ppm/electron) deviates in its slope from the empirically derived value of −160 ppm/electron. Both groups found essentially the same results for a comparison between RBS and the PRSB, i.e., odd carbons lose electron density on going from RSB to PRSB and show a downfield chemical shift, while even carbons gain electron density and show an upfield chemical shift. Recently, Birge et al. used NMR measurements and INDO-DSDCI calculations to analyze level ordering reversed in protonated and unprotonated retinal Schiff bases.[17]

In this work, charge densities were calculated with CNDO/S-DCI employing the Ohno formula for the electron repulsion integrals,[18] together with two-electron configuration interaction (CI) using only π orbitals in the CI. This method has been shown to give good results for delocalized polyenes.[19] All bond angles were assumed to be 120°. Carbon–carbon and carbon–nitrogen bond lengths were calculated by using PPP, and C–H bond lengths were set to 1.08 Å. All molecules were assumed to be planar and all-trans except for the ring–chain conformation, which is set to 45° in the model compounds RET, RSB, and PRSB, but is allowed to vary in the models for bR. The β-ionone ring and all methyl groups are replaced by hydrogens. The environments of the molecules are modified by placing point charges around the chromophore to represent counterions in solution and charged amino acids in bR. Absorption maxima were calculated with the PPP method, parameterized to account for the spectroscopic properties of delocalized polyenes.[20]

Harbison et al.[8] proposed that the upfield shift of C8 in bR relative to PRSB is due to a γ effect. γ effects are, however, not accounted for by calculated charge densities. The γ effect is due to a steric interaction where the hydrogens on nonbonded carbons are in close contact, as is the case for the 1,4-carbons in a cis bond. The carbons involved in such a contact exhibit a large upfield chemical shift relative to the values they would have if they were in a trans configuration. This shift is believed to be due to polarization of the H–C bond as a result of the repulsive forces between the nonbonded hydrogens. Unfortunately, such polarization effects are not well accounted for by semiempirical methods. Therefore, carbons experiencing γ effects are not included in the correlations.

(10) van der Steen, R.; Biesheuvel, P. L.; Mathies, R. A.; Lugtenburg, J. *J. Am. Chem. Soc.* **1986**, *108*, 6410–6411.
(11) Martin, G. J.; Martin, M. L.; Odiot, S. *Org. Magn. Reson.* **1975**, *7*, 2–17.
(12) Spiesecke, J.; Schneider, W. G. *Tetrahedron Lett.* **1961**, *14*, 468–472.
(13) Lauterbur, P. C. *J. Am. Chem. Soc.* **1961**, *83*, 1838–1852.
(14) Tokuhiro, T.; Fraenkel, G. *J. Am. Chem. Soc.* **1969**, *91*, 5005–5013.
(15) Shriver, J.; Abrahamson, E. W.; Mateescu, G. D. *J. Am. Chem. Soc.* **1976**, *98*, 2407–2409.
(16) Inoue, Y.; Tokitô, Y.; Tomonoh, S.; Chûjô, R.; Miyoshi, T. *J. Am. Chem. Soc.* **1977**, *99*, 5592–5596.
(17) Birge, R. R.; Murray, L. P.; Zidovetzki, R.; Knapp, M. H. *J. Am. Chem. Soc.* **1987**, *109*, 2090–2100.
(18) Ohno, K. *Theor. Chim. Acta* **1964**, *2*, 219.
(19) Dinur, U.; Honig, B.; Schulten, K. *Chem. Phys. Lett.* **1980**, *72*, 493–497.
(20) Honig, B.; Greenberg, A. D.; Dinur, U.; Ebrey, T. G. *Biochemistry* **1976**, *15*, 4593–4599.

When models of bR are derived based on the ^{13}C NMR data, PRSB is used as the reference molecule. Since PRSB and the chromophore of bR are chemically identical, using PRSB as the reference maximizes the similarity of the corresponding atoms in these two molecules and thus minimizes the error from factors other than charge density in determining the chemical shift.

Results

I. **Testing the Method.** In order to determine whether good correlations can be obtained, the methods discussed above are first applied to model compounds that differ only in the C15 substituent.

RET and RSB. Figure 1a and the first column of Table II show the correlation between charge density differences and chemical shift differences for RET and RSB.[21] The results are quite good: the correlation coefficient is very high (0.98), the standard deviation fairly low (2.13 ppm), the slope close to that obtained by Spiesecke and Schneider[12] (−186 ppm/electron), and the y_{int} is near zero (−1.6). (Including $y = 0$ in the data causes insignificant changes in the results: a correlation coefficient of 0.98, a standard deviation of 2.08 ppm, a slope of −187 ppm/electron, and a y_{int} of −1.5.)

For comparison, Figure 1b and the last two columns of Table II show the correlations obtained when the absolute charge densities of each molecule are compared with absolute chemical shift (reported relative to C5 to facilitate comparison with column 1). These correlations are lower and the standard deviations much higher than those found when differences in charge densities and chemical shifts are compared. Although the correlation for RET actually appears quite high, as can be seen in Figure 1b, this is largely due to the contribution of C15. If this atom is left out, the correlation drops to 0.65, and the standard deviation rises to 5.98 ppm. The correlation for RSB (which is only 0.85 for the entire molecule) is even lower without C15. In contrast, there is still a linear correlation among C5–C14 in RSB–RET [albeit smaller than with the full data set (0.83); the standard deviation, however, remains low (2.20 ppm)], as is evident from Figure 1a. These results highlight the importance of using charge density and chemical shift differences as opposed to absolute values.

It is important to note that while good correlations are obtained between charge density differences and the isotropic chemical shift differences, we find little correlation (correlations of about 0.6 and standard deviations about 6.5 ppm) between charge density differences and differences in the components of the chemical shift tensor (see Table I in Harbison et al.[7] for the chemical shift tensors of RET and RSB). In some carbons the three components of the chemical shift tensor change in the same direction in going from RSB to RET (e.g., C13 and C15), in other carbons σ_{22} and σ_{33} shift in the same direction but in the opposite direction as σ_{11} (e.g., C10), and in some carbons σ_{22} and σ_{33} shift in opposite directions (e.g., C5 and C14). The lack of an apparent pattern makes it difficult to draw conclusions about the molecules in question based on the tensor element differences. *It is remarkable that such high correlations can be obtained between charge density differences and isotropic chemical shift differences when the components of the chemical shift tensor seem to vary independently.*

PRSB and RSB. A major problem that arises in considering PRSB is that the ^{13}C chemical shifts of PRSB, unlike those of RET and RSB, are sensitive to both the solvent and the counterion. However, there is a pattern in the way the chemical shifts of the carbons in PRSB change as a function of environment. Shriver et al.,[15] Inoue et al.,[16] and Harbison et al.[7] all report that as the counterion to the Schiff base becomes "weaker" (e.g., chloride to bromide) the odd-numbered carbons shift downfield and the even-numbered carbons shift upfield. The existence of this regularity in the data suggests that appropriate placement of the counterion in the charge density calculations might permit the data to be reproduced.

In order to test this idea, charge densities were calculated for PRSB with four different counterion distances ranging from 3.0 to 3.7 Å. The charge density differences between each of these 4 PRSB models and the corresponding atoms of RSB were compared with the chemical shift differences between PRSB (with 9 different counterions or solvents) and RSB in the same solvent

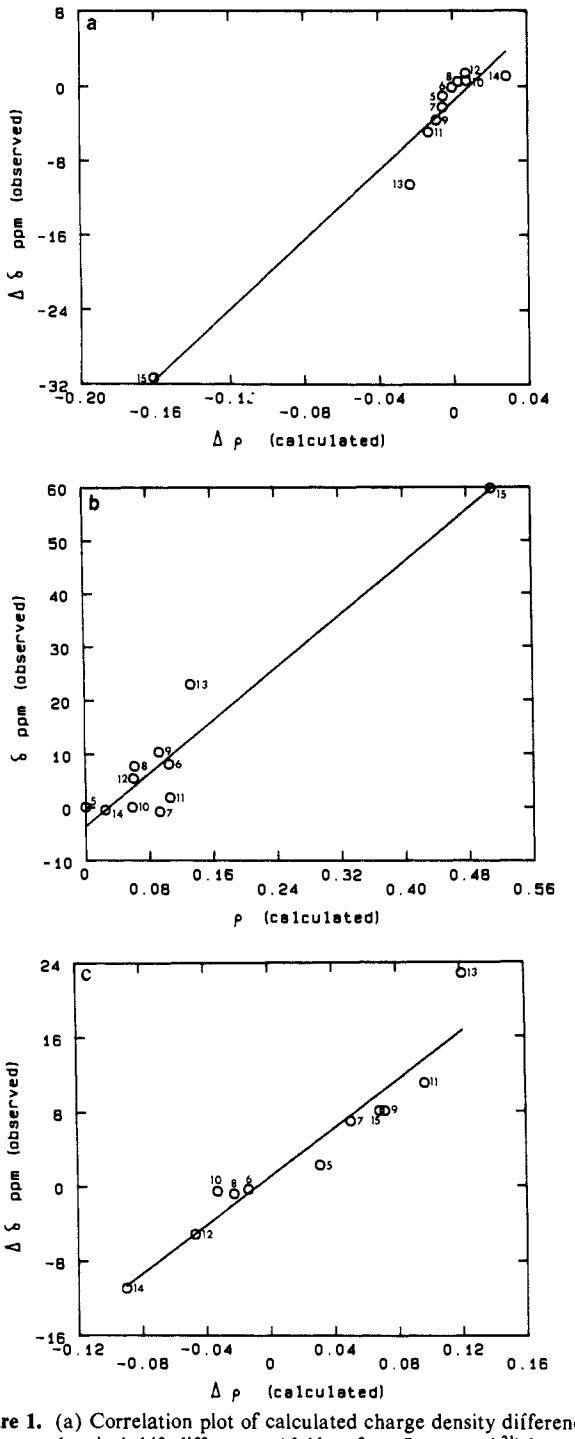

Figure 1. (a) Correlation plot of calculated charge density differences $\Delta\rho$ versus chemical shift differences $\Delta\delta$ (data from Inoue et al.[21]) between RSB and RET. The results show a correlation of 0.98, standard deviation of 2.13 ppm, slope of −186 ppm/electron, and a y_{int} of −1.6. (b) Correlation plot of absolute calculated charge densities ρ versus absolute chemical shifts δ (data from Inoue et al.[16]) in RET (referenced to C5). The results show a correlation of 0.95, standard deviation of 5.64 ppm, slope of −124 ppm/electron, and a y_{int} of −3.6 including all carbons, and a correlation of 0.65, standard deviation of 5.98 ppm, slope of −119 ppm/electron, and a y_{int} of −3.2 excluding C15 from the correlation. (c) Correlation plot of calculated charge density differences $\Delta\rho$ of PRSB with a counterion 3.7 Å from the Schiff base nitrogen and RSB versus chemical shift difference $\Delta\delta$ (data from Inoue et al.[16]) of PRSB in CCl_4 with a TFA counterion and RSB in CCl_4. The results show a correlation of 0.96, standard deviation of 2.43 ppm, slope of −125 ppm/electron, and a y_{int} of 1.0.

(21) Inoue, Y.; Tokitô, Y.; Tomonoh, S.; Chûjô, R. *Bull. Chem. Soc. Jpn.* **1979**, *52*, 265–266.

(i.e., 36 comparisons were made). The PRSB data include the following: the solid-state data from Harbison et al.,[7] with chloride, bromide, dichloroacetate (DCA), and trichloroacetate (TCA); the solution data of Inoue et al.,[16] with PRSB in CD_3OD, and PRSB in CCl_4 with TSA, trifluoroacetate (TFA), and iodide counterions; and Shriver and co-worker's[15] data with PRSB in $CDCl_3$ with a chloride counterion.

The calculations with counterion distances between 3.3 and 3.7 Å yielded higher correlations and lower standard deviations between the calculated charge density differences and the chemical shift differences than those with smaller distances. Comparisons between the calculated values and the solution NMR data yielded higher correlations (0.96), low standard deviations (2.5 ppm), slopes averaging −125 ppm/electron, and y_{int} of about 1.0 than did comparisons with the solid-state data. As the counterion moves further from the Schiff base nitrogen, the odd-numbered carbons become more positive and the even-numbered carbons become more negative. This is consistent with the observed changes in chemical shift as a function of counterion. In particular, a counterion distance of 3.5 Å yielded the best results (e.g., highest correlation and lowest standard deviation) with PRSB and RSB in CD_3OD, which has the properties of a "strong" counterion.[22] Equally good results are obtained for PRSB with TCA, TFA, and iodide counterions, which are weaker counterions than CD_3OD, and RSB in CCl_4 when the counterion distance in the model for PRSB is changed to 3.7 Å. Thus, as expected, the longer counterion distance yields better results for the "weaker" counterion (see Figure 1c).

Most of the solid-state data were not as well accounted for by the charge densities. The solid-state chloride, bromide, and TCA counterions all resulted in correlations of about 0.91 and standard deviations greater than 3.5 ppm. In particular, C5, C10, and C13 exhibited large deviations from the linear correlation. However, the DCA counterion solid-state data gave results comparable with the solution data.

Although it is not clear why better correlations are obtained with the solution data than with the solid-state data, some factors suggest themselves: (a) In solution the molecules are free to assume different conformations. The chemical shifts are therefore an average of the chemical shifts of each different conformation. On average the structure of PRSB and RSB may be quite similar. In the crystals, however, the molecules assume a single conformation. Differences in the conformation between the molecules in the PRSB crystal and the RSB crystal may produce changes in the chemical shift that cannot be accounted for with charges densities. (b) The local environment of the chromophore in the crystal is different than that in solution. In particular, there may be interactions between the counterion of one molecule (or extra ions in the crystal) with parts of other molecules. (Unfortunately, there are no crystal structures for these molecules at this time.) (c) The solid-state data used for RSB was RSB–propylimine, while the PRSB molecules are all PRSB–butylimine. This was necessary because RSB–butylimine appears to crystallize with an s-trans ring–chain conformation,[8] causing large deviations in the chemical shifts of the carbons near the ring compared with the s-cis ring–chain conformation. Since chemical shifts in RSB are sensitive to the imine group,[7] comparing PRSB and RSB with different imine groups could have also led to lower correlations.

***s-cis*- and *s-trans*-Retinoic Acid and 13-*cis*-Retinal.** The last point that needs to be addressed before considering bR is the effect of the ring–chain conformation on chemical shift and charge density. Retinoic acid and 13-*cis*-retinal both crystallize in s-cis and s-trans forms. Harbison et al.[4] measured the chemical shifts of C5 and C8 in these molecules and showed that there is a 7–10 ppm downfield shift in C5 and a 6–8 ppm upfield shift in C8 in going from the s-cis to the s-trans conformation. The explanation given for the C8 shift is a γ effect due to crowding at this position by the C1 methyls in the trans conformation. The change in the chemical shift of C5 is attributed to greater delocalization of the π electrons along the conjugated chain in the s-trans than in the s-cis isomer. In order to test this suggestion, charge densities for retinoic acid and retinal calculated using a 45° ring–chain twist were compared with those obtained with a planar s-trans conformation. The results of the calculations indicate that only about a 1 ppm downfield shift in this position in going from the s-cis to the s-trans conformation (assuming the standard slope of −160 ppm/electron) could be accounted for by charge density differences.[23,24] The downfield shift in C5 on going from the s-cis to the s-trans conformation, therefore, is unlikely to be due entirely to changes in electron delocalization resulting from ring–chain planarity.

In order to determine the origin of the downfield chemical shift on C5 the charge density calculations were repeated with the crystal structures[23,24] rather than the idealized geometries discussed in the Methods. The results of these calculations indicate that about half of the chemical shift change on going from the s-cis to the s-trans conformation can be accounted for by charge density differences (3 ppm in retinoic acid and 4 ppm in 13-*cis*-retinal, assuming a coefficient of −160 ppm/electron). (The difference in these results from those using idealized geometries is probably due to the electronic effects of the twisted C5–C6 double bond in the crystal structure.) The other half of the chemical shift difference is probably due to factors other than changes in electron delocalization. These might include, for example, steric effects and subtle changes in electronic structure brought about by geometric differences.

There is little change in the charge density at C8 in going from the s-cis to the s-trans isomers (equivalent to about −0.3 ppm). This is consistent with the upfield shift on C8 being steric in nature, since calculated charge densities do not account for these types of effects (see Methods). It is clear from the crystal structure that the hydrogen on C8 is crowded in the s-trans conformation, since it lies only 2.1 Å from the hydrogen on the C1 methyl. Thus, the upfield shift of C8 in the s-trans structure is most likely due to a γ effect (as suggested by Harbison et al.[7]). It appears then that changes in the ring–chain angle effect the chemical shift at carbons 5 and 8 both by changing the degree of electron delocalization and through more subtle and poorly understood effects on electronic structure. The effects on C5 are particularly hard to understand. For example, it is not at all clear why the shift in C5 in going from the s-cis to the s-trans conformation is restricted to the σ_{33} component of the chemical shift tensor (see Discussion).

II. Modeling Bacteriorhodopsin. There are two sets of data to be accounted for in modeling the chromophore and its binding site in bR: the dihydro absorption data upon which the "point charge" model of Spudich et al.[2] was based (see Table I) and the ^{13}C NMR data of Harbison et al.[7,8] (summarized in Table III). As is apparent from Table III, there are wide variations in chemical shift differences for a given atom, depending on the PRSB data set with which bR is compared. However, since the calculated charge densities for PRSB and RSB account well for the solution data, it would not be unreasonable to expect that the same will hold when modeling bR.

Correlations were calculated between chemical shift differences of bR and each of the nine different PRSB data sets discussed above (i.e., all the data in Table III) and the charge density differences of the bR model in question and PRSB models with counterion distances of 3.0–3.7 Å.[25] A model is considered good if it yields results comparable with the best model compound results (see columns 1 and 2 of Table II), for at least two sets of PRSB data (see Table III), and significant results for the rest of

(22) For example, PRSB in CD_3OD has a λ_{max} of 445 nm, corresponding to chloride counterion in CCl_4.

(23) Stam, C. H. *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem.* **1972**, *B28*, 2936–2945.

(24) Simmons, C. J.; Liu, R. S. H.; Denny, M.; Seff, K. *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem.* **1981**, *B37*, 2197–2205.

(25) Note that two values are given for C6, C13, and C15 of bR in Table III. Harbison et al.[5] were unable to assign these values to either the all-trans or 13-cis forms of the chromophore in dark-adapted bR. The correlations reported use the chemical shifts that yielded the highest correlations and lowest standard deviations.

Table III. Differences between Chemical Shift (ppm) of Bacteriorhodopsin[a] and PRSB–Butylimine with Different Counterions (CA) and Solvents

	solid state CA[b]				solution CA[c]				
					CD_3OD,	CCl_4			$CDCl_3$,
atom	Cl^-	Br^-	DCA	TCA	Cl^-	TCA	TFA	I^-	Cl^-
5	16.1	15.4	14.1	13.9	11.9	11.8	11.8	11.8	13.0
6	−3.4	−3.4	−4.0	−3.8	−4.2	−3.4	−3.5	−4.0	−2.0
6′	−3.9	−3.9	−4.5	−4.3	−4.7	−3.9	−4.0	−4.5	−2.5
7	0.7	1.0	−0.2	−2.0	−5.6	−6.3	−6.2	−6.2	−2.0
8	−8.1	−8.0	−6.0	−6.6	−7.2	−6.4	−6.4	−6.4	−4.2
9	4.3	3.8	2.4	3.3	−0.2	0.2	−0.2	−0.4	1.1
10	−2.0	−1.8	2.6	−2.9	0.8	1.3	1.3	1.2	3.5
11	0.2	−0.6	3.7	0.1	−0.5	−1.4	−1.4	−0.6	1.7
12	−0.7	−0.5	−1.1	−1.6	−0.4	1.1	1.1	0.9	0.7
13	7.2	6.3	8.7	8.2	5.2	3.7	3.5	2.1	6.7
13′	3.5	2.6	5.0	4.5	1.5	0.0	−0.2	−1.6	3.0
14	−0.5	1.3	0.9	0.3	−1.0	−0.3	−0.2	0.6	1.9
15	−3.8	−1.6	−1.7	1.2	−2.8	−5.3	−5.5	−5.1	−0.4
15′	−6.6	−4.4	−4.5	−1.6	−5.6	−8.1	−8.3	−7.9	−3.2

[a] Solid-state data for bR is from Harbison et al.[8] The atoms listed twice (e.g., 6 and 6′) are the results of both the 13-cis and the all-trans isomers of dark-adapted bR, correct assignment is not known. [b] Solid-state PRSB data is from Harbison et al.[7] [c] Solution PRSB data is from Inoue et al.[16] and Shiver et al.[15]

the PRSB data. (Significant means correlations of at least 0.8, where the correlation is not destroyed by the removal of a single data point, and slopes that are in the range of −100 to −200 ppm/electron.)

There are many ways to account for the λ_{max} of bR, ranging from a weak counterion to the more complex models such as those proposed by Spudich et al.[2] and Harbison et al.[8] In modeling bR no assumptions were made about the ring–chain conformation or the counterion distance. The "dihydro" data were initially ignored in order to determine what could be learned from the ^{13}C NMR data alone (sections A and B below). The dihydro data are then introduced in order to increase the constraints on the model.

(A) bR Modeled as PRSB with a Weakly Interacting Counterion and a Planar Ring–Chain Conformation. The simplest way to account for the 568-nm λ_{max} of bR is to assume a weakly interacting counterion and a planar ring–chain conformation. A planar ring–chain conformation permits the electrons of the ring to delocalize throughout the molecule, causing a red shift. The λ_{max} of bR could be reproduced with a counterion distance of 3.9 Å and either a planar s-cis or an s-trans ring–chain conformation. The planar ring–chain conformation also accounts for the large upfield shift of C8 in bR compared with PRSB. In the s-trans conformation the hydrogen of C8 interacts with the C1 methyls, while in the s-cis conformation this hydrogen interacts with the C5 methyl. Therefore in either conformation C8 should undergo a γ effect.

High correlation coefficients (>0.92) and low standard deviations (<2.0 ppm) can be obtained with this model, using PRSB with a counterion distance of 3.7 Å as a reference, however, the slope of the linear fit is very large (>−600 ppm/electron). This means that although a correlation between charge density differences and chemical shift differences is observed, the magnitude of the charge density differences are too small to account for the full chemical shift differences. The largest error occurs at C5. Using the standard coefficient of −160 ppm/electron, the charge density difference at this position between the planar s-trans model for bR and the model PRSB yields only a 3 ppm downfield shift. The actual downfield chemical shift difference at this position varies between 12 and 16 ppm depending on the PRSB data used in the comparison (see Table III). There must therefore be an additional perturbation in bR to account for this large chemical shift difference.

One possible explanation for the large downfield shift in C5 compared with PRSB is that the structure of the chromophore in the protein is distorted compared with the structure in solution. As discussed above for *s-cis*- and *s-trans*-retinoic acid and 13-*cis*-retinal, these distortions can lead to large chemical shift differences at this position. When C5 is left out, however, the slope of the linear fit reduces to only about −500 ppm/electron. Therefore, either the entire chromophore is highly distorted in the protein compared with solution or there are other (or additional) factors influencing the chemical shifts of the chromophore in the protein.

Another possible explanation for the shift on C5 is that there is a negative charge placed near C5 in bR.[8] This negative charge would reduce the electron density at this position relative to PRSB and thus produce a downfield chemical shift. This charge would also influence the other carbons in the molecule and could potentially lower the slope of the correlation of carbons 6–15 as well. It is worth noting that the dihydro data[2] also points to a negative charge near C5.

(B) bR Modeled with a Negative Charge Near C5. Bacteriorhodopsin was modeled as PRSB with a negative charge near C5 and a variety of ring–chain conformations, counterion distances, and locations of this second charge. The slopes of the charge density–chemical shift lines were sensitive to the position of the charge near C5. The configuration of charges that yielded the best agreement was the original bR model of Nakanishi et al.,[1] where the charge density differences were calculated relative to PRSB with a counterion 3.0 Å away. If C7 is excluded from the calculations, the correlation coefficient (0.96) and standard deviation (2.03 ppm) are comparable with the best model compound results. The slope (−126 ppm/electron) is also in the correct range. The charge density on C7 is, however, too positive (including C7 reduces the correlation coefficient to 0.89). This suggests, as first pointed out by Harbison et al.[8], that a positive charge may lie near C7 in bR, making its charge density more negative.

(C) bR Modeled with a Charge Pair Near the β-Ionone Ring. s-Cis Models. The dihydro data are easily accounted for with many different models that have an s-cis ring–chain conformation. It is necessary, however, to include both a negative charge near C5 and a positive charge near C7 to fit these data. The models include a counterion distance of 3.9 Å, a negative charge placed 3.2–4.3 Å from C5, a positive charge placed 3.0–4.2 Å from C7, and ring–chain angles between 20 and 45°. Many of these models, however, do not agree well with the ^{13}C NMR data. This is because the charge densities are very sensitive to the placement of the negative charge near C5. The models that place the negative charge at least 3.6 Å from C5 and the positive charge at least 3.8 Å from C7, however, yield good fits between the calculated charge density differences and chemical shift differences (correlation coefficients ~0.95, standard deviations ~2.3 ppm, and slopes ~−125 ppm/electron). These results are comparable with our model compound results (see Table II and Figure 1, parts a and c).

The s-cis model that best fits both the dihydro data and the ^{13}C NMR data (see Table IV) is illustrated in Figure 2a. The

Table IV. Comparison of the Results of the s-Cis and s-Trans Models of Bacteriorhodopsin (λ_{max} in nm, Opsin Shifts in cm^{-1}, Standard Deviations (SD) in ppm, and Slope in ppm/Electron)

	exptl[a]	s-cis	s-trans
all-trans	567	571	572
opsin shift	4830	4800	4840
5,6-H_2	484	487	483
opsin shift	2540	2450	2280
7,8-H_2	440	437	435
opsin shift	2780	2630	2520
corr		0.99	0.98
SD		0.82	1.15
slope		-125	-134
y_{int}		-1.3	-1.1

[a] Data from Spudich et al.[2]

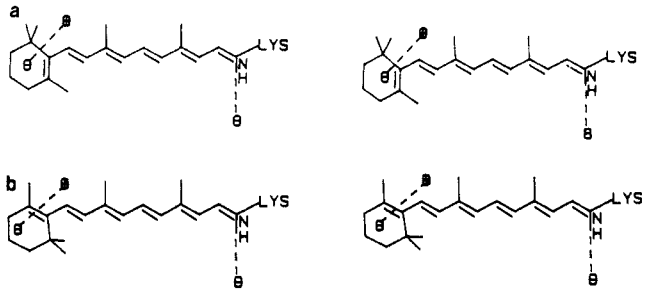

Figure 2. s-Cis (a) and s-trans (b) models for the chromophore and its binding site in bR. These models optimally account for both the dihydro data of Spudich et al.[2] and all the ^{13}C NMR data of Harbison et al.[8] The exact location of the charges is described in the text. Stereo pairs are provided in the figure.

corresponding correlation plot of charge density differences versus chemical shift differences is shown in Figure 3. The model of Figure 2a has a ring–chain angle of 25° and a counterion 3.9 Å from the Schiff base nitrogen. The negative charge by the ring is 3.85 Å from C5 and 3.75 Å from C6. The angle between this charge, C5, and C6 is 75° and the dihedral angle between this charge, C5, C6, and C7 is 130°. This places the charge above the ring portion of the molecule. The positive charge is 3.3 Å from the negative charge and 4.0 Å above both C6 and C7. The best results derived with the model were obtained when comparing the chemical shifts of bR with (1) PRSB in CCl_4 (the different counterions yielded indistinguishable results; the results shown in Table IV and Figure 3 use PRSB with a TFA counterion) and using a counterion distance of 3.7 Å for the charge densities of PRSB and (2) PRSB in CD_3OD and a counterion distance of 3.5 Å for the charge densities of PRSB. (These data also yielded the highest correlation coefficients for the comparison of PRSB and RSB.) The resulting correlation coefficients are all 0.99, the standard deviations average 0.89 ppm, the slopes average −125 ppm/electron, and the y_{int}'s average −1.4. It should be noted that comparable results can be obtained with any ring–chain angle greater than or equal to 25°, as long as the upfield shift of C8 can be attributed to a γ effect (see below).

These results do not include C8 in the statistics. The charge density difference on C8 is too positive to account for the full 6.4 ppm upfield shift of this carbon in bR relative to PRSB. The upfield shift on C8 may be due to a γ effect resulting from a steric interaction between the methyl group on C5 (C18) and the hydrogen on C8. This is consistent with the use of a smaller ring–chain angle than is found in solution. In this regard it is worth noting that the energy of retinal with a ring–chain angle of 25° is approximately equal to retinal with an s-trans ring–chain conformation.[12] Therefore, a γ effect on C8 of the same magnitude as found in *s-trans*-retinoic acid and 13-*cis*-retinal (i.e., about 6–8 ppm[7]) is expected for this position. The charge density difference on C8 accounts for a 1.3 ppm upfield shift, leaving a γ effect on 5.1 ppm. Thus, a ring–chain angle of 25° is consistent with the magnitude of the γ effect. The ring–chain angle of 25° should not, however, be taken too literally. Any ring–chain angle

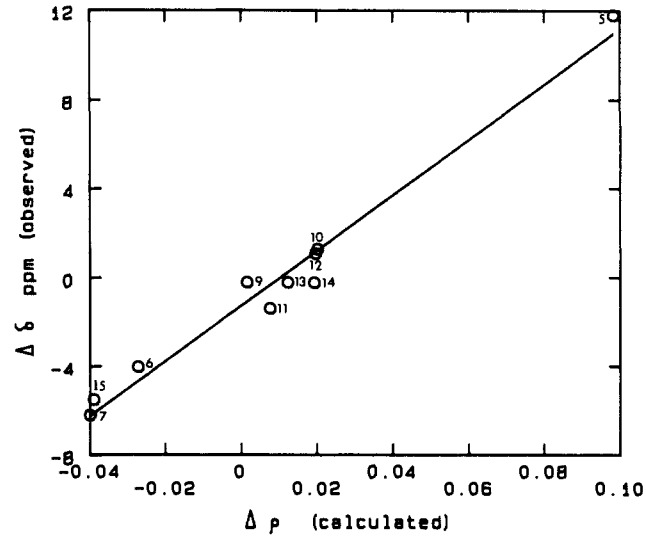

Figure 3. Correlation plot of calculated charge density differences $\Delta\rho$ of the bR model of Figure 4 and PRSB with a counterion 3.7 Å from the Schiff base nitrogen versus chemical shift difference $\Delta\delta$ of bR[5] and PRSB in CCl_4 with a TFA counterion.[18] The chemical shift differences used in this correlation for C6, C13, and C15 are the ones labeled 6′, 13′, and 15 in Table III. The results show a correlation of 0.99, standard deviation of 0.82 ppm, slope of −125 ppm/electron, and a y_{int} of −1.3.

somewhat lower than about 40° (the smallest ring–chain angle found in crystals of *s-cis*-retinal analogues[23]) would be expected to cause a γ effect on C8. This can be seen by noting that the closest distance between the hydrogen on the C1 methyl groups and the hydrogen on C8 in *s-trans*-retinoic acid is 2.1 Å, while the distance between the hydrogens on the C5 methyl and the hydrogen on C8 in *s-cis*-retionic acid (which has a ring–chain angle of 42°) is 2.4 Å.[22]

s-Trans Models. In contrast to the ease in which models with an s-cis ring–chain conformation can be derived, it is very difficult to find models that satisfy the dihydro data with an s-trans ring–chain conformation. The difficulty stems from the fact that the λ_{max} of *s-trans*-PRSB is shifted about 1000 cm^{-1} to the red of the λ_{max} of the model *s-cis*-PRSB. This red shift adds to any shifts that result from the placement of charges about the chromophore as required to fit the dihydro data and the ^{13}C NMR data. For example, placing the counterion and the charge near C5 is positions identical with those of the original point charge model of bR (Nakanishi et al.[1]), but making the ring–chain conformation s-trans with a ring–chain angle of 170°, results in a λ_{max} of 610 nm, which is 1200 cm^{-1} to the red of the λ_{max} of bR. This difficulty is compounded when a weakly interacting counterion, which is necessary to reproduce the λ_{max} of the 7,8-dihydro pigment, becomes part of the model. The basic problem is one of finding a placement of charges that does not overestimate the λ_{max} of the full chromophore while not underestimating the λ_{max} of the dihydro chromophores.

The first s-trans model considered was that of Spudich et al.[2] This model has a planar s-trans ring–chain conformation, a negative charge 3.0 Å from C5, and a positive charge above C1 of the β-ionone ring. The negative charge in this model is very close to C5, causing this atom to become very positive. The resulting chemical shift difference on this carbon is 30–60 ppm, using a range of coefficients of −100 to −200 ppm/electron. Likewise, the calculated chemical shift differences of all the carbons near the ring show large deviations from those observed. For example, the calculated chemical shift of C6 is −10 to −20 ppm and C7 is 6 to 13 ppm as compared with the measured chemical shift differences of −3 to −5 ppm and −6 to +1 ppm, respectively (see Table III). Moving the positive charge, which lies above C1, closer to C7 reduces the error in the calculated chemical shifts. However, the resulting model no longer fits the dihydro data. For example, placing the positive charge nearer C7 causes large blue shifts in the 5,6- and 7,8-dihydro pigments.

It is clear from these considerations that the point charges in this model do not produce the proper range of charge densities needed to fit ^{13}C NMR data.

After extensive testing it was found that only one satisfactory model with a planar s-trans ring–chain conformation could be found. As is the case for the model of Spudich et al.,[2] the fit to the dihydro data is very sensitive to the exact position of the charges. The new model does, however, give excellent agreement with the NMR data (see Table IV). This s-trans model has an arrangement of charges very similar to that of the s-cis model discussed above (see Figure 2b); the differences being that the ring–chain angle is 170° (the middle of the range of ring–chain angles found in s-trans crystal structures[23]), the negative and positive charges are both 0.1 Å further from C6, and the distance between the positive and negative charges by the ring is 3.8 Å. As can be seen in Table IV, excellent agreement with the data is also obtained with this model. This model shows a slightly smaller correlation coefficient and a larger standard deviation than the s-cis model. This difference is the result of small deviations of several carbons from the linear fit, rather than a large deviation in a single carbon. This s-trans model also has a slightly larger λ_{max} than the s-cis model but smaller λ_{max}'s for the dihydro pigments.

Discussion

The models presented in Figure 2a,b are similar to the model previously proposed by Harbison et al.,[8] which was based solely on ^{13}C and ^{15}N NMR, and the model of Spudich et al.,[2] which was based on dihydro data and the conclusions of Harbison et al.[8] concerning the s-trans ring–chain conformation. Each of these models include a weakly interacting counterion and an ion pair near the β-ionone ring. It is important to emphasize that these dual requirements can be deduced independently from either the NMR or dihydro data. The most important new features of the models proposed in this work is that they satisfy both the NMR and dihydro data simultaneously and that the NMR data are now accounted for in a quantitative fashion. Developing models consistent with both sets of data was the major goal of this work. An additional feature introduced in this work is that we have allowed for the possibility that the ring–chain conformation is s-cis. Since this constitutes a deviation from current thinking, it is important to justify our retention of the possibility that the ring–chain conformation in bR is s-cis model in some detail.

The s-trans assignment of the ring–chain conformation in bR is based on the NMR measurements of Harbison et al.[7,8] and the interpretation as given by these workers. The original NMR evidence pointing to the s-trans assignment is based on a comparison between the chemical shift tensor of C5 in bR and the chemical shift tensor of C5 in both *s-cis* and *s-trans*-retinoic acid. Harbison et al.[8] noted that the σ_{33} component of the chemical shift tensor of C5 in bR is shifted downfield from the σ_{33} component of C5 in *s-cis*-retinoic acid by about 20 ppm. The σ_{33} component of C5 in bR is, however, quite close to σ_{33} of C5 in *s-trans*-retinoic acid, thus suggesting that bR has an s-trans ring–chain conformation.

There are, however, a number of difficulties with this assignment. First, conclusions drawn for bR, which contains a protonated Schiff base, based on model compounds that contain acid, aldehyde, or unprotonated Schiff base substituents at C15 are suspect, since it is necessary to assume that the substituent at C15 could not be responsible for the chemical shift difference at C5. The justification for this assumption is that ten C–C bonds intervene between the two atoms.[8] However, in some cases, the chemical shift of C5 is, in fact, sensitive to changes in the C15 substituent. For example, there is a −3 ppm change in C5 in going from *s-cis*-RSB to *s-cis*-retinal. This downfield shift is due *entirely* to the σ_{33} component of the chemical shift tensor, which shifts by −14 ppm.[7] In this case, it is clear that the σ_{33} component of the chemical shift tensor of C5 is quite sensitive to substituent effects at C15.

A second problem with using σ_{33} as a marker for the ring–chain conformation is, as discussed above, that the source of the upfield shift in the s-trans structure is unknown. It could, for example, be due to twisting about C5=C6, a distortion that would be expected to occur in near-planar s-cis conformers as well. This highlights the serious uncertainty associated with identifying a single element of the chemical shift tensor with a particular molecular property. Moreover, the σ_{22} component of C5 is shifted in bR relative to *s-cis*- and *s-trans*-retinoic acid by 30 ppm. Harbison et al. attribute this shift to a charge near C5, thus invoking different sources for the shifts in σ_{22} and σ_{33}. However, given the large shift in σ_{22} due to a charge it is unlikely that σ_{33} be totally unaffected by the same interaction. Comparing, for example, the tensor elements of *all-trans*-retinal and various unprotonated Schiff bases (Table I in Harbison et al.[7]), for the carbons where there is a large change in chemical shift (>5 ppm), *both* σ_{22} and σ_{33} are affected by the change in substituent at C15. In general, it appears that using individual elements of the chemical shift tensor as a basis for assignments may have to await a more through theoretical understanding of the factors that contribute to each of the elements.

The second piece of evidence for an s-trans conformation in bR comes from the large (7 ppm) upfield shift of C8 in bR relative to PRSB. As mentioned above, upfield shifts of this magnitude are indicative of γ effects. There is usually no γ effect at C8 in retinal analogues because the ring–chain conformation is usually a twisted s-cis conformation with a ring–chain angle greater than 40°.[24] Consequently, there is no interaction between the hydrogen on C8 and the methyl groups on the β-ionone ring. Harbison et al.,[7] however, pointed out that an upfield shift in C8 of 8 ppm is observed in going from *s-cis*- to *s-trans*-retinoic acid. In *s-trans*-retinoic acid the hydrogen on C8 interacts with the methyls on C1 (i.e., C16 and C17). Therefore the upfield shift in C8 of the chromophore in bR is consistent with an s-trans ring–chain conformation. However, a γ effect on C8 can occur as well in an s-cis form with a smaller ring–chain angle than is found in solution. In this conformation the hydrogen on C8 would interact with the methyl group on C5 (C18). Therefore, although the aforementioned data indicate a γ effect on C8, they do not distinguish between s-cis and s-trans conformations.

The final piece of evidence for an s-trans ring–chain conformation in bR is the long T_1 of C18 in bR. Harbison et al.[8] measured the T_1 of the methyls in several s-cis and s-trans isomers of retinal analogues and demonstrated that long T_1's are due to steric interactions between the methyls and the adjacent hydrogens on the conjugated chain. Indeed, in all cases where there are short hydrogen–hydrogen contacts in the crystal structures (less than 2.3 Å), the T_1's are long. Since C18 in bR has a long T_1, Harbison et al.[8] suggested that in bR, in analogy to s-trans model compounds, C18 interacts strongly with the proton on C7. However, it is also possible that the long T_1 of C18 in bR is caused by an s-cis conformation with a small ring–chain angle. In this case C18 should interact strongly with the proton on C8, which should also produce long T_1's. As discussed above, this same interaction could account for the γ effect on C8. In summary, although the work of Harbison et al.[7,8] clearly demonstrates that the ring–chain angle in bR is smaller than is found in solution, our analysis indicates the NMR data cannot unambiguously distinguish between the s-trans and s-cis conformers.

However, a recent model compound study by van der Steen et al.[10] does support the suggestion of an s-trans ring–chain conformation in bR. In this work, locked *s-cis*- and *s-trans*-retinals were incorporated into bR. Both analogues produced pigments with opsin shifts of 3800 cm^{-1}; however, the locked s-trans compound formed more rapidly than the s-cis compound (at a rate 50% lower than retinal) and had a higher pumping efficiency. On the other hand, the fact that both s-cis and s-trans conformers did form pigments with large opsin shifts renders the interpretation of these results as suggestive rather than conclusive. Moreover, if the ring and chain in bR are not coplanar, conclusions based on planar-locked s-cis and locked s-trans analogues could turn out to be misleading.

Thus, while available data favor the existence of an s-trans conformation in bR, in our view the s-cis conformer cannot be

definitively ruled out. For this reason, we have presented both s-cis and s-trans models in Figure 2.

Summary

The models illustrated in Figure 2a,b for the chromophore and its protein environment in bR are the ones that optimally fit both the dihydro and all the ^{13}C NMR data. The essential features of these model are (1) a weak counterion to reproduce the large opsin shift of 7,8-dihydro-bR, (2) a negative charge near C5 to reproduce the opsin shift of the bR and the large downfield chemical shift of C5 relative to PRSB, (3) a positive charge near C7 to depress the opsin shift of 5,6-dihydro-bR relative to 7,8-dihydro-bR and native bR and to account for the upfield chemical shift of C7 in bR relative to PRSB, and (4) a ring-chain conformation that has a ring methyl in contact with the hydrogen on C8 to produce a γ effect on C8, accounting for its large upfield chemical shift. Both the s-cis and the s-trans configurations about the ring produce excellent agreement with the experimental data.

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Registry No. *all-trans*-RET, 116-31-4; 5,6-H_2-RET, 11907-28-9; 7,8-H_2-RET, 75917-44-1; 9,10-H_2-RET, 72535-17-2.

Bacterial Organomercurial Lyase: Novel Enzymatic Protonolysis of Organostannanes†

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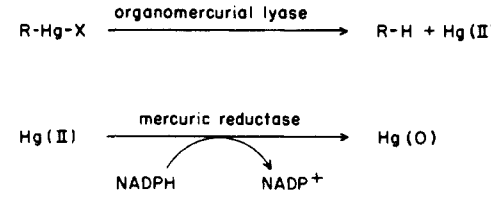
Abstract: Pure bacterial organomercurial lyase has been found to catalyze a protonolytic cleavage of the carbon-tin bond in certain organostannanes. Of the compounds tested tetravinyltin is turned over with the highest specific activity, yielding ethylene as the organic product. Similarly, triethylvinyltin undergoes turnover by the lyase to yield ethylene and ethane in a 97:3 ratio, at $^1/_{60}$th the rate of tetravinyltin turnover. Finally, tetramethyltin and trimethyltin fluoride yield small amounts of methane (2-5 turnovers/mol of enzyme) prior to eventual loss of enzyme activity. The decrease in activity observed during turnover of the organostannanes is consistent with the observed inhibition of the enzyme by dimethyltin dibromide.

Bacterial organomercurial lyase catalyzes a remarkable protonolytic cleavage of the carbon-mercury bond in organomercurials.[1] This reaction constitutes the first detoxification step of the "broad spectrum" bacterial mercury resistance pathway, yielding inorganic Hg(II) and the corresponding hydrocarbon RH (Scheme I). The detoxification sequence is completed in a second step by the flavoenzyme mercuric reductase, which effects reduction of Hg(II) to Hg(0). The Hg(0) subsequently evaporates from the cellular microenvironment, thereby completing removal of the mercury. Recent reports from this laboratory have described purification of these two enzymes and elucidation of the mechanisms by which they carry out their transformations.[2,3]

The widespread use of organometallic compounds as antimicrobial agents and the resulting ubiquitous environmental distribution of these compounds are well documented.[4,5] Organomercurials have found extensive use as bactericides, fungicides, and slimicides, and a range of organostannanes have been developed for use as marine antifouling agents, wood preservatives, polymer stabilizers, germicides, and fungicides. Mechanisms for microbial resistance to these compounds have evolved[6-10] and constitute a challenge to the development of new organometal-based antimicrobials. While the details of microbial degradation of organomercurials are fairly well understood, less is known about the corresponding degradation of organostannanes. For example, bacterial and fungal degradation of tributyltin oxide to give mono- and dibutyltin compounds has been noted, although the mechanism of this degradation has not been determined.[9,10]

In order to test our hypothesis that the enzymatic C-Hg bond cleavage that confers bacterial resistance to organomercurials may be paradigmatic for microbial detoxification of other organometallic compounds, we have screened a number of compounds possessing a carbon-metal bond as substrates for pure organomercurial lyase. We report here our finding that certain organostannanes are indeed substrates for the lyase, resulting in an apparent protonolytic cleavage of the carbon-metal bond. The specificity of the enzyme for organostannanes is significantly

Scheme I



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(1) (a) Summers, A. O. *Trends Biotechnol.* **1985**, *3*, 122. (b) Robinson, J. B.; Tuovinen, O. H. *Microbiol. Rev.* **1984**, *48*, 95. (c) Silver, S.; Kinscherf, T. In *Biodegradation and Detoxification of Environmental Pollutants*; Chakrabarty, A. M., Ed.; CRC: Boca Raton, FL, 1982. (d) Summers, A. O.; Silver, S. *Annu. Rev. Microbiol.* **1978**, *32*, 637.
(2) (a) Begley, T. P.; Walts, A. E.; Walsh, C. T. *Biochemistry* **1986**, *25*, 7186. (b) *Ibid.*, **1986**, *25*, 7192.
(3) (a) Schultz, P. G.; Au, K. G.; Walsh, C. T. *Biochemistry* **1985**, *24*, 6840. (b) Fox, B. S.; Walsh, C. T. *Biochemistry* **1983**, *22*, 4082. (c) Fox, B. S.; Walsh, C. T. *J. Biol. Chem.* **1982**, *257*, 2498.
(4) Evans, C. J.; Karpel, S. *J. Organomet. Chem. Lib.* **1985**, *16*, 258.
(5) Thayer, J. S. *Organometallic Compounds and Living Organisms*; Maitlis, P. M., Stone, F. G. A., West, R., Eds.; Academic: Orlando, FL, 1984; Chapter 10.
(6) Reference 5, Chapters 5, 9.
(7) Traxler, R. W.; Wood, E. M. *Dev. Ind. Microbiol.* **1980**, *22*, 521.
(8) Hallas, L. E.; Cooney, J. J. *Dev. Ind. Microbiol.* **1980**, *22*, 529.
(9) Barug, D. *Chemosphere* **1981**, *10*, 1145.
(10) Maguire, R. J.; Wong, P. T. S.; Rhamney, J. S. *Can. J. Fish. Aquat. Sci.* **1984**, *41*, 537.

0002-7863/88/1510-1950$01.50/0