Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 1706–1709, March 1984
Biophysics

Dark-adapted bacteriorhodopsin contains 13-*cis*,15-*syn* and all-*trans*,15-*anti* retinal Schiff bases

(**^{13}C solid-state NMR/magic-angle sample spinning/γ effect/ketimine/geometrical isomerism**)

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Communicated by John S. Waugh, November 28, 1983

ABSTRACT **^{13}C NMR spectra of lyophilized dark-adapted [14-^{13}C]retinyl-labeled bacteriorhodopsin show a large anomalous upfield shift for the ^{13}C-14 resonance assigned to the 13-*cis* isomer, relative to both the all-*trans* isomer and model compounds. We attribute this to the so-called γ effect, which results from a steric interaction between the C-14 retinal proton and the protons on the ε CH_2 of the lysine. As a consequence of this observation, we infer that dark-adapted bacteriorhodopsin is composed of a mixture of all-*trans*,15-*anti* (*trans* or *E*) and 13-*cis*,15-*syn* (*cis* or *Z*) isomers. These occur in an approximate 4:6 ratio and are commonly identified as bR_{568} and bR_{548}. This conclusion is based on an examination of the isotropic and anisotropic chemical shifts and a comparison with ^{13}C shifts of the carbons adjacent to the C=N linkage in protonated ketimines. Other possible origins for the anomalous shift are examined and shown to be insufficient to account for either the size of the shift or the nature of the shift tensor. We discuss the consequences of this finding for the structure and photochemistry of bacteriorhodopsin.**

Bacteriorhodopsin (bR), the single protein of the purple membrane (PM) of *Halobacterium halobium* (1), has been the subject of considerable experimental scrutiny for some time. Like rhodopsin (2), it contains as its chromophore the polyene aldehyde retinal, connected via a Schiff base linkage to the ε-amino group of a lysine side chain (3). Although bR appears to resemble visual pigments (4), its function is quite different. In response to the absorption of light, this protein undergoes a photocycle during which protons are pumped across the cell membrane (5).

Isomerization about carbon–carbon double bonds of retinal plays an important and pivotal role in the photocycle of bR (6–8). The presence of 13-*cis*-retinal in both dark-adapted bR_{548} and the photointermediate M_{412} was demonstrated by Pettei *et al.* (6). Since then, indirect (7) and direct (8) experiments have indicated that the photointermediate K_{625} also has a 13-*cis* chromophore. On the other hand, on the basis of Raman spectroscopy evidence, the chromophore in the photointermediate O_{640} was recently shown to be all-*trans* (9), as it is in light-adapted bR_{568} (6).

It has been appreciated for many years that C=N bonds can also exhibit geometrical isomerization (10). In particular, Schiff bases of both ketones (11) and aldehydes (12, 13) can exist as both *syn* (i.e., *cis* or *Z*) and *anti* (i.e., *trans* or *E*) isomers with lifetimes of several weeks at room temperature (13). *syn* isomers of aldimines, while being energetically less favored than *anti* isomers, can be produced photochemically by UV irradiation (12) and occur naturally for certain highly substituted aromatic aldimines (13). However, with one exception (14), the configuration of the C=N Schiff base linkage in bR has not been considered. In addition, the interesting possibility that isomerization about this bond might occur during the photocycle has been largely overlooked.

The inattention to this important question can be attributed in part to the absence of an experimental means to discriminate definitively between *syn* and *anti* isomers in bR. In a recent paper (15) we demonstrated that high-resolution solid-state ^{13}C NMR is a potent means of establishing configuration about C=C bonds in bR. Here, we present evidence that it is equally effective in determining the C=N bond configuration and evinces that dark-adapted bR contains all-*trans*,15-*anti* and 13-*cis*,15-*syn* isomers in an approximately 4:6 ratio. The implications of this finding for both the structure of the chromophore and the bR photocycle will also be discussed.

MATERIALS AND METHODS

^{13}C-14-labeled retinal was prepared by the method of ref. 16 and incorporated into white membrane as described (8, 15). The reconstituted PM was then lyophilized at 0.1 mm Hg (1 mm Hg = 133 Pa) and packed into a Kel-F rotor of the Andrew–Beams design (17). ^{13}C magic-angle sample spinning (MASS) spectra were obtained at various spinning frequencies between 1.9 and 3.2 kHz, with a ^{13}C frequency of 79.9 MHz. Typically, $(\omega_1/2\pi) = 50$ kHz was used for cross-polarization. Subsequently, the magnetization was sampled in the presence of ^{1}H decoupling fields of 125 kHz. Usually, 15,000 transients were accumulated, with a recycle delay of 2.5 sec. Isotropic chemical shifts were referenced to external tetramethylisilane (Me_4Si). Chemical shift tensors (σ) were calculated from several spectra taken at different spinning speeds, by the method of Herzfeld and Berger (18), using the computer program described in their publication.

N-Retinylidene butylimmonium bromide and other retinal Schiff base salts were prepared as described (19). Isopropylidene propylimine and 3-pentylidene propylimine were prepared by the method of Weingarten *et al.* (20). 2,4-Heptadien-6-one was synthesized essentially according to Heilbron *et al.* (21). Its butylamine ketimine was prepared by azeotropic distillation in pentane and rendered pure by three vacuum distillations: bp 59–63°C at 0.2 mm Hg. Purity was checked by thin-layer chromatography and ^{1}H NMR. The compound exists as a mixture of *anti* and *syn* isomers in an approximately 5:1 ratio. Ketimines were protonated in 10% solution in $C^2H_2Cl_2$, using a 10% excess of trifluoroacetic acid. ^{1}H and ^{13}C high-resolution NMR spectra were obtained immediately with a commercial Bruker 270-MHz spectrometer and assigned by using narrow-band decoupling and selective nuclear Overhauser effect methods.

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Abbreviations: bR, bacteriorhodopsin; PM, purple membrane; MASS, magic-angle sample spinning; Me_4Si, tetramethylsilane.

RESULTS

Spectra a and b in Fig. 1 show the downfield part of the ^{13}C MASS spectra of [14-^{13}C]retinyl-PM, in its lyophilized, dark-adapted state, obtained at rotation frequencies of 3.10 and 1.99 kHz, respectively. The sharp ($\Delta\nu_{1/2} \approx 30$ Hz) pair of lines from the labeled retinyl moiety is quite apparent; the broader resonances in the spectra derive from natural-abundance ^{13}C at carbonyl and aromatic sites in the apoprotein. Both retinyl and apoprotein signals consist of centerbands (arrowed) flanked by a series of rotational sidebands spaced at integral multiples of the rotation frequency (18, 22). The increased sideband intensities apparent in the slower spinning case reflect the less complete averaging of the chemical shielding tensor.

We assign the upfield line of the retinyl pair at 110.5 ppm to the 13-*cis* isomer and the downfield line at 122.0 ppm to the all-*trans* isomer on the following grounds. First, the upfield line is markedly more intense than the downfield line, in accord with Pettei *et al.* (6), who showed a 6:4 ratio of 13-*cis* to all-*trans* isomers in dark-adapted PM, and with Korenstein and Hess (23), who showed dark adaptation to be unaffected by drying. Moreover, the results are consistent with our previous ^{13}C NMR results, which demonstrated the presence of this ratio of 13-*cis* to all-*trans* isomers in several other ^{13}C-labeled PM samples prepared in an identical fashion.

Second, lyophilization of light-adapted [14-^{13}C]retinyl-PM in intense light caused no more than a transient increase in the intensity of the downfield line, with the isomer ratio becoming equal to that of dark-adapted within 12 hr. This allows us to discount the possibility that partial light adaptation might be "frozen" in by lyophilization. Third, when retinals are extracted from the dark-adapted lyophilized material at 0°C by the method of Pettei *et al.* (6) and thin-layer chromatography is performed (silica gel plate, 30% diethyl ether in hexane), 13-*cis*-retinal is clearly seen to be present in larger quantity than all-*trans*. Thus, all our results are consistent with the assignment of the upfield line to the 13-*cis* isomer.

Spectrum c in Fig. 1 is the ^{13}C MASS spectrum of the bromide salt of *N*-retinylidene butylimine. The ^{13}C-14 resonance (arrowed) can be identified by its characteristic isotropic chemical shift of 120.7 ppm, which is quite close to the 122-ppm line assigned to the all-*trans* species in bR.

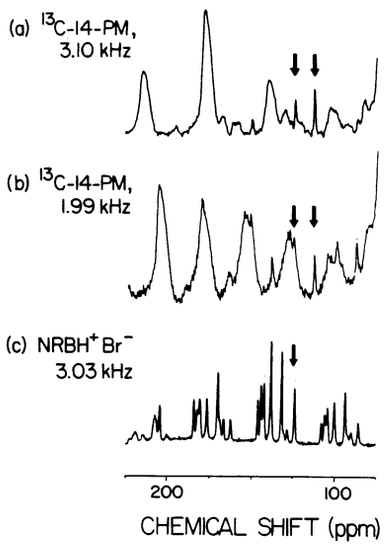

FIG. 1. ^{13}C MASS spectra of lyophilized dark-adapted bR, at 3.10-kHz rotation frequency (spectrum a); lyophilized dark-adapted bR at 1.99-kHz rotation frequency (spectrum b); and crystalline *N*-all-*trans*-retinylidene butylimmonium bromide, at 3.03-kHz rotation frequency (spectrum c). The arrows denote the ^{13}C-14 centerbands in each spectrum.

Table 1. Isotropic chemical shifts of retinal Schiff bases

Schiff base	Shift, ppm σ_I (^{13}C-14)	Shift, ppm σ_I (^{15}N)*
Lyophilized dark-adapted bR		
13-*cis*	110.5	144.9
All-*trans*	122.0	151.6
N-Retinylidene propyliminium trifluoroacetate, solution		
13-*cis*	118.4	
All-*trans*	119.8	
All-*trans* *N*-retinylidene butylimmonium salts, solid		
Chloride	122.6	171.7
Bromide	120.7	166.1
Dichloroacetate	121.7	174.7
Trichloroacetate	121.1	154.2

^{13}C and ^{15}N chemical shifts of dark-adapted bR and model compounds were measured relative to Me_4Si (^{13}C) or 5 M NH_4Cl in H_2O (^{15}N).
*Taken from refs. 19 and 24.

In Table 1 we compare the isotropic chemical shifts measured for [14-^{13}C]retinyl-bR with the isotropic shifts of protonated retinal Schiff bases in solution and in the solid state. Also included in this table are the ^{15}N isotropic chemical shifts for bR and for the solid all-*trans* butylimmonium salts. These data will be used to establish that the large upfield shift for the ^{13}C-14 position is not due to isomerization at the C-13, C-14 double bond or to counterion effects. In Table 2 are compiled the ^{13}C-14 anisotropic shifts for dark-adapted bR and for *N*-retinylidene butylimmonium bromide. The changes in these components provide additional evidence that C=N isomerization is the principal cause of the change in the ^{13}C-14 isotropic shift. For further comparison, Table 3 lists the ^{13}C solution chemical shifts of several protonated ketimines, prepared and assigned as described above, for the position adjacent to the C=N bond and both *syn* and *anti* to the alkylimine residue. These results demonstrate the effect of isomerization on the ^{13}C chemical shifts.

DISCUSSION

The difference between the isotropic chemical shifts of the 13-*cis* and all-*trans* isomers in dark-adapted [14-^{13}C]retinyl-labeled-PM is the largest observed in any position yet studied by us in the protein (ref. 15; unpublished results). We shall argue that a *syn* configuration about the C=N bond in the 13-*cis* isomer is both necessary and sufficient to account for the large upfield shift at the C-14 position in this isomer. Therefore, we first examine other possible sources for the phenomenon, and we show that they are incapable of accounting for either the size or the nature of the upfield shift.

Isomerization about the C-13, C-14 double bond itself does not give rise to large changes in the chemical shifts of the C-

Table 2. Chemical shift tensors for [14-^{13}C]retinal Schiff base

Schiff base	Shift, ppm σ_{33}	σ_{22}	σ_{11}
Lyophilized dark-adapted bR, 13-*cis*	186.8 ± 1.3	109.5 ± 0.5	35.2 ± 1.5
N-Retinylidene butylimmonium bromide	192.8 ± 1.0	123.2 ± 2.2	45.4 ± 1.2

^{13}C chemical shift tensor principal values were measured in bR and in a typical protonated retinyl Schiff base, relative to external Me_4Si; errors given are ±1 standard deviation, from several measurements.

Table 3. Solution chemical shifts of protonated ketimines

R	R'	Shift, ppm σ_{syn}	σ_{anti}	$\Delta\sigma$
CH_3	CH_3	20.7	26.4	5.7
CH_3CH_2	CH_3CH_2	25.6	30.7	5.1
CH_3	$CH_3CH{=}CHCH{=}CH$	14.8	122.6	5.8 (=CH—)
$CH_3CH{=}CHCH{=}CH$	CH_3	116.8	19.7	4.9 (CH_3)

^{13}C solution chemical shifts of the α carbons of several protonated *N*-butyl ketimines $RR'C{=}NC_4H_9$ were measured relative to internal Me_4Si, showing the change in chemical shift on going from *syn* to *anti* to the aklylimine residue. Note that the $\Delta\sigma$ values for 2,4-heptadien-6-ylidene are obtained by comparing shifts of the two $—CH_3$ groups and the two pentadienyl residues.

14 position. As shown in Table 1, the shift difference between the all-*trans* and 13-*cis* protonated Schiff bases in solution is a mere 1.4 ppm at the C-14 position, in accordance with Englert (25), who reported changes of 1–2 ppm for seven retinal derivatives on 13-*cis* isomerization. Similarly, isomerization about the C-9, C-10 and C-11, C-12 double bonds causes only small changes in the shifts of the carbons of the isomerized bond itself (25–27), about an order of magnitude too small to account for the change observed here.

The nature of the Schiff base counterion might also be expected to affect the isotropic shift of protonated Schiff bases at the C-14 position. We have already shown (19, 24) that substituting a weakly hydrogen-bonding counterion for a strongly hydrogen-bonding one can cause a change of over 20 ppm in the chemical shift of the Schiff base nitrogen in the solid state. We have therefore measured the chemical shifts at the ^{13}C-14 position for a series of protonated retinal Schiff base salts spanning the entire range of ^{15}N chemical shifts, with the results given in Table 1. It is apparent that, while there are large counterion-induced effects in the ^{15}N shifts there are only small effects observed at the ^{13}C-14 position, entirely inadequate to account for the magnitude of the upfield shift in bR.

Perhaps most significant, however, is the composition of the anomalous isotropic shift in the chemical shift tensor. As seen in Table 2, approximately 35% of the change in the isotropic shift arises from a change in the upfield principal value, σ_{11}. For double-bonded carbons, this tensor element is aligned perpendicular to the plane of the double bond (28). On theoretical grounds, this principal value is expected to be almost completely unaffected by shifts that are paramagnetic in origin—i.e., due to changes in the electron distribution in the conjugated system (29); only the in-plane tensor elements should be affected by such changes. This expectation is borne out by model-compound studies, which have shown that the large changes in isotropic shifts caused, for example, by protonation, are usually due to changes in σ_{22} and σ_{33} of the chemical shift tensor (unpublished results).

In contrast to the above, it is quite well documented that, in retinal derivatives, and in olefins and polyenes generally, double bond isomerization can create upfield chemical shift changes of the size observed in PM, but that such changes are experienced *one carbon down the chain* from the double bond in question. Specifically, Englert (25) shows upfield shifts of ≈6–8 ppm at C-12 on going from all-*trans* to 13-*cis* isomers in seven retinal derivatives, and at C-8 in the case of C-9, C-10 isomerization. Recently, we showed that the C-12 position of *N*-(13-*cis*-retinylidene)propyliminium trifluoroacetate has a shift 9.1 ppm upfield of the all-*trans* derivative (15), a shift actually larger than any observed by Englert. These upfield shifts are typical of those observed in simple and conjugated olefins (30) and are considered to be steric in origin (25), arising from a close contact between the proton bonded to the shifted carbons and that on the carbon three bonds distant across the *cis* linkages [the so-called γ effect (31)]. In the case of *syn–anti* isomerization about the C=N bond, the steric interaction should be between the C-14 proton and the ε protons of the lysine side chain (cf. Fig. 2), and should be comparable in magnitude to the interactions experienced within the conjugated chain itself.

To verify that *syn-anti* isomerization can actually cause a large chemical shift change on the carbon adjacent to the Schiff base linkage, we measured the ^{13}C chemical shifts of several protonated ketimines. As indicated in Table 3, we observed that the shift of the carbon adjacent and *syn* to the C=N bond is indeed 5–6 ppm upfield of the *anti* carbon in immonium salts of this sort. Thus, a 6:4 mixture of 13-*cis*,15-*syn* and all-*trans*,15-*anti* isomers can in fact account for the isotropic chemical shifts observed in dark-adapted PM. It also accounts for the shift tensor observed for the 13-*cis* isomer. Steric interactions influence chemical shifts via a diamagnetic shielding mechanism, and thus can be expected to affect σ_{11}, as well as σ_{22} and σ_{33}. We have already observed this phenomenon for the C-12 position in the 13-*cis* isomer of dark-adapted PM, in which σ_{11} is considerably upfield of its value in the all-*trans* derivative (16). The C-14 tensor reported here shows similar behavior of σ_{11} and thus provides additional strong evidence for a 15-*syn* configuration.

What are the consequences of this isomerization for the structure and function of PM? First, the 15-*syn* bond in the 13-*cis* isomer allows the retinal moiety to be accommodated in an approximately linear binding site of about the same dimensions as is necessary for the all-*trans*,15-*anti* isomer. It can be seen in Fig. 2 that a single *cis* double bond would introduce an ≈60° bend in a conjugated chain; the *syn* linkage removes the bend, while shortening the chain and shifting it somewhat along its length. Thus, there is no requirement for a large change in the protein conformation between dark- and light-adapted bR. Our previous NMR results for carbons in the middle of the conjugated chain indeed show consistent small upfield chemical shifts in the 13-*cis* isomer (15), in accord with its having a somewhat different relation-

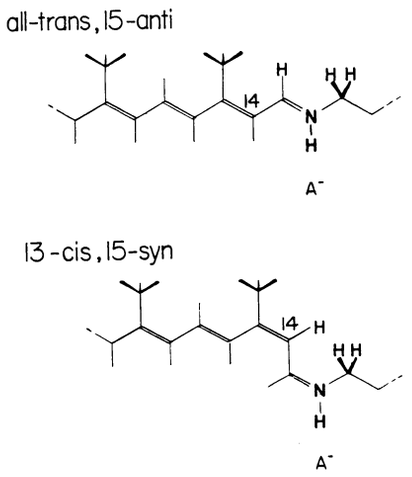

FIG. 2. Conformation of the retinal Schiff bases in dark-adapted bR.

ship with amino-acid side chains of the apoprotein. Significantly, the change in configuration of the C=N bond from *anti* to *syn* permits the relationship between Schiff base and its counterion in the all-*trans* isomer to be preserved in the 13-*cis* isomer. Thus, we observed essentially identical ^{15}N chemical shifts for both isomers in spectra of dark-adapted bR (19).

The presence of energetically unfavorable *cis* and *syn* double bonds at equilibrium in dark-adapted PM makes it clear that the binding site fits a 13-*cis*,15-*syn* isomer rather better than it fits the all-*trans*,15-*anti* version. This suggests that during the photocycle it might have the function of directing a 15-*anti*–*syn* isomerization at some suitable point. So far we have not obtained MASS spectra of bR photointermediates. Fortunately, subsequent analysis of the resonance Raman results (31) has shown that vibrational spectroscopy can also be used to discriminate between *syn* and *anti* isomers of the bR Schiff base (32). Besides providing completely independent corroboration of the present results, the latter study also reveals that the K_{625}, L_{550}, and O_{640} intermediates in the photocycle also possess a 15-*anti* configuration. However, it is still possible that M_{412} or some short-lived intermediate between M_{412} and O_{640} might have a *syn* configuration. We anticipate that when the *complete* chemical state of the photointermediates is characterized, clarification of the mode of operation of the proton pump will be considerably more straightforward.

The authors thank Dr. D. Ruben and Dr. J. E. Roberts for technical assistance, Dr. S. K. Das Gupta for valuable discussions, and Ms. V. Elms for preparation of the manuscript. This research was supported by the U.S. National Institutes of Health (GM-22316, GM-23289, EY-02051, and RR-00995), the U.S. National Science Foundation (DMR-8216959 and CHE-8116042), the Netherlands Foundation for Chemical Research (SON), and the Netherlands Organization for the Advancement of Pure Research (ZWO). J.H. and R.M. are recipients of an American Cancer Society Faculty Research Award and a National Institutes of Health Research Career Development Award, respectively.

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