nosine and two cytidine residues is GC:GC is more stable than GG:CC which is more stable than CG:CG.

(2) The greater stability of a GC:GC core over a CG:CG core can be outweighed by the stabilization imparted by four 3′ double-dangling bases.

(3) The application of the RY model for prediction of RNA secondary structure stability to the tetramers studied was reasonably successful. Base composition of the interstrand stacks is obviously important and is a logical extension of the model.

Registry No. GGCC, 56399-78-1; CCGG, 55048-62-9; GCGC, 89873-22-3; CGCG, 89873-23-4; GCCG, 73942-16-2; CGGC, 89435-89-2; GCC, 3184-24-5; CCG, 3960-32-5; CGGA, 67147-82-4; AGGC, 56399-93-0.

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Solid-State ^{13}C NMR Studies of Retinal in Bacteriorhodopsin†

Gerard S. Harbison, Steven O. Smith, Johannes A. Pardoen, Patrick P. J. Mulder, Johan Lugtenburg, Judith Herzfeld,* Richard Mathies, and Robert G. Griffin

ABSTRACT: Solid-state ^{13}C magic-angle sample spinning (MASS) NMR has been used to study lyophilized dark-adapted purple membrane containing ^{13}C-labeled retinals. C-10-, C-11-, and C-12-labeled derivatives each showed two lines, assigned to the coexisting 13-cis and all-trans isomers. The isotropic chemical shifts, particularly of C-11, indicate that the Schiff base is protonated. Shift anisotropies are also similar to those of model compounds, indicating that this part of the chromophore is rigid and immobile and possesses the same degree of in-plane bending as crystalline retinal derivatives. Purple membrane samples labeled on the C-19- and C-20-methyl groups both give single lines from the retinal, upfield shifted by 2.1 and 1.0 ppm, respectively, from model compounds. In all cases, high-quality spectra were obtained from ~50-mg samples in modest signal-averaging times. These results suggest that it is now practical to exploit the enormous potential of MASS NMR for structural studies of ^{13}C-labeled membrane proteins.

Bacteriorhodopsin (bR),[1] the single protein of the purple membrane of *Halobacterium halobium* (Oesterhelt & Stoeckenius, 1971, 1973) is a source of considerable interest to biochemists as an integral membrane protein, an ion pump, a transducer of light, and an analogue of mammalian visual pigments. A wide variety of spectroscopic techniques have been employed to examine the structure and function of bR. Electron and X-ray diffraction (Henderson & Unwin, 1975; Henderson, 1975) have provided a low-resolution structure of the protein, and neutron diffraction combined with specific ^{2}H labeling (Trewhella et al., 1983) has permitted some amino acid residues to be localized within that structure. In addition, flash spectroscopy has been widely used to study photointermediate absorption and kinetics [see, for example, Nagle et al. (1982)], and resonance Raman has been used to study the structure of the retinal prosthetic group in many of the

† From the Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115 (G.S.H. and J.H.), the Department of Chemistry, University of California, Berkeley, California 94720 (S.O.S. and R.M.), the Department of Chemistry, Rijksuniversiteit te Leiden, 2300 RA Leiden, The Netherlands (J.A.P., P.P.J.M., and J.L.), and the Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (R.G.G.). *Received October 21, 1983.* This research was supported by the National Institutes of Health (GM-22316, GM-23289, EY-02051, and RR-00995) and the National Science Foundation (C-670 and CHE-8116042) and by the Netherlands Foundations 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 an NIH Research Career Development Award, respectively.

[1] Abbreviations: bR, bacteriorhodopsin; CP, cross-polarization; HPLC, high-pressure liquid chromatography; MASS, magic-angle sample spinning; NOE, nuclear Overhauser enhancement; PM, purple membrane; TFA, trifluoroacetic acid.

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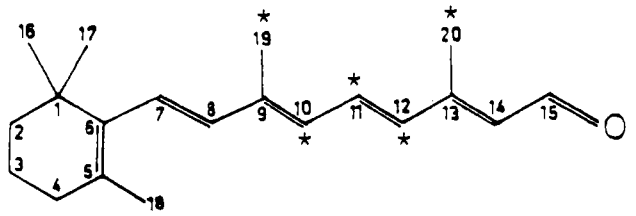

FIGURE 1: Structure of *all-trans*-retinal, showing the numbering of atoms and the positions labeled in this study.

bR photointermediates (Lewis et al., 1974; Aton et al., 1977; Stockburger et al., 1979; Hsieh et al., 1981; Smith et al., 1983).

NMR spectroscopy offers another promising means of studying bR. By use of specific labeling to offset its inherently low sensitivity, the technique can probe the structure and dynamics of membrane proteins. To date, several attempts have been made to obtain NMR spectra of bR. Most have used solution NMR techniques to acquire the data, and the associated problem of the low rotational correlation time of the purple membrane has been sidestepped by using detergent "solubilized" samples. While this approach has yielded some spectroscopic results (Yamaguchi et al., 1981; Mateescu et al., 1983b), it has also been beset with problems. First, it has invariably employed ^{13}C-labeled retinal incorporated into chemically bleached PM samples, which has resulted in the inclusion of some nonspecifically bound retinal. Due to its high mobility, the nonspecifically bound retinal gives narrower lines with shorter T_1's and larger NOE's than the specifically bound retinal and is thus selectively observed by solution NMR (Shriver et al., 1977; Yamaguchi et al., 1981). Second, the detergents perturb the protein structure and cause release of retinal. Finally, the considerable dilution required to prevent aggregation leads, despite the enrichment, to NMR spectra with poor signal-to-noise ratios.

Recently, we have shown that these problems can be overcome by employing high-resolution solid-state NMR techniques. Specifically, we employed MASS to obtain ^{15}N spectra of bR containing [ϵ-^{15}N]lysine (Harbison et al., 1983). Since we studied the intact PM, we encountered none of the decomposition problems mentioned above. Also, because the samples consisted of lyophilized bR and were thus highly concentrated, it was possible to obtain spectra with good signal-to-noise ratios even in the demanding case of ^{15}N.

This successful observation of ^{15}N spectra of bR suggested that similar approaches might be applicable to ^{13}C NMR studies provided that signals from the labeled site were visible above the much more significant natural abundance ^{13}C background. We report here that this is indeed the case for bR containing retinals that are ^{13}C enriched at five different positions in the olefinic chain: C-10, C-11, C-12, C-19, and to some extent C-20 (see Figure 1). As will be seen, the ^{13}C spectra for a system with an effective molecular weight of $\sim$70 000[2] are of excellent quality and permit measurement, for the olefinic carbons, of the shift anisotropy, as well as the isotropic chemical shifts. The spectra clearly show the presence of the 13-cis and all-trans isomers of retinal in dark-adapted bR, and the chemical shifts are consistent only with the presence of a protonated Schiff base. The size of the shift anisotropies indicates that the retinal in these samples is immobile on the ^{13}C time scale and suggests a retinyl moiety that is structurally quite similar in the regions studied to that found in model compounds. Finally, we discuss the implications of the chemical shifts for the electronic structure of the protein-bound retinal chromophore.

[2] The molecular weight of bR is 26 000. Accounting for the fact that the membrane is $\sim$25% lipid brings the number to $\sim$35 000. Also, since lyophilized PM is in the dark-adapted state consisting of two retinal isomers, which are observed as two distinct lines, we are studying a protein with an effective molecular weight of $\sim$70 000.

Materials and Methods

^{13}C-Labeled *all-trans*-retinals ($\sim$90% enriched) were prepared by recently published procedures (Pardoen et al., 1983). These were used to regenerate purple membrane from "white membrane" (isolated from a retinal-deficient strain of *Halobacterium*, JW5), as described by Smith et al. (1983). The regenerations were followed spectrophotometrically, and retinal was added to regenerate only 90–95% of the bacteriorhodopsin in order to avoid excess nonspecifically bound retinal. The regenerated membranes were purified via sucrose density gradient centrifugation and extensive dialysis in order to remove any residual labeled material. They were then lyophilized in the dark at 0.1 Torr and transferred to rotors at room temperature humidity. Solid-state NMR was conducted at 6.9 T with MASS rotors of an Andrew-Beam design on a home-built spectrometer. The particular batch of Kel-F that was used as a rotor material contained a minor protonated hydrocarbon, which gave signals in the 20–50 ppm region. This results in some apparent variation in our spectra in this area. Nevertheless, the rotor was free of resonances in the regions important for this study. Typically, cross-polarization from ^1H to ^{13}C spin systems was achieved with rotating fields of 25 and 100 G, respectively, and a mixing time of 2 ms. ^1H decoupling was performed with a 65-kHz H_1, and a flipback pulse (Tegenfeldt & Haeberlen, 1979) was applied after the pulse train to accelerate data acquisition. Typically, 30 000 transients were accumulated for the chain-labeled samples and 8000 for the methyl-labeled samples. All chemical shifts were referenced to external (neat) Me_4Si, and no correction was made for bulk susceptibility effects, which we expect to be small.

13-*cis*-Retinylidenepropylimine was prepared as follows. A 100-mg aliquot of 13-*cis*-retinal (prepared by photoisomerizing *all-trans*-retinal in hexane, followed by HPLC) was dissolved in 10 mL of diethyl ether, and to it was added, at 0 °C, 2 g of 3-Å molecular sieves, followed by 100 μL of *n*-propylamine (Eastman-Kodak). After 1 h in the dark at 0 °C, the ether and excess amine were removed in vacuo at −30 °C; the residue was dissolved in 1.5 mL of [$^2H_2$]methylene chloride and its purity checked by ^{13}C NMR at −20 °C. The spectra of this unprotonated Schiff base are essentially identical with those reported by Inoue et al. (1979). The Schiff base was converted to its TFA salt by addition, in the dark, of 27 μL of trifluoroacetic acid in 0.5 mL of CD_2Cl_2, at −10 °C. ^{13}C solution NMR spectra at 67.9 MHz were recorded immediately at −20 °C, although the material was found to be stable under these conditions for at least 4 h. Spectra were referenced to an internal TMS standard and assigned by using off-resonance and narrow-band proton decoupling, the proton spectra being immediately and directly assignable. The differences between 13-cis and all-trans isomers agree closely with those found for other retinal derivatives by Englert (1975).

Results

Figure 2 shows the ^{13}C MASS spectrum of lyophilized unlabeled dark-adapted purple membrane, at a spinning speed of 2.1 kHz. The upfield resonances between 15 and 70 ppm have very weak rotational sidebands, while the carbonyl and aromatic centerbands (at 173 and 128 ppm, respectively) are flanked by strong sidebands, reflecting the fact that these groups have small and large ^{13}C shift anisotropies, respectively.

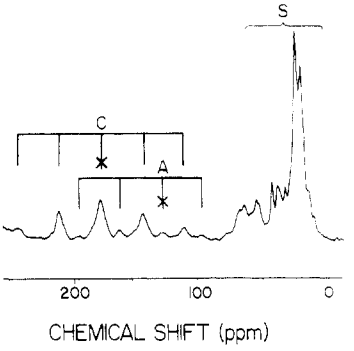

FIGURE 2: ^{13}C CP-MASS NMR spectrum of lyophilized unlabeled purple membrane: S, resonances from saturated aliphatic carbons and residual signal from the Kel-F; A, aromatic centerbands and rotational sidebands; C, carbonyl centerbands and rotational sidebands. The centerbands in the latter two cases are marked with an asterisk.

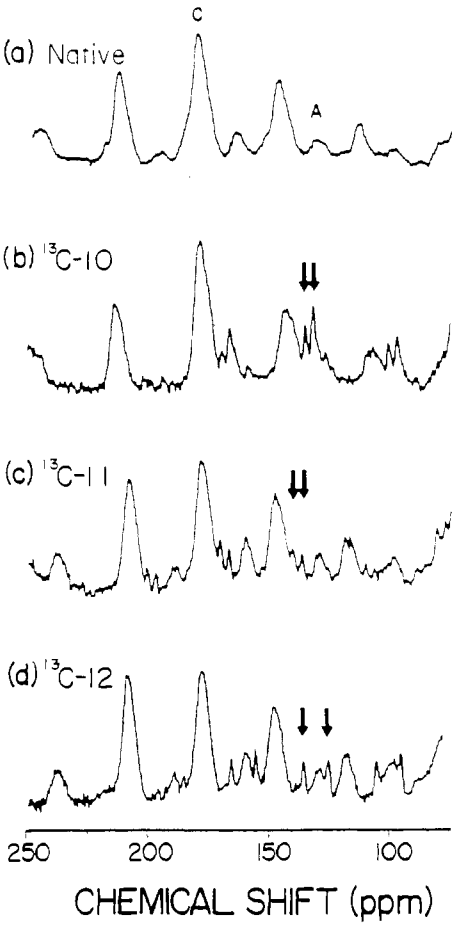

FIGURE 3: (a) Expanded view of the downfield region of the unlabeled PM spectrum in Figure 2. (b) ^{13}C CP-MASS NMR spectrum of purple membrane regenerated with ^{13}C-10-labeled retinal, spinning at ~2.4 kHz. (c) PM labeled with [11-^{13}C]retinal, spinning at 2.1 kHz. (d) PM labeled with [12-^{13}C]retinal, spinning at 2.4 kHz. The two additional sharp centerbands in (b)–(d) (identified by arrows) are due to labeled retinal in 13-cis and all-trans isomeric forms: note the presence in each case of strong rotational sidebands spaced at multiples of the spinning frequency.

Figure 3a shows the downfield region of this same spectrum in more detail. Since both the carbonyl and the aromatic signals include a large number of overlapping lines, there are no narrow resonances in this spectrum. In addition, the carbonyl carbon lines are further broadened by a dipolar coupling to the peptide ^{14}N's, which is not removed by sample rotation (Hexem et al., 1981; Menger et al., 1982). Thus, the sharp ($\Delta\nu_{1/2} \simeq 0.5$ ppm) lines in Figures 3b–d, from the white membrane regenerated with ^{13}C-10-, ^{13}C-11-, and ^{13}C-12-labeled *all-trans*-retinal, respectively, stand out in vivid contrast. In each spectrum, the two additional sharp centerbands (marked with arrows in the figure) are flanked by strong first and weak second sidebands. For reasons to be discussed below, we assign these lines to the all-trans and 13-cis isomers of the protonated retinal Schiff base, which have been reported to be present in dark-adapted bR. In Table I, we give the isotropic chemical shifts of these resonances, together with the summed integrated intensities of the sidebands and centerbands corresponding to each resonance, as a percentage of the total retinylic carbon intensity observed for that sample. For each sample, these were measured at two or more spinning speeds, and both the shifts and the intensities were consistent and reproducible to ±0.2 ppm and 5%, respectively. Note that in all cases the ratio of the relative intensities falls close to the 6:4 ratio observed by Pettei et al. (1977); this ratio is reported

Table I: Isotropic Chemical Shifts and Assignments of [^{13}C]Retinyl Resonances in PM

position of label	resonance frequencies	relative intensity (%)	assignment	chemical shift in solution[a]
10	133.0	43	all-trans	129.5
	129.7	57	13-cis	129.5
11	139.1	45	all-trans	137.4
	135.4	55	13-cis	137.8
12	134.3	41	all-trans	133.6
	124.2	59	13-cis	124.9
19	11.3	100	all-trans and 13-cis	13.4 13.4
20	13.3	~45	all-trans	14.25
	b		13-cis	22.2

[a] All-trans values are for the chloride salt of the *n*-propyl Schiff base (Shriver et al., 1976). 13-Cis values were obtained as in the text. [b] Not observed.

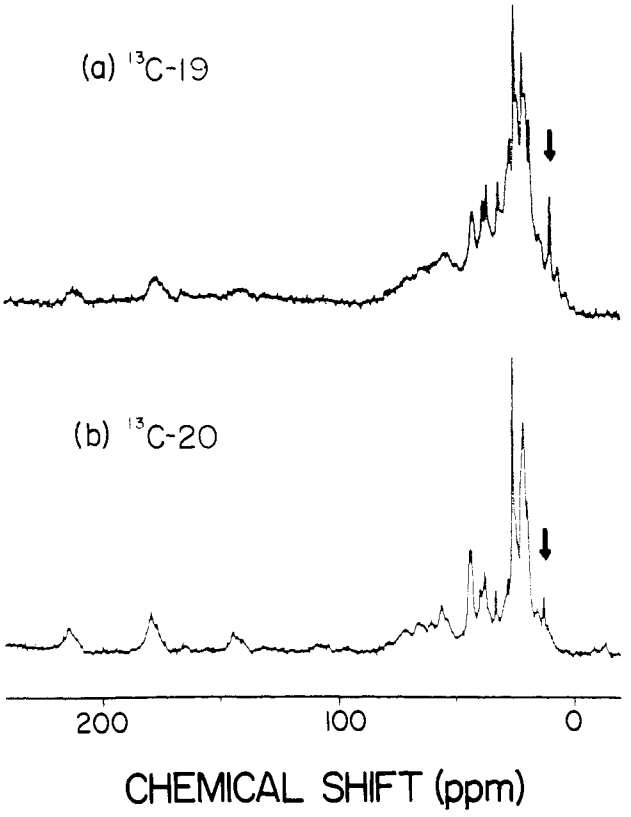

FIGURE 4: ^{13}C CP-MASS spectra of methyl-^{13}C-labeled retinyl-PM: (a) ^{13}C-19-labeled PM; (b) ^{13}C-20-labeled PM. The labeled methyl resonance is identified by an arrow.

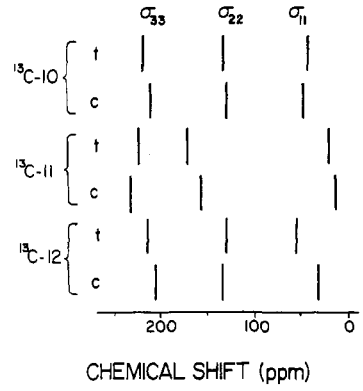

FIGURE 5: Chemical shift tensor principal values of chain-labeled retinals in bR reconstructed from sideband intensities by the method of Herzfeld & Berger (1980). σ_{11} is aligned perpendicular to the plane of the double-bonded system, σ_{22} is parallel to the double bond, and σ_{33} is orthogonal to σ_{11} and σ_{22} (Wolff et al., 1977).

to be unchanged by lyophilization (Korenstein & Hess, 1977).

Parts a and b of Figure 4 show the spectra of [19-^{13}C]-retinyl- and [20-^{13}C]retinyl-labeled PM, respectively. In both cases, the retinylic methyl is seen as a sharp line without sidebands at the extreme upfield edge of the aliphatic region of the spectrum. In the case of ^{13}C-19-labeled PM, 13-cis and all-trans derivatives may be expected to resonate in the same region. The absence of an obvious splitting into two components implies that the chemical shifts of these isomers fall within 0.4 ppm of each other. In the protonated Schiff base of 13-*cis*-retinal, the C-20 line falls 7–9 ppm downfield of the all-trans line, which puts it in the region dominated by natural abundance methyl groups of the amino acid side chains. Thus, only the all-trans signal is visible, at approximately 45% of the intensity of the ^{13}C-19 (combined) resonance.

Figure 5 depicts the principal values of the chemical shift tensors of the ^{13}C-10-, ^{13}C-11-, and ^{13}C-12-labeled PM's, in both their isomeric forms. These were calculated from the rotational sideband intensities by the method of Herzfeld & Berger (1980), with the computer program described in that paper. Because of the difficulty of accurately subtracting base-line contributions to the line intensities in complex spectra of this kind, the expected errors in the shift tensor principal values (~7 ppm) are much larger than those in the isotropic chemical shifts.

Discussion

The clearest and most notable feature of the C-10 through C-12 spectra is the obvious doubling of the resonance lines of the labeled retinylic carbons with an intensity ratio of approximately 6:4. We believe this to be the clearest spectroscopic corroboration to date of the extraction experiments of Pettei et al. (1977), who noted a similar ratio of 13-cis to all-trans isomers in dark-adapted purple membrane. In addition, Korenstein & Hess (1977) reported the shape of the visible absorption spectrum remains unchanged by dehydration under conditions similar to ours, implying that the ratio of 13-cis to all-trans is unaffected by this process. These results are also consistent with our observations.

In order to further confirm that no significant change in either isomer composition or state of protonation occurs under *our* lyophilization conditions, we have obtained resonance Raman spectra of the bR samples lyophilized from H_2O and D_2O (S. O. Smith and R. Mathies, unpublished results). These are very similar to solution spectra of dark-adapted bR and, in particular, exhibit the expected Schiff base vibration at 1639 cm^{-1} (H_2O) or 1623 cm^{-1} (D_2O) with no indication of Schiff base deprotonation.

In Table I we compare the shifts we obtain for ^{13}C-labeled bacteriorhodopsin, with the published data for a protonated all-trans Schiff base (Shriver et al., 1976) and with the chemical shifts obtained by us for 13-*cis*-retinylidenepropyliminium trifluoroacetate. Comparison with these values allows us to assign the 134.3 ppm resonance in ^{13}C-12 purple membrane to the all-trans chromophore and the 124.2 ppm resonance to the 13-cis chromophore, which is shifted even further upfield than the solution value. Moreover, this assignment agrees with that based on the isomer ratio as determined from the line intensities.

In the case of C-10 and C-11, the ^{13}C chemical shift differences between the two lines are smaller (~4 ppm) and therefore do not permit us to clearly differentiate between 13-cis and all-trans isomers. We have therefore used the line intensities rather than the shifts to make these two assignments. It should be emphasized that we regard these assignments as tentative. With this proviso, our evidence suggests that for all three olefinic positions examined here, the 13-cis isomer is upfield shifted 1.3–4.1 ppm farther from the all-trans isomer than might be expected from isomerization alone.

Regardless of how we assign the spectra, the data are consistent *only* with the presence of a protonated Schiff base in lyophilized, dark-adapted PM. Although the ^{13}C chemical shifts of even-numbered carbons in the middle of the chain are not particularly sensitive to protonation, the shifts of the odd-number carbons are. Thus, the observation of shifts of 139.1 and 135.4 ppm for C-11 is compelling evidence for the presence of a protonated Schiff base. The chemical shift of the deprotonated form would be expected to appear 6–10 ppm upfield from these values (Tokito et al., 1975; Inoue et al., 1979). This result is in agreement with our previous ^{15}N MASS experiments, which also indicated that lyophilized dark-adapted bR contains a protonated Schiff base (Harbison et al., 1983), and with results from vibrational spectra of fully hydrated samples (Lewis et al., 1974; Bagley et al., 1982; Rothschild & Marrero, 1982).[3]

The chemical shielding tensors for the labeled olefinic carbons show both the large anisotropy expected for unsaturated carbons in general (Mehring, 1982) and the large asymmetry parameter previously observed for a carbon–carbon double bond in a single crystal study (Wolff et al., 1977). The tensors of all-trans C-12 and of all-trans and 13-cis C-10 are identical within experimental error, with $\delta \equiv -80$ ppm and $\eta \simeq 1$.[4] The C-11 tensors are somewhat more anisotropic and

[3] Mateescu et al. (1983a,b) have presented preliminary MASS NMR results suggesting that lyophilization of bR causes deprotonation of the Schiff base. Thus, in ^{13}C spectra of bR containing retinal labeled at C-13, a shift of 143.7 ppm has been observed (Mateescu et al., 1983b). This result is not consistent with our ^{13}C and ^{15}N chemical shifts. Under some conditions, it has been reported that deprotonation is transient. Specifically, ^{15}N MASS spectra were interpreted in terms of a deprotonated Schiff base that converts to a protonated form in 12 h at room humidity (Mateescu et al., 1983a,b). This result is inconsistent with our ^{15}N results (Harbison et al., 1983). In addition, deprotonated imines have shift anisotropies of about 600 ppm. Thus, in a MASS experiment significant signal intensity resides in the rotational sidebands, and in some cases, the sidebands are more intense than the centerbands. The spectra of Mateescu et al. (1983a) did not show the sideband patterns characteristic of unprotonated Schiff bases (Harbison et al., 1983; Figure 2). Furthermore, because of the large shift anisotropy, the ratio of the centerband intensities in the protonated vs. nonprotonated forms should be ~3:1. In the spectra of Mateescu et al. (1983b), the ratio, with the intensity of the amide backbone as a standard, is 1:1. It is possible that such discrepancies are partly due to different conditions of lyophilization and that under extreme dehydrating conditions, at vacuums much higher than those used in this study, deprotonation of the Schiff base might occur. Nevertheless, it appears that substantial inconsistencies in the spectroscopic results remain to be resolved.

much more symmetric ($\delta \equiv -110$ ppm, $\eta = 0.4$). Similar differences between C-11 and its neighbors have been noted in all retinal derivatives studied and in β-carotene. In contrast, in straight-chain conjugated polyene Schiff bases, the shift tensors are roughly invariant with chain position. The differences may be attributable to the in-plane bending of methyl-substituted polyenes, which causes the CCC bond angle at C-11 to be less than, and those at C-10 and C-12 to be greater than, 125°. This bending should affect the relative size of the in-plane tensor elements σ_{22} and σ_{33}. Thus, the in-plane bending in bR appears to be similar to that noted in crystallographic studies of model compounds [e.g., Hamanaka et al., 1972].

The shift anisotropies can also be used to obtain information on the dynamic properties of molecules (Spiess, 1979; Rice et al., 1981). Specifically, when there is molecular motion, the size of the anisotropy is reduced to an extent determined by the rate and the mechanism of the motion. The ^{13}C anisotropies in bR are too large to allow for any substantial motion by the main part of the retinyl moiety. Thus in lyophilized PM, the chromophore must be essentially static on the ^{13}C NMR time scale ($\sim 10^{-3}$ s).

Interpretation of the isotropic shifts observed in these samples is hindered by the relatively small shift differences between the labeled bR chromophores and the protonated *all-trans*-retinal Schiff bases. The positions studied here are distant both from the ionone ring and the Schiff-base linkage, where, on the basis of current models of the electronic structure of the bR chromophore (Kakitani et al., 1983; Sheves & Nakanishi, 1983), the largest differences may be expected. Nevertheless, the 3.5 ppm downfield shift at the C-10 position in the all-trans isomer is not inconsistent with, and might indicate, a negative point charge near the ionone ring, as proposed by Nakanishi et al., (1980). The upfield shift of the C-19 methyl group is also outside the range of the C-19 shifts observed in model compounds in the solid state (G. S. Harbison, J. Herzfeld, and R. G. Griffin, unpublished results) and might, for example, result from the proximity of a positively charged or aromatic amino acid.

The final interpretation of these retinal ^{13}C shifts must be based on a complete set of data from all positions of the chromophore, particularly those near the Schiff base and the ionone ring. Studies of other ^{13}C-labeled PM's are in progress, and we are compiling an extensive set of ^{13}C shift tensors for model compounds. When this work is complete, detailed comparison of both isotropic and anisotropic shifts may be made. This should allow us to study more completely the structure of the bR chromophore and to determine the location and nature of key protein perturbations.

Acknowledgments

We thank H. J. Weber (University of California at San Francisco) for providing the retinal-deficient strain of *H. halobium* and V. Elms for preparation of the manuscript.

Registry No. *all-trans*-Retinal, 116-31-4; 13-*cis*-retinylidenepropylimine, 89824-24-8; 13-*cis*-retinal, 472-86-6; *n*-propylamine, 107-10-8; 13-*cis*-retinylidenepropyliminium trifluoroacetate, 89824-25-9.

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[4] The chemical shift anisotropy is described by two parameters, δ and η, defined by Haeberlen (1976) as $\delta = \sigma_{11} - (Tr\bar{\sigma})/3$ and $\eta = (\sigma_{22} - \sigma_{33})/\delta$.

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Differential Light Scattering and Absorption Flattening Optical Effects Are Minimal in the Circular Dichroism Spectra of Small Unilamellar Vesicles†

David Mao and B. A. Wallace*

ABSTRACT: The large size of membrane particles and the high local concentration of proteins in these particles give rise to differential scattering and absorption flattening effects which result in significant distortions of the circular dichroism spectra of membrane proteins and produce erroneous estimates of secondary structure. In an attempt to find a membrane system in which scattering and flattening are minimal, but in which native protein conformation is retained, several methods of fragmentation, including sonication, solubilization, and incorporation into small unilamellar vesicles (SUVs), were examined. Bacteriorhodopsin in purple membrane sheets was used as a test system for the effectiveness of the procedures since its secondary structure is known from independent physical measurements and these large membranes produce considerable distortions, as seen by comparison of observed and calculated spectra for the protein. While sonication decreased differential scattering, it had little effect on the total distortion; solubilization in octyl glucoside tended to decrease both differential scattering and flattening but induced some conformational change in the protein. However, when bacteriorhodopsin was incorporated into small unilamellar vesicles, which both decrease particle size and dilute the local concentration of protein, the spectrum produced was nearly identical with the calculated one, suggesting that SUVs may be appropriate vehicles for use with membrane proteins and may be a facile method for eliminating optical artifacts.

The circular dichroism (CD)[1] spectra of particulate samples such as membrane suspensions are distorted by the optical artifacts of differential light scattering (Bustamante et al., 1983) and differential absorption flattening (Gordon & Holzwarth, 1971; Duysens, 1956). These distortions must be removed in order to analyze the data accurately.

Differential light scattering is the difference in the extent of scattering of left and right circularly polarized light by an optically active sample, which will be detected by a spectropolarimeter as if it were differential absorption and leads to distortion of the protein spectrum. The inherent difference in the indices of refraction for left and right circularly polarized light exhibited by an optically active particle is the primary source of this differential scattering. Results of differential light scattering are abnormally large or small CD peaks, a dependence of the CD spectrum on the detector acceptance angle, and CD signals outside the absorption range of the chromophore. The angular dependence and the magnitude of differentially scattered intensities are functions of the particle size and relative orientation and distance between the scattering units within each particle (Bustamante et al., 1983).

A complete correction for differential scattering is potentially possible by using fluorescence-detected circular dichroism (FDCD), which permits measurement with an effective acceptance angle of 4 sr (Reich et al., 1980). Alternatively, if the scattering is concentrated in the forward direction, as is the case for spherical particles, an end-window photomultiplier detector placed directly behind the sample cell should also be sufficient to collect most of the scattered light (Schneider & Harmatz, 1976).

Absorption flattening occurs when chromophores are closely packed, resulting in a smaller total physical cross-sectional area than if the molecules were uniformly dispersed. Consequently, the probability for protein molecules to encounter photons is decreased, and the protein absorption is correspondingly reduced. The extent of flattening is a function of the size of the particles and the concentration of chromophores within each particle.

An optically active particulate sample will exhibit differential flattening of the absorption of the left and right circularly polarized light because chiral particles absorb oppositely polarized light differently. Because the degree of flattening is directly proportional to absorption, the reduction in the CD signal varies with the wavelength and is maximal at wavelengths corresponding to absorption peaks. Consequently, a significant distortion in the shape of the CD spectrum may result.

† From the Department of Biochemistry and Molecular Biophysics, Columbia University, New York, New York 10032. *Received November 21, 1983.* This work was supported by Grant GM-27292 from the National Institutes of Health. B.A.W. is the recipient of a Hirschl Career Scientist Award.

[1] Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; DMPC, dimyristoylphosphatidylcholine; SUV, small unilamellar vesicle; NOG, *n*-octyl glucoside; SDS, sodium dodecyl sulfate; CD, circular dichroism; FDCD, fluorescence-detected circular dichroism.

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