Ioannou, P. V., & Golding, B. T. (1979) *Prog. Lipid Res. 17*, 279-318.
Jori, G., Tamburro, A. M., & Azzi, A. (1974) *Photochem. Photobiol. 19*, 337-345.
Kimbelberg, H. K., & Lee, C. P. (1970) *J. Membr. Biol. 2*, 252-262.
Margoliash, E., & Schejter, A. (1966) *Adv. Protein Chem. 21*, 113-286.
Marsh, D., & Powell, G. L. (1988) *Bioelectrochem. Bioenerg. 20*, 73-82.
Marsh, D., & Watts, A. (1988) in *Lipid Domains and the Relationship to Membrane Function* (Aloia, R. C., Curtain, C. C., & Gordon, L. M., Eds.) pp 163-200, Alan R. Liss, New York.
Müeller, M., Moser, R., Chenerval, D., & Carafoli, E. (1985) *J. Biol. Chem. 260*, 3839-3843.
Reitveld, A., Sijens, P., Verkleij, A. J., & de Kruijff, B. (1983) *EMBO J. 2*, 907-913.
Robinson, N. C., Strey, F., & Talbert, L. (1980) *Biochemistry 19*, 3656-3661.
Seelig, J., Tamm, L., Hymel, L., & Fleischer, S. (1981) *Biochemistry 20*, 3922-3932.
Smith, D. W., & Williams, R. J. P. (1970) *Struct. Bonding 7*, 1-45.
Smith, I. C. P., & Ekiel, I. H. (1984) in *Phosphorus-31 NMR, Principles & Applications* (Gorenstein, D. G., Ed.) pp 447-475, Academic Press, New York.
Spooner, P. J. R., & Watts, a. (1991) *Biochemistry* (preceding paper in this issue).
Sullivan, M. J., & Marciel, G. E. (1982) *Anal. Chem. 54*, 1606-1615.
Tamm, L. K., & Seelig, J. (1983) *Biochemistry 22*, 1474-1483.
Vik, S. B., Georgevich, G., & Capaldi, R. A. (1981) *Proc Natl. Acad. Sci. U.S.A. 78*, 1456-1460.
Vincent, J. S., Kon, H., & Levin, I. W. (1987) *Biochemistry 26*, 2312-2314.
Waltham, M. C., Cornell, B. A., & Smith, R. (1986) *Biochim. Biophys. Acta 862*, 451-456.
Watts, A. (1987) *J. Bioenerg. Biomembr. 19*, 625-653.
Watts, A. (1989) *Curr. Opin. Cell Biol. 1*, 691-700.
Yoshimura, T. (1988) *Eur. J. Biochem. Biophys. 264*, 450-461.

# High-Resolution $^{13}C$ NMR Study of the Topography and Dynamics of Methionine Residues in Detergent-Solubilized Bacteriorhodopsin†

Michel Seigneuret,* Jean-Michel Neumann, Daniel Levy, and Jean-Louis Rigaud
*URA-CNRS 1290 and Département de Biologie, CEN Saclay, 91191 Gif-sur-Yvette, Cedex, France*
*Received May 23, 1990; Revised Manuscript Received December 28, 1990*

ABSTRACT: The proton transport membrane protein bacteriorhodopsin has been biosynthetically labeled with [*methyl*-$^{13}C$]methionine and studied by high-resolution $^{13}C$ NMR after solubilization in the detergent Triton X-100. The nine methionine residues of bacteriorhodopsin give rise to four well-resolved $^{13}C$ resonances, two of which are shifted upfield or downfield due to nearby aromatic residues. Methionine residues located on the hydrophilic surfaces, on the hydrophobic surface, and in the interior of the protein could be discriminated by studying the effects of papain proteolysis, glycerol-induced viscosity increase, and paramagnetic broadening by spin-labels on NMR spectra. Such data were used to evaluate current models of the bacteriorhodopsin transmembrane folding and tertiary structure. $T_2$ and NOE measurements were performed to study the local dynamics of methionine residues in bacteriorhodopsin. For the detergent-solubilized protein, hydrophilic and hydrophobic external residues undergo a relatively large extent of side chain wobbling motion while most internal residues are less mobile. In the native purple membrane and in reconstituted bacteriorhodopsin liposomes, almost all methionine residues have their wobbling motion severely restricted, indicating a large effect of the membrane environment on the protein internal dynamics.

Bacteriorhodopsin (BR),[1] the light-driven proton pump from *Halobacterium halobium*, appears as the best-characterized membrane transport protein to date [for a review, see Dencher (1983)]. However, many aspects of its structure are still a matter of debate, including the polypeptide chain transmembrane folding (Huang et al., 1982; Fimmel et al., 1989), the secondary structure (Jap et al., 1983; Nabedrik et al., 1985), the tertiary structure (Agard & Strout, 1982; Trewhella et al., 1986), and the protein internal dynamics (Herzfeld et al., 1987; Bowers & Oldfield, 1988). New data on BR tridimensional structure have recently been obtained from mutagenesis (Mogi et al., 1989; Soppa et al., 1989) and neutron (Popot et al., 1989) or electron (Henderson et al., 1990) diffraction experiments and need to be confirmed and extended by other approaches. Nuclear magnetic resonance (NMR) is a promising technique for investigation of the structure and dynamics of BR due to the large possibilities of stable isotope labeling of the protein. Presently, most NMR studies have used solid-state techniques to investigate the protein in the

† This work was supported by grants from the Commissariat à l'Energie Atomique and the Centre National de la Recherche Scientifique.
* To whom correspondence should be addressed.

[1] Abbreviations: BR, bacteriorhodopsin; [Met, $^{13}C$]-BR, [*methyl*-$^{13}C$]methionine-labeled BR; $^{13}C$-$^{1}H$ COSY, $^{13}C$-$^{1}H$ heteronuclear chemical shift correlation spectroscopy; DEPC, dielaidoylphosphatidylcholine; HPLC, high-performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate.

0006-2960/91/0430-3885$02.50/0 

membrane and have yielded important results concerning protein dynamics (Keniry et al., 1984; Lewis et al., 1985) and chromophore conformation and environment (Harbison et al., 1983, 1984, 1985). High-resolution NMR has, on the other hand, been rarely employed (Arseniev et al., 1988). However, due to its ability to resolve features from single amino acid residues, this technique has the potential for providing relatively precise structural and dynamic data on BR. In the present study, we have biosynthetically incorporated [*methyl*-$^{13}$C]methionine into BR and studied the labeled protein by high-resolution $^{13}$C NMR after solubilization in detergent. This approach allowed us to obtain new data on both the topography and dynamics of specific amino acid residues in the protein.

## Materials and Methods

*Materials.* L-[*methyl*-$^{13}$C]methionine was from Merck Sharp & Dohme or CEA. Triton X-100 and hydrogenated Triton X-100 were from Sigma, and sodium dodecyl sulfate (SDS) was from Bio-Rad. Papain was from Boehringer-Mannheim; tempamine was from Molecular Probes; 16-doxylstearic acid was from Aldrich; dielaidoylphosphatidylcholine (DEPC) was from Avanti. Other chemicals were reagent grade.

*Labeled BR Sample Preparation. H. halobium* strain S9 was grown by using the medium of Ohnishi et al. (1965) except that 0.185 g/L L-[*methyl*-$^{13}$C]methionine was substituted for unlabeled methionine. Purple membrane was isolated by using the method of Oesterhelt and Stoeckenius (1974) including the sucrose density gradient step. Proteolysis of BR by papain in the purple membrane was performed as described (Abdulaev et al., 1978; Liao et al., 1983) using papain to BR ratios of 1:200 or 1:20 (w/w) and incubation times at 37 °C of 2 and 120 h, respectively, for low and high proteolysis conditions. After proteolysis had been stopped with 50 mM iodoacetamide, the membranes were washed once with 1 M NaCl and 3 times with water. Such a procedure removes small peptides generated by proteolysis (Fimmel et al., 1989). It was checked by SDS–polyacrylamide gel electrophoresis that the expected proteolytic cleavages had occurred at a level of at least 85%. Detergent-solubilized BR was prepared by suspending purple membrane (10 mg/mL) in 5% (w/v) Triton X-100/10 mM MES, pH 5, followed by 15-s sonication and stirring in the dark for 12 h at room temperature. Unsolubilized material (less than 5% protein) was removed by 30-min centrifugation at 250 000*g*. Control HPLC measurements performed with a TSK 3000 SW gel filtration column (Toyo Soda) according to Le Maire et al. (1986) indicated that the Triton X-100 (solubilized BR eluted as a single peak corresponding to a Stokes radius of 5.5 ± 0.5 nm, the amount of aggregated material at the void volumn being less than 5%. The protein was thus monodisperse and monomeric, in agreement with previous data (Reynolds & Stoeckenius, 1977; Dencher & Heyn, 1982). Control electrophoresis indicated that no endogenous proteolysis occurred during detergent solubilization. Reconstituted BR liposomes were prepared from purple membrane and DEPC at a total lipid to BR ratio of 1.3:1 (w/w) in 125 mM potassium phosphate, pH 7, using the reverse-phase evaporation technique (Rigaud et al., 1983) and checked by freeze–fracture electron microscopy according to Gulik-Krzywicky et al. (1987). All samples for NMR contained 10% (v/v) $D_2O$ for deuterium locking.

*NMR Experiments.* $^{13}$C NMR spectra were recorded at 20 °C on Bruker MSL 300 or Bruker AM 600 spectrometers at 75.4 MHz in 10-mm sample tubes or at 150.8 MHz in 5-mm sample tubes. For single-pulse $^{13}$C NMR experiments, the following conditions were used: a pulse length of 6 μs (150.8 MHz) or 9 μs (75.4 MHz), an acquisition time of 0.3 s, and a recycling delay of 3 s. Two-level broad-band proton decoupling was used except for spectra without NOE enhancement for which inverse gated decoupling was used. Chemical shifts were determined from external aqueous dioxane. Calculation of the extent of $^{13}$C labeled and of the number of methionine residues contributing to each resonance was performed from spectral integrals using the 57.5 ppm natural-abundance line of Triton X-100 in solubilized samples and the 25.5 ppm natural-abundance line of endogenous lipids in membrane samples as internal standards since these had $T_2$ values similar to those of methionine lines. Integrals were corrected for NOE effects. The concentration of BR determined spectrophotometrically directly on the NMR sample was also used. Such absorption measurements also indicated that for noncleaved BR, at most 3% bleaching occurs during solubilization, and NMR experiments, in agreement with previous works (Dencher & Heyn, 1982; Miercke et al., 1989). Transverse relaxation times, $T_2$, were measured by using a Hahn spin-echo sequence. $^{13}$C–$^1$H heteronuclear chemical shift correlation (COSY) spectra were recorded at 150 MHz by using the pulse sequence described by Wilde and Bolton (1984). A total of 256 FIDs were collected, and each FID consisted of 4096 data points. Sine-bell weighting was used in both dimensions before Fourier transformation.

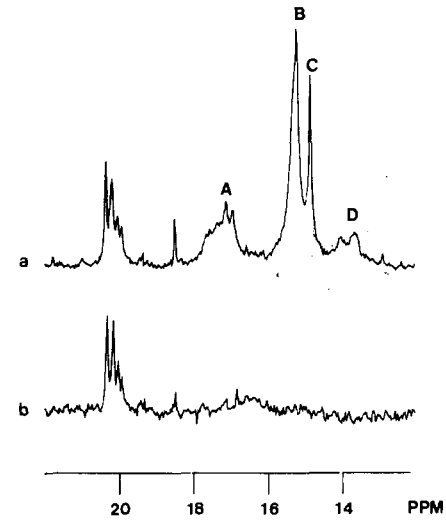


FIGURE 1: 150.8-MHz $^{13}$C NMR spectrum of 10 mg/mL [Met, $^{13}$C]-BR (a) and unlabeled BR (b) solubilized in 5% (w/v) Triton X-100/10 mM MES, pH 5, recorded at 20 °C.

## Results

*$^{13}$C NMR Spectrum of Detergent-Solubilized Labeled BR.* Figure 1 shows the 150.8-MHz $^{13}$C NMR spectrum of BR biosynthetically labeled with [*methyl*-$^{13}$C]methionine (hereafter referred to as [Met, $^{13}$C]-BR) after solubilization in Triton X-100. The corresponding natural-abundance spectrum is also shown for comparison. There appears to be no overlapping of natural-abundance lines with the labeled protein spectrum. The integral of this spectrum was estimated (see Materials and Methods) as corresponding to 8.6 $^{13}$C atoms per BR molecule, i.e., 95% labeling of the 9 methionine residues of the protein. The small resonance at 18.5 ppm is due to an impurity present in variable amounts in the Triton X-100 and is not related to $^{13}$C incorporation. Only one smaller $^{13}$C-enriched resonance was present at 53.5 ppm, presumably due to metabolism of methionine to an unknown component.

The spectrum of [Met, $^{13}$C]-BR in Triton X-100 shown in Figure 1 displays four well-resolved resonances, the chemical

Table I: Chemical Shifts and Integrals of Methionine Resonances in Triton X-100 Solubilized [Met, $^{13}$C]-Br

| resonance[a] | chemical shift[b] (ppm) | integral[c] |
|---|---|---|
| A | 17.2 | 2.6 ± 0.4 |
| B | 15.4 | 3.8 ± 0.6 |
| C | 14.9 | 1.4 ± 0.2[d] |
| D | 14.0 | 1.1 ± 0.15 |

[a] See Figure 1. [b] With respect to tetramethylsilane. [c] Corrected for NOE and normalized to a total value of 9. [d] Value overestimated due to the overlap with resonance B.

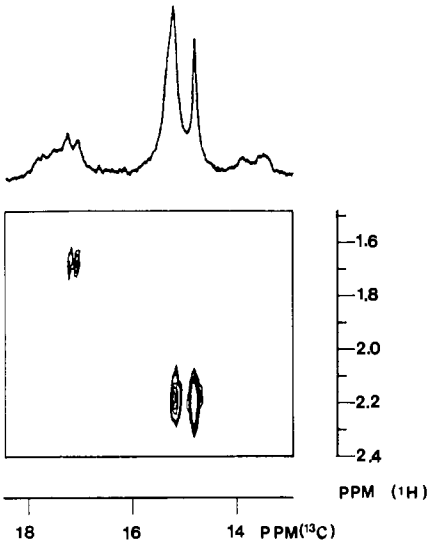


FIGURE 2: 150.8-MHz $^{13}$C–$^{1}$H heteronuclear COSY spectrum of [Met, $^{13}$C]-BR solubilized in 5% (w/v) Triton X-100/10 mM MES, pH 5, recorded at 20 °C.

shifts and NOE-corrected integrals (normalized to nine methionine residues) of which are given in Table I. These integrals indicate that resonances A–D correspond to approximately 3, 4, 1 and 1 of the 9 methionine residues of BR. Resonance A appears to be composed of one broad and two narrow components and resonance D of two broad components that are only partially resolved. It is obvious from Figure 1 that each of the two narrow components within resonance A, as well as the two broader components forming resonance D, corresponds to less than one methionine residue. This indicates that a limited heterogeneity is present among the BR molecules in the sample and splits the $^{13}$C NMR lines of two methionine residues (see Discussion).

*Chemical Shifts of Methionine Resonances.* The two narrow central resonances B and C in the $^{13}$C spectrum of Figure 1 appear to have chemical shift values (Table I) that are close to that of free methionine [15.04 ppm according to Blakey et al. (1978)]. On the other hand, the mostly broader resonances A and D are strongly shifted upfield and downfield, respectively. Such differences are also reflected in the corresponding methyl proton chemical shifts that were obtained from the $^{13}$C–$^{1}$H heteronuclear COSY spectrum (Figure 2). Resonances B and C yield a $^{1}$H chemical shift of 2.2. ppm, i.e., in the range expected for methionine residues with only minor environmental effects (Wüthrich, 1986). On the other hand, the narrow double peak from resonance A, which is shifted downfield in the $^{13}$C scale, is, interestingly, shifted upfield in the $^{1}$H scale. Other broader components did not give detectable cross-peaks in the $^{13}$C–$^{1}$H COSY spectrum, due to their small $T_2$ values (see below).

The unusual chemical shifts of resonances A and D are likely to be due to environmental effects of the protein structure on the corresponding methionine residues (e.g., nearby aromatic residues). However, the possibility remained that such chemical shifts were due to ring-current effects from the phenyl moiety of the Triton X-100 molecules surrounding BR in the solubilized state. This was ruled out by the fact that substitution of hydrogenated Triton X-100 for the normal detergent had no effect on the chemical shifts of resonances A and D (not shown).

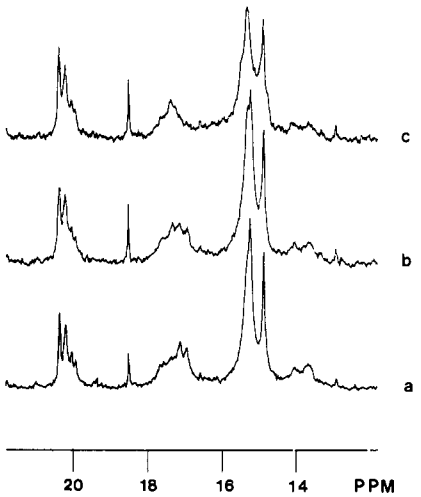


FIGURE 3: Effect of papain pretreatment on the 150.8-MHz $^{13}$C NMR spectrum of [Met, $^{13}$C]-BR in 5% (w/v) Triton X-100/10 mM MES, pH 5, recorded at 20 °C. (a) No proteolysis; (b) low proteolysis; (c) high proteolysis (see Materials and Methods).

*Effect of Papain Proteolysis on the $^{13}$C NMR Spectrum of Detergent-Solubilized [Met, $^{13}$C]-BR.* With the goal of assigning the resonance of one methionine residue, we watched the effect of proteolysis of [Met, $^{13}$C]-BR by papain in the purple membrane on the $^{13}$C NMR spectrum of the Triton X-100 solubilized protein. As shown in Figure 3, mild papain treatment has virtually no effect on the spectrum, consistent with the fact that it only removes the 232–248 C-terminal segment of BR (Abdulaev et al., 1978) containing no methionine. On the other hand, a heavier papain treatment stongly reduces the intensity of the $^{13}$C NMR spectrum, to an extent that corresponds to about one methionine per BR as judged from the NOE-corrected integrals. The main intensity decrease appears to be located on resonance B. A smaller decrease in also found on resonances A and D. However, the latter effect appears to be due to the fact that papain-treated BR is slightly unstable and undergoes 15% bleaching during detergent solublization and NMR acquisition. Indeed, partial bleaching on nonproteolyzed BR with SDS appears to promote a similar modification of resonances A and B which is due to a broadening or a shift of part of the resonance and does not change by itself the spectral integral (not shown). Since under such conditions segment 67–72 is removed from BR (Abdulaev et al., 1978), one may conclude that one of the four methionine residues that contributes resonance B is Met-68, i.e., a residue from one of the hydrophilic surfaces of the protein.

*Determination of BR Surface Methionine Residues by Paramagnetic Broadening.* In order to obtain information concerning the location of methionine residues in the BR structure, we studied the effect of site-directed paramagnetic reagents on the $^{13}$C NMR spectrum of [Met, $^{13}$C]-BR. Two spin-labels were used: the aqueous positively charged tempamine, in order to assess residues from the two hydrophilic surfaces of BR; and the amphiphilic 16-doxylstearic acid, in order to probe residues from the protein hydrophobic surface in contact with the detergent–nonpolar moiety. The broadening of Triton X-100 natural-abundance $^{13}$C resonances in

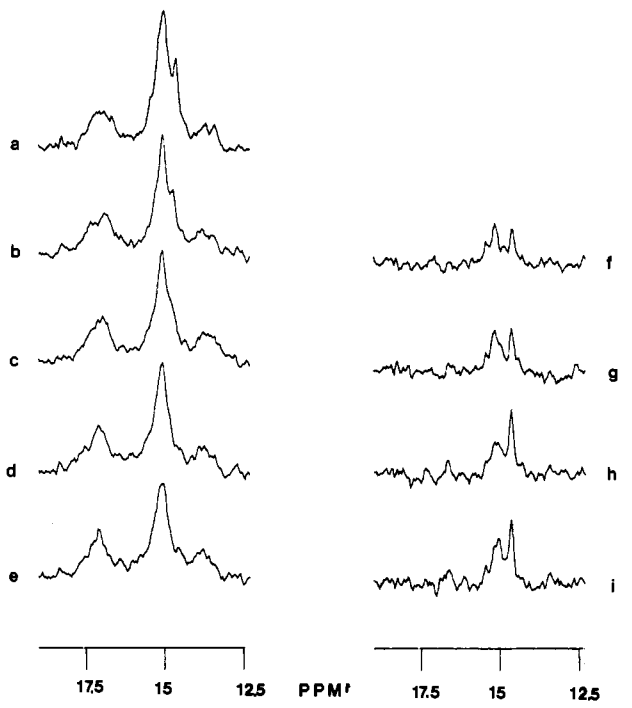


FIGURE 4: Effect of tempamine on the 75.4-MHz $^{13}C$ NMR spectrum of [Met, $^{13}C$]-BR in 5% (w/v) Triton X-100/10 mM MES, pH 5, recorded at 20 °C (a–e) and corresponding (no tempamine minus with tempamine) difference spectra (f–i). (a) 0, (b, f) 5, (c, g) 10, (d, h) 15, and (e, i) 20 mM tempamine.

the solubilized [Met, $^{13}C$]-BR spectrum could be used as an internal test of the spin-label selectivity. With 16-doxylstearic acid, much more broadening occurred for the hydrophobic portion than for the polar head group of the detergent (66% and 23% intensity decreases, respectively, for methyl and oxyethylene resonances at 2 mM spin-label) while a reverse effect was effected by tempamine (34% and 59% intensity decreases for the same resonances at 20 mM spin-label). There is thus a limited nonselectivity of the spin-labels.

The effect of increasing concentrations of tempamine on the methionine resonances of [Met, $^{13}C$]-BR is shown in Figure 4 in the form of both direct and difference spectra. The number of affected methionine residues was deduced from NOE-corrected integrals. Tempamine appears to selectively decrease the intensity of resonances B and C. A small effect may also occur on resonance A but would correspond to less than one-fourth of a methionine residue. The maximum effect is reached at 15–20 mM spin-label and was calculated to correspond to 1.7 ± 0.3 methionine residues per BR.

A similar experiment performed with 16-doxylstearic acid is depicted in Figure 5. Here, a drawback was that the natural abundance of the methyl resonance of ethanol from the spin-label stock solution partially overlapped with resonance A. Nevertheless, it can clearly be observed from the difference spectra that 16-doxylstearic acid selectively decreases the intensity of the narrow component of resonance A as well as of resonances B and C. The maximum effect is reached at 1.5–2 mM spin-label [i.e., 1.8–2.4% (mol/mol) relative to Triton X-100] and corresponds to about 4.2 ± 0.6 residues per BR (the ethanol contribution to the integrals was estimated from the values at 1.5 and 2 mM spin-label for which paramagnetic broadening is identical). Resonance C, which corresponds to a single methionine residue, appears to be broadened both by tempamine and by 16-doxylstearic acid. On the other hand, for resonance B, the single methionine residue affected by tempamine appears to be distinct from the two residues perturbed by 16-doxylstearic acid. Indeed, as shown in Figure

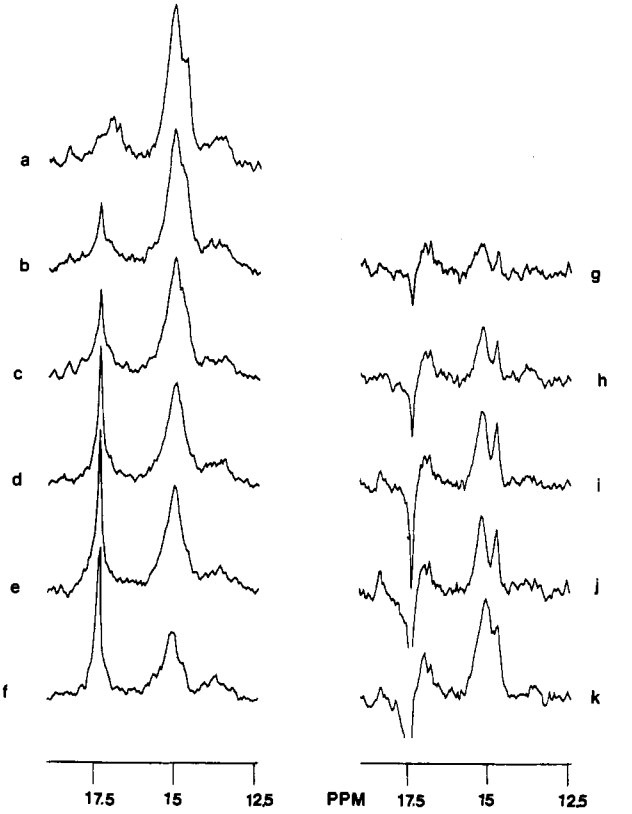


FIGURE 5: Effect of 16-doxylstearic acid on the 75.4-MHz $^{13}C$ NMR spectrum of [Met, $^{13}C$]-BR in 5% (w/v) Triton X-100/10 mM MES, pH 5, recorded at 20 °C (a–f) and corresponding (no 16-doxylstearic acid minus with 16-doxylstearic acid) difference spectra (g–k): (a) 0, (b, f) 5, (c, h) 1, (d, i) 1.5, and (e, j) 2 mM doxylstearic acid; (f, h) 2 mM doxylstearic acid/20 mM tempamine. The methyl natural-abundance $^{13}C$ resonance of ethanol from the 16-doxylstearic acid stock solution [maximum final concentration 1% (v/v)] occurs at 17.4 ppm.

5 (f and k), tempamine, when added after 16-doxylstearic acid, promotes an additional intensity decrease of resonance B, equivalent to that produced by tempamine alone; i.e., five methionine residues were affected by the two spin-labels used together.

These results indicate that five methionine residues among nine are located at the periphery of the BR molecule. Among these five residues, one can be assigned to the hydrophilic surface and three to the hydrophobic surface without ambiguity since these are broadened by only one of the two spin-labels. The fact that the fifth accessible methionine residue is broadened by both spin-labels may be attributed to their noncomplete spatial selectivity. However, this nonselectivity appears to be limited to only one methionine residue. It is thus probable that this residue is located near the boundary of the hydrophilic and hydrophobic surfaces of BR.

*Dynamics of Methionine Residues in Detergent-Solubilized BR.* The $^{13}C$ NMR spectrum of [Met, $^{13}C$]-BR in Triton X-100 displays rather different apparent line widths for the four resolved resonances (see Figure 1). However, since most of these resonances correspond to several methionine residues, it was not certain whether such features correspond to motional differences between residues or to superimposition of single lines with very close chemical shifts. For further information, $T_2$ and NOE measurements were performed for the detergent-solubilized [Met, $^{13}C$]-BR, the results of which are summarized in Table II. The broad part of resonance A, as well as resonance D, appears to have much smaller $T_2$ and NOE values than resonances B and C. For these former resonances, NOE values even reach the lower limit of 1.1

Table II: Transverse Relaxation Times and NOE Values for the Methionine Resonance of [Met, $^{13}$C]-BR in Detergent and Membrane Systems

| sample | no. of Met residues[a] | chemical shift[b] (ppm) | NOE | $T_2$ (ms) |
|---|---|---|---|---|
| Triton X-100 solubilized BR | 9 | 17.2 | 1.1[c] | 13[c] |
| | | | 1.3 | 33 |
| | | 15.4 | 1.5 | 40 |
| | | 14.9 | 1.6 | 50 |
| | | 14.0 | 1.1 | 17 |
| Triton X-100 solubilized BR in 60% glycerol | ~3 | 15.5 | 1.4 | 50 |
| purple membrane | 0.3–0.7 | 15.1 | *d* | 35 |
| reconstituted liposomes at 5 °C | ~1 | 15.2 | 1.3 | 33 |
| reconstituted liposomes at 25 °C | ~1 | 15.2 | 1.3 | 34 |

[a] Determined from the NOE corrected integrals of $^{13}$C resonances. [b] With respect to tetramethylsilane. [c] The two NOE and $T_2$ values refer to the broad and narrow components of resonance A, respectively (see Figure 1). [d] Not determined.

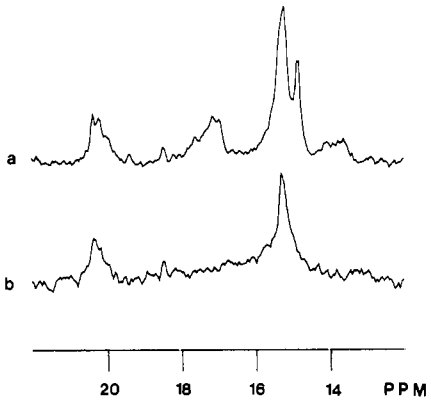


FIGURE 6: Effect of glycerol on the 75.4-MHz $^{13}$C NMR spectrum of [Met, $^{13}$C]-BR in 5% (w/v) Triton X-100/10 mM MES, pH 5, recorded at 20 °C. (a) No glycerol (b) 60% (v/v) glycerol.

(Doddrell et al., 1972). The narrower component of resonance A displays intermediate $T_2$ and NOE values. These data indicate that effective differences occur between the motional behavior of the various methionine residues in detergent-solubilized BR. Clearly, the motion of each methionine residue cannot be described by a single isotropic correlation time. For example, resonances B and C have very similar measured NOEs of 1.5 and 1.6 while values of 1.8 and 2.4 would be expected from an isotropic $\tau_c$ calculated from the $T_2$ (Doddrell et al., 1972). Rather, the above NMR parameters may depend on three types of motions: overall protein tumbling, wobbling motion of the methionine side chain, and terminal methyl rotation (Richarz et al., 1980). As explained under Discussion, only the wobbling motion is likely to differ significantly between methionine residues. To investigate further these features, similar experiments were performed in the presence of 60% (w/v) glycerol in order to assess the influence of the medium viscosity. Glycerol had no effect on the visible absorption spectrum of detergent-solubilized BR. As shown in Figure 6, in the presence of glycerol, part of the resonances of [Met, $^{13}$C]-BR disappear, being replaced by a broad featureless line. The fact that the broader resonances A and D are not longer detectable confirms the idea that the corresponding methionine residues undergo only a limited extent of wobbling motion. Such motion appears insufficient to yield relatively narrow $^{13}$C NMR lines after slowing down of the overall protein tumbling. On the other hand, the disappearance in part of resonance B and in total of resonance C can only be explained if the viscosity increase also has the effect of slowing down the initially high wobbling motion of the corresponding methionine residues. This is in agreement with the above spin-label data which indicated that these resonances contain the contribution of the two methionine residues from the hydrophilic BR surfaces that are broadened by tempamine. Finally, the remaining part of resonance B in the presence of glycerol displays line-width, $T_2$, and NOE values (Table II) that are little affected. This corresponds to methionine residues undergoing high wobbling motion so that their spectral parameters depend only slightly on the overall tumbling motion. In addition, these methionines must also be removed from contact with the aqueous medium. Although the presence of the broad component in Figure 6b precludes a precise integration of the narrow resonance, the corrected integral would be consistent with a contribution from three methionines per BR expected from spin-label experiments.

*Dynamics of Methionine Residues in Membrane-Bound BR.* Although detergent-solubilized BR is the main subject of this work, it was worth investigating whether the protein dynamic properties inferred above were still valid in a membrane-embedded state. Indeed, the previous glycerol experiment indicated that at least those methionine residues having a high extent of wobbling motion would yield narrow resonances even in the absence of rapid protein tumbling. A drawback was that membrane samples exhibited broad and intense natural-abundance lines. In the case of [Met, $^{13}$C]-BR in the native purple membrane, a narrow methionine methyl $^{13}$C resonance was observed above the natural-abundance signal (now shown). The chemical shift and $T_2$ values of this resonance were similar to those of the central resonances B and C in the detergent-solubilized protein spectrum (Table II). The intensity of this resonance varied from sample to sample and corresponded to approximately 0.3–0.7 methionine residue per BR. Such variability was due presumably to partial aggregation of purple membrane sheets since the results depended critically on sonication of the samples.

We also investigated the case of [Met, $^{13}$C]-BR reconstituted into liposomes with DEPC as phospholipid. DEPC undergoes a fluid–gel-phase transition at 13 °C (Silvius & Mc Elhaney, 1979). Freeze–fracture electron micrographs (not shown) indicated that BR was respectively dispersed and aggregated in patches above and below the phase transition as already reported with other lipids (Cherry et al., 1978). The $^{13}$C NMR spectra corresponding to both situations are shown in Figure 7 for both [$^{13}$C, Met]-BR and unlabeled BR. A small methionine resonance corresponding to one residue per BR is present in the labeled protein spectra both at 5 °C and at 25 °C. Again the chemical shift, $T_2$, and NOE values (Table II) are close to those of the central resonances B and C in the Triton X-100 solubilized [Met, $^{13}$C]-BR spectrum. Additionally, these NMR parameters appear markedly insensitive to the lipid-phase transition, that is, to the dispersed or aggregated character of the protein in the membrane.

## Discussion

The present high-resolution $^{13}$C NMR study of [Met, $^{13}$C]-BR was performed with the protein solubilized in the detergent Triton X-100. This detergent was chosen because of its high solubilizing power for purple membrane (Dencher & Heyn, 1982), its absence of natural-abundance $^{13}$C NMR

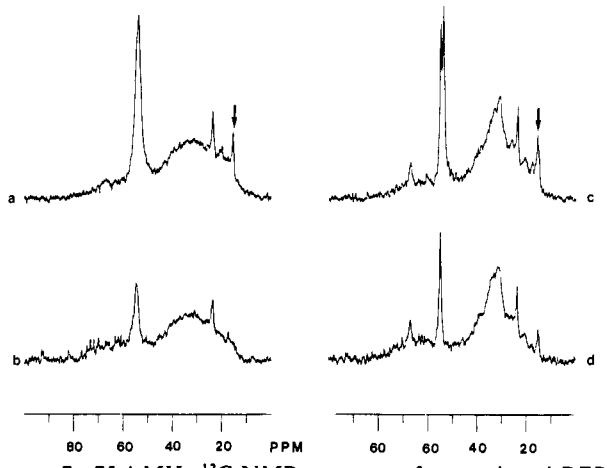


FIGURE 7: 75.4-MHz $^{13}C$ NMR spectrum of reconstituted DEPC liposomes containing [Met, $^{13}C$]-BR (a, c) or unlabeled BR (b, d) at a total lipid to protein ratio of 1.3:1 (mol/mol) in 125 mM potassium phosphate, pH 7, recorded at 5 °C (a, b) and 25 °C (c, d). The arrows indicate the position of the enriched [*methyl*-$^{13}C$]methionine resonance.

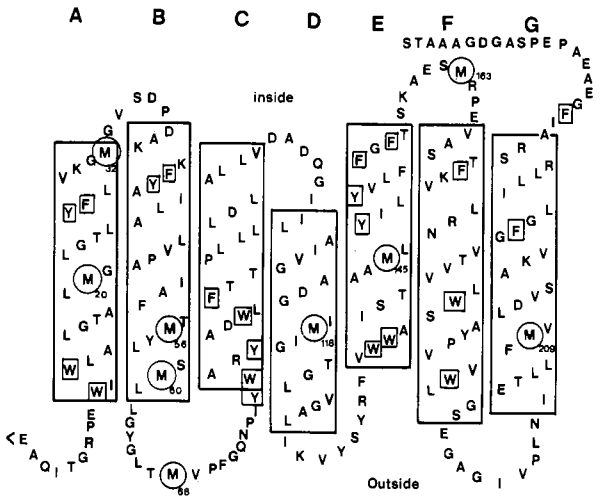


FIGURE 8: Transmembrane folding model of BR according to Henderson et al. (1990). The letters A–G correspond to $\alpha$-helical transmembrane segments. The nine methionine residues of BR are circled, and aromatic residues inside $\alpha$-helices are boxed.

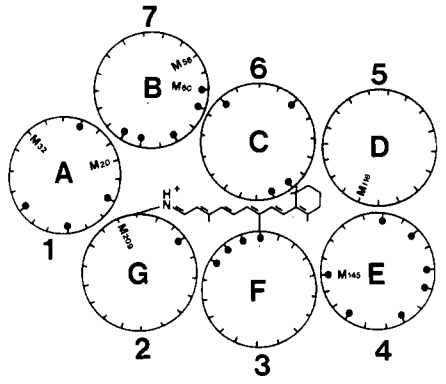


FIGURE 9: Helical wheel projection map of the tertiary structure of BR. Methionine residues are positioned in agreement with the rotational orientations of $\alpha$-helices proposed by Mogi et al. (1989). Closed circles correspond to the positions of aromatic residues.

lines in the spectral region of interest (unlike all other common detergents), and its ability to maintain the protein in a stable native state for several hours at room temperature. Previous studies have indicated that the Triton X-100 solubilized BR preserves most of the features of the membrane-associated protein, including secondary structure, retinal visible absorption spectrum around 560 nm, light–dark adaptation, and photocycling activity (Casadio et al., 1980; Mantele et al., 1981; London & Khorana, 1983). This indicates that the results obtained in the present study are relevant to the native protein.

Since it is interesting to discuss information on the dynamics and environment of methionine residues in light of their topography in the BR molecule, these latter data will be considered first. These were mainly derived from paramagnetic broadening experiments using spin-labels, the selectivity of which must be evaluated. The hydrophilic spin probe tempamine seems to slightly bind to protein–detergent micelles, as previously shown for membranes (Melhorn et al., 1982). However, since tempamine is positively charged at pH 5, such binding must be limited to the surface of the micelles so that the slight broadening effect of the detergent hydrophobic chains may be due to a markedly disordered character of the latter. The amphiphilic spin-label 16-doxylstearic acid locates only in the detergent–protein micelles but is probably not anchored at the interface at pH 5, again as already determined in membranes (Feix et al., 1984), so that it may probe the whole micelle. Nevertheless, the fair spatial selectivity of both spin-labels is indicated by the fact that BR methionine residues that were broadened by one probe were not broadened by the other except for one residue that seems to bear a particular location. Additionally, the paramagnetic broadening results are in agreement with those from proteolysis and glycerol effects. Namely, only the resonances that are affected by papain treatment and/or glycerol are those which are broadened by the hydrophilic spin-label.

The spin-label experiments indicated that two methionines residues of BR are located at or near the hydrophilic surfaces of BR. The most hydrophilic residue appears to be Met-68 from the cytoplasmic second loop of BR since it is the one removed by papain. The second hydrophilic residue is closer to the hydrophobic surface (being accessible to both tempamine and 16-doxylstearic acid) but is definitely water-exposed since its mobility is affected by glycerol. We tentatively assign this residue as Met-163 from the external fifth loop, on the basis of the BR folding model depicted in Figure 8 which is reproduced from Henderson et al. (1990). This model agrees with our data in that it assumes the presence of two hydrophilic methionine residues, Met-32 and Met-68. Of course, our data on methionine residue do not allow us to evaluate the whole folding model. Additionally, it is unlikely that BR residues that are water-exposed in the membrane become burried in the detergent micelle since, as shown here, the mobility of such residues is more restricted in the membrane.

Among the nine methionine residues of BR, seven thus appear to be located within the membrane-embedded segments. Our data indicate that three of these are located on the hydrophobic surface of the protein and the remaining four in the interior of the protein. Such locations are in fact in total agreement with the BR tertiary structure model recently proposed by Mogi et al. (1989) on the basis of mutagenesis (Mogi et al., 1988; Soppa et al., 1989) and neutron diffraction (Trewhella et al., 1986; Seiff et al., 1986) experiments. In Figure 9, we have reproduced this model and indicated the expected locations of the seven $\alpha$-helical methionine residues. Indeed, Met-32, Met-56, and Met-60 are found on the hydrophobic surface and Met-20, Met-118, Met-145, and Met-209 in the protein interior. The inward position of Met-20 is also in agreement with results by Popot et al. (1989) and Henderson et al. (1990). Additionally, Henderson et al. (1990) could not decide upon the orientations of helices B and D on the basis of electron diffraction data alone and had to invoke functional arguments. Our data appear consistent with the

outward orientation of Met-56 and the inward orientation of Met-118 that these authors inferred.

It is interesting to discuss in this context the chemical shift of methionine resonances in the $^{13}C$ NMR spectrum of detergent-solubilized [Met, $^{13}C$]-BR. It appears that one of the three hydrophobic surface methionine residues and three of the four interior methionine residues yield $^{13}C$ resonances that are strongly either downfield- or upfield-shifted. Such shifts are likely to be due to ring-current effects from a nearby aromatic amino acid (since a role for Triton X-100 has been excluded). Although transfer of electron density from the sulfur atom of methionine to a nearby positive charge has also been proposed as a downfield shifting effect (Blakey et al., 1978), in our case this is uncompatible with the fact that the corresponding $^1H$ chemical shifts are displaced upfield as judged from the $^{13}C$–$^1H$ COSY spectrum. In Figures 8 and 9, we have indicated the positions of $\alpha$-helical aromatic residues expected from folding and tertiary structure models. It seems that one hydrophobic surface methionine (Met-56) and three interior methionines (Met-20, Met-60, and Met-145) are expected to be close to such aromatic residues, again in agreement with our data. For two of these methionine residues, the corresponding resonances appear to be split in two, indicating heterogeneity among the BR molecules. This is likely to be due to the all-trans and 13-cis configurations of the retinal chromophore present in dark-adapted BR (Marcus & Lewis, 1978) that may induce small changes in the BR structure and modify the interaction between methionine and aromatic residues.

The local dynamics of methionine residues were also investigated by $^{13}C$ NMR for both detergent-solubilized and membrane-embedded [Met, $^{13}C$]-BR. Our analysis assumed very fast (i.e., $\tau_c < 10^{-10}$ s) methyl rotation for all residues in both types of samples. This is expected for any protein methyl group in solution, membrane, or crystal (Richarz et al., 1980; Keniry et al., 1983, 1984; Colnago et al., 1987). In addition, for the Triton X-100 solubilized BR, all methionine residues possess a significant degree of wobbling motion due to partial segmental rotations along the side chain and/or the peptide backbone. Indeed, with an overall tumbling correlation time of 150 ns for the detergent–protein complex [calculated by using the Stokes radius of 4.1 nm from Reynolds and Stoeckenius (1977) and a viscosity of 2.1 cP at 20 °C for the 5% Triton X-100 solution (unpublished results)], a $T_2$ value of 4.4 ms would be expected for methionine residues undergoing only fast methyl rotation (Doddrell et al., 1972). This value is much lower than those measured for detergent-solubilized [Met, $^{13}C$]-BR. Two motional classes of methionine residues appear to be present. For each BR, six residues appear to have a higher rate and amplitude of wobbling motion than the remaining three residues. As expected, the two residues located at the hydrophilic surface have a high extent of wobbling motion. This is also the case for the three residues of the hydrophobic surface, seemingly due to the highly fluid and disordered character of the hydrophobic interior of detergent micelles. Most residues located in the protein interior undergo, however, a much more limited extent of such wobbling motion as expected from the tight packing of transmembrane $\alpha$-helices (Henderson & Unwin, 1975). One interior methionine seems, however, to possess more wobbling motion. Flexibility of some parts of the BR molecule is indeed suggested by the fact that the protein can accommodate retinal analogues longer than the natural chromophore (Zinogi et al., 1986). High mobility of an internal methionine has also been found in myoglobin that also contains a prosthetic group (Jones et al., 1976). Taken together, these data indicate that the internal dynamics of detergent-solubilized BR are rather similar to those of a soluble protein, surface residues being, on the average, more mobile than internal residues.

A rather different situation is found for membrane-embedded BR in which only one methionine residue retains a high extent of wobbling motion. It is likely to be one of the two hydrophilic surface methionine residues, namely, Met-68 or Met-163. All other methionine residues appear to have their motion restricted by the membrane environment. Such restrictive effects appear to be due to embedding of the protein into a bilayer per se rather than to protein–protein interactions since it is observed in purple membrane as well as in liposomes whenever the protein is dispersed or aggregated. This means that the bulky lipid head groups, the relatively ordered paraffinic chains, and the membrane lateral pressure strongly restrict the BR surface and internal dynamics. However, it is probable that all methionine residues retain some wobbling motion in the membrane as shown by $^2H$ NMR for other residues in the purple membrane (Kinsey et al., 1981; Keniry et al., 1984). Such motions would not be detectable by conventional NMR experiments.

## Conclusions

The present work indicates that high-resolution $^{13}C$ NMR in association with biosynthetic labeling can be performed on detergent-solubilized BR. Relatively simple NMR experiments can yield information on the transmembrane folding, the tertiary structure, and the dynamics of the protein. Such an approach can be extended to other $^{13}C$-labeled positions and possibly also to other membrane proteins provided that sufficient local mobility is present to yield high-resolution NMR spectra.

## Acknowledgments

We are grateful to Dr. Tadeus Gulik-Krzywicki for performing electron microscopy experiments and to Drs. Mireia Duñach and Esteve Padrós for their help in setting up bacterial culture conditions. We sincerely thank Miss Aline Bluzat for skillful technical assistance.

**Registry No.** Met, 63-68-3.

## References

Abdulaev, N. G., Feigina, M. Y., Kiselev, A. V., Ovchinnikov, Y. A., Drachev, L. A., Kaulen, A. D., Khitrina, L. V., & Skulachev, V. P. (1978) *FEBS Lett. 90*, 190–194.

Agard, D. A., & Stroud, R. M. (1982) *Biophys. J. 37*, 589–602.

Arseniev, A. S., Maslennikov, I. V., Bystrov, V. F., Koznich, A. T., Ivanov, V. T., & Ovchinnikov, Y. A. (1988) *FEBS Lett. 231*, 81–88.

Blakey, R. L., Coco, L., London, R. E., Walker, T. E., & Matwiyoff, N. A. (1978) *Biochemistry 17*, 2284–2292.

Bowers, J. L., & Oldfield, E. (1988) *Biochemistry 27*, 5156–5161.

Cherry, R. J., Muller, U., Henderson, R., & Heyn, M. P. (1978) *J. Mol. Biol. 121*, 283–298.

Colnago, L. A., Valentine, K. G., & Opella, S. J. (1987) *Biochemistry 26*, 847–854.

Dencher, N. A. (1983) *Photochem. Photobiol. 38*, 753–767.

Dencher, N. A., & Heyn, M. P. (1982) *Methods Enzymol. 88*, 5–10.

Doddrell, D., Glushko, V., & Allerhand, A. (1972) *J. Chem. Phys. 56*, 3681–3689.

Feix, J. B., Popp, C. A., Venkataraman, A. H., Beth, J. A., & Hyde, J. S. (1984) *Biochemistry 23*, 2293–2299.
Fimmel, S., Choli, T., Dencher, N. A., Buldt, G., & Wittmann-Liebold, B. (1989) *Biochim. Biophys. Acta 978*, 231–240.
Gulik-Krzywicki, T., Seigneuret, M., & Rigaud, J. L. (1987) *J. Biol. Chem. 262*, 15580–15588.
Gutowitz, H., Mowery, P., Taylor, M., & Stoeckenius, W. (1980) *Biochim. Biophys. Acta 590*, 13–23.
Harbison, G. S., Herzfield J., & Griffin, R. G. (1983) *Biochemistry 22*, 1–5.
Harbison, G. S., Smith, S. O., Pardoen, J. A., Mulder, P. P., Lugtenburg, J., Herzfeld, J., Mathies, R. & Griffin, R. G. (1984) *Biochemistry 23*, 2662–2667.
Harbison, G. S., Smith, S. O., Pardoen, J. A., Courtin, J. M., Lugtenburg, J., Herzfeld, J., Mathies, R. A., & Griffen, R. G. (1985) *Biochemistry 24*, 6955–6962.
Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F.,, Beckman, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899–929.
Huang, K. S., Radakrishnan, R., Bayley, H., & Khorana, H. G. (1982) *J. Biol. Chem. 257*, 13616–13623.
Jap, K. J., Maestre, M. F., Hayward, S. B., & Glaeser, R. M. (1983) *Biophys. J. 43*, 81–89.
Jones, W. C., Rothgeb, T. M., & Gurd, F. R. N. (1976) *J. Biol. Chem. 251*, 7452–7460.
Keniry, M. A., Rothgeb, T. M., Smith, R. L., Gutowsky, H. S., & Oldfield, E. (1983) *Biochemistry 22*, 217–226.
Keniry, M. A., Kintanar, A., Smith, R. L., Gutowsky, H. S., & Oldfield, E. (1984) *Biochemistry 23*, 288–298.
Kinsey, R. A., Kintanar, A., & Oldfield, E. (1981) *J. Biol. Chem. 256*, 4146–4149.
Le Maire, M., Aggerbeck, L. P., Monteilhet, C., Andersen, J. P., & Moller, J. V. (1986) *Anal. Biochem. 154*, 524–535.
Lewis, B. A., Harbison, G. S., Herzfeld, J., & Griffin, R. G. (1985) *Biochemistry 24*, 4671–4679.
Liao, M. J., London, E., Khorana, H. G. (1983) *J. Biol. Chem. 258*, 9949–9955.
London, E., & Khorana, H. G. (1982) *J. Biol. Chem. 257*, 7003–7011.
Mantele, W., Siebert, F., & Kreutz, W. (1981) *FEBS Lett. 128*, 249–254.
Marcus, M. A., & Lewis, A. (1978) *Biochemistry 17*, 4722–4735.
Melhorn, R. J., Landau, P., & Packer, L. (1982) *Methods Enzymol. 88*, 751–762.
Miercke, L. J. W., Ross, P. E., Stroud, R. M., & Dratz, E. A. (1989) *J. Biol. Chem. 264*, 7531–7535.
Mogi, T., Stern, L. J., Chao, B. H., & Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5595–5599.
Mogi, T., Marti, T., & Khorana, H. G. (1989) *J. Biol. Chem. 264*, 14197–14201.
Nabedrik, E., Bardin, A. M. & Breton, J. (1985) *Biophys. J. 48*, 873–876.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667–678.
Ohnishi, H., McCance, M. E., & Gibbons, N. E. (1965) *Can. J. Microbiol. 11*, 365–373.
Popot, J. L., Engelman, D. M., Gurel, O., & Zaccai, G. (1989) *J. Mol. Biol. 210*, 829–847.
Reynolds, J. A., & Stoeckenius, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2803–2804.
Richarz, R., Nagayama, K., & Wuthrich, K. (1980) *Biochemistry 19*, 5189–5195.
Seiff, F., Westherhausen, J., Wallat, I., & Heyn, M. P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7746–7750.
Silvius, J. R., & Mc Elhaney, R. M. (1979) *Chem. Phys. Lipids 25*, 125–134.
Soppa, J., Otomo, J., Straub, J., Tittor, J., Meessen, S., & Oesterhelt, D. (1989) *J. Biol. Chem. 264*, 13049–13056.
Trewhella, J., Popot, J. L., Zaccai, G., & Engelman, D. M. (1986) *EMBO J. 5*, 3045–3049.
Wilde, J. A., & Bolton, P. H. (1984) *J. Magn. Reson. 59*, 343–346.
Zinogi, J., Or, Y. S., Crouch, R. K., Hang, C. H., Govindjee, R., & Ebrey, T. G. (1986) *Biochemistry 25*, 2022–2027.