# Dramatic in situ conformational dynamics of the transmembrane protein bacteriorhodopsin

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ABSTRACT The conformational dynamic capabilities of the in situ bacteriorhodopsin (bR) can be studied by determination of the changes of the bR net helical segmental tilt angle (the angle between the polypeptide segments and the membrane normal) induced by various perturbations of the purple membrane (PM). The analysis of the far-UV oriented circular dichroism (CD) of the PM provides one means of achieving this. Previous CD studies have indicated that the tilt angle can change from ~10° to 39° depending on the perturbants used with no changes in the secondary structure of the bR. A recent study has indicated that the bleaching-induced tilt angle can be enhanced from ~24° to 39° by cross-linkage and papain-digestion perturbations which by themselves do not alter the tilt angle. To add further credence, this study has been repeated using midinfrared (IR) linear dichroic spectral analysis. In contrast to the CD method, analysis by the IR method depends on the orientation of the amide plane of the helix assumed. Excellent consistency is achieved between the two methods only when it is assumed that the structural characteristics of the α-helices of the bR are equally $\alpha_I$ and $\alpha_{II}$ in nature. Furthermore, the analysis of the IR data becomes essentially independent of the three amide transitions utilized. The net tilt angle of segments completely randomized relative to the incident light must be 54.736 in view of helix symmetry. A value of 54.735° ± 0.001° was achieved by the IR method for the ethanol-treated PM film, establishing this kind of film as an ideal random state standard and demonstrating the accuracy potential of the IR method.

## INTRODUCTION

The bacteriorhodopsin, bR, which is a chromoprotein with a Schiff-base bound retinylidene prosthetic group, is presently the best-characterized example of a transmembrane protein. It is considered by many to be the paradigm of transmembrane transport proteins (for comprehensive reviews, see Henderson, 1977; Eisenback and Caplan, 1979; Stoeckenius et al., 1979, 1981; Ottolenghi, 1980; Stoeckenius and Bogomolni, 1982; Dencher, 1983; Henderson et al., 1990, and references cited therein). For well over a decade the major research thrust of this laboratory has been directed toward an understanding of the molecular dynamical capabilities of this protein in its native lipid bilayer environment in the purple membrane, PM, of *Halobacterium halobium* (Becher and Cassim, 1976, 1977; Muccio and Cassim, 1979*a*, *b*; Draheim and Cassim, 1985*a*; Draheim et al., 1988*a*; Gibson and Cassim, 1989*a*, *b*).

The bR protein consists of a single 26,866 D polypeptide chain of 248 amino acids. It is the sole protein in the PM, comprising 75% of its dry weight with the remainder being lipids. Approximately 80% of the amino acids of the bR are enclosed within the bilayer of the PM.

The bilayer-spanning polypeptide segments are mostly α-helical in secondary structure whereas those positioned outside are mostly aperiodic (Henderson, 1977). However, the α-helices are not exclusively of the $\alpha_I$-geometric type in which the amide planes are oriented nearly parallel to the helical axis. They exhibit equal $\alpha_I$ and $\alpha_{II}$ structural characteristics which result in the amide planes being on the average oriented at a significant angle to the helix axis (Krimm and Dwivedi, 1982; Vogel and Gärtner, 1987; Gibson and Cassim, 1989*a*).

The sevenfold folding of the polypeptide forms the tertiary structure of the bR. The resulting transmembrane polypeptide segments are close packed (Henderson, 1977). Three-dimensional imaging of the molecular structure of the bR in the PM at progressively improved resolutions have been realized over the last decade and a half due in large part to the electron imaging and diffraction studies of Henderson and co-workers (Henderson and Unwin, 1975; Henderson et al., 1990). The proposed structural model of the bR emerging from these studies indicates a tilt of some of these segments away from the normal of the membrane resulting in an average tilt angle per segment of ~11°. However, it is not clear whether this view of the segmental orientation of the bR is a characteristic of the native PM or of a structurally modified form of the PM induced by the experimental conditions imposed on the PM during these studies (Henderson and Unwin, 1975; Hayward

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and Stroud, 1981; Jaffe and Glaeser, 1987; Henderson et al., 1990; Mitra and Stroud, 1990). There is evidence from the analysis of the oriented far-UV circular dichroism, CD, of the PM that subjection of the PM to some of the similar conditions can result in significant segmental tilting (Draheim et al., 1988*a*; Gibson and Cassim, 1989*c*). The orientations of the segments of the bR has also been studied by midinfrared, IR, linear dichroism. However, calculation of the net segmental tilt angle from the observed dichroic ratio of the amide bands are strongly dependent on the model of the α-helix chosen. If the parameters of a normal type $\alpha_I$-helix are used in calculations, a net tilt angle of $\sim 29°$ is obtained (Rothschild and Clark, 1979; Nabedryk and Breton, 1981; Aldashev, 1985). On the other hand, if the parameters of a slightly distorted type of $\alpha_I$-helix with hydrogen bonds somewhat longer than in the normal type are used, the calculated net tilt angle is reduced to $\sim 11°$ (Nebadryk and Breton, 1981), while the use of the parameters of an equal combination of normal $\alpha_I$- and $\alpha_{II}$-helices yields a net tilt angle of about zero degrees (Draheim et al., 1988*b*). This result is consistent with the profile of the oriented film far-UV CD spectrum of the PM which also suggests a net tilt angle of about zero degrees independent of the α-helix type chosen for the segments (Muccio and Cassim, 1979*a*).

The bR molecules are organized as trimeric clusters arranged in a hexagonal lattice forming a two-dimensional crystal. In over a decade and a half an impressive body of experimental and theoretical evidence has been accumulated consistent with an exciton model of interactions among the retinylidene $\pi - \pi^*$ induced transition dipole moments of the bR's in the PM (Becher and Cassim, 1975*a*; Heyn et al., 1975, 1977*a*, *b*; Bauer et al., 1976; Becher and Ebrey, 1976; Kriebel and Albrecht, 1976; Cherry et al., 1977, 1978; Ebrey et al., 1977; Dencher and Heyn, 1978; Muccio and Cassim, 1979*a*, *b*; Casadio and Stoeckemius, 1980; Papadopoulos and Cassim, 1981*a*; Klausner et al., 1982; Draheim and Cassim, 1985*a*,*b*; Draheim et al., 1988*a*). Recently, El-Sayed and co-workers have discussed a number of experimental observations which they claim could cast serious doubts on the viability of this exciton model of the PM (El-Sayed et al., 1981; El-Sayed et al., 1989; Jang et al., 1990). These observations are (*a*) fluorescence and absorption of the $K_{610}$ photointermediate of the bR are highly polarized with respect to the initial polarized excitation, (*b*) absence of a biphasic magnetic circular dichroism (MCD) spectrum for $bR_{568}$, (*c*) $K_{610}$ and $L_{550}$ photointermediates, in which the trimeric structure is destroyed by the isomerization of one of the three retinals, exhibit CD spectra similar to that of $bR_{568}$ in 58–65% glycerol solutions based on a study of Zimányi et al. (1987), and (*d*) the mutagenic substitution of Tyn (185) by Phe is found to greatly change the CD in Y185F as compared with eBR. However, these observations can be shown not to present any serious determent to this model if interpreted properly in conjunction with a number of other published observations, respectively. These are (*a*) relatively large interchromophoric distances in the PM which are indicative of weak-coupling excitons and slow energy transfer time that suggest photochemical processes occur faster than energy transfer (King et al., 1980; Muccio and Cassim, 1981), (*b*) expectation that the magnetic moment of the double-degenerate excitonic state of the bR trimer may be insignificantly small (El-Sayed et al., 1989), (*c*) the monomeric bands of the bR exhibit negligible rotational power in 58–65% glycerol solutions (Draheim and Cassim, 1985*b*) and the excitonic-induced CD of dimers is not expected to be significantly different from that of the cyclic trimers (Ebrey et al., 1977), and (*d*) the sign of the bR monomeric CD band can be changed by a reversal of the sign of the net static charge distribution surrounding the chromophore or by a reversal of the screw sense of the physical twist imposed on the chromophore as a result of a mild structural perturbation of the bR (Draheim and Cassim, 1985*b*).

Analysis of the oriented film far-UV CD spectra of the PM based on the molecular geometry of the PM and formalism of the exciton model of α-helical proteins have indicated that the segments of bR can tilt significantly as a result of certain perturbations of the PM. The results are summarized in Table 1. The tilt-inducing effects of almost all of these perturbants are reversible. Also, in all cases studied, the secondary structure of the bR is completely invariant to these perturbants.

The fact that an electron imaging and diffraction analysis of the PM failed to detect any significant structural differences due to the induction of the $M_{412}$ state from the ground $bR_{568}$ state is not surprising in view of the experimental conditions used which included one if not more of the tilt-causing perturbants listed in Table 1 (Glaeser et al., 1986). The effects of the $M_{412}$ transformation were probably masked by the stronger effects of these perturbants. Initial polarized IR spectroscopic studies failed to detect any significant segmental tilt angle changes (Bagley et al., 1982; Earnest et al., 1986; Nabedryk and Breton, 1986). However, a more recent study detected changes attributable to alterations in the bR backbone (Braiman et al., 1987). It was concluded that these changes were inconsistent with the CD detected tilt-angle change unless there were no significant changes in the bR secondary structure. Both solution and film far-UV spectral studies of the PM have clearly demonstrated no observable secondary structure

TABLE 1 **Analysis of oriented far-UV circular dichroic spectra of purple membrane: calculated bacteriorhodopsin segmental tilt angles, $\Theta_\alpha$, for various perturbations**

| Perturbation | $\Theta_\alpha$ in degrees‡‡ |
|---|---|
| None‡ | ~0 |
| Light bleached† | 24 |
| Cross-linked‡ | ~0 |
| Cross-linked light bleached*‡ | 32 |
| Mildly papain digested‡ | ~0 |
| Mildly papain digested light bleached‡ | 24 |
| Moderately papain digested‡ | ~0 |
| Moderately papain digested light bleached*‡ | 35 |
| Extensively papain digested‡ | ~0 |
| Extensively papain digested light bleached*‡ | 39 |
| Vacuum dehydrated§ | 21 |
| Dry glycerol impregnated§ | 19 |
| Vacuum dehydrated glycerol impregnated§ | 22 |
| Glucose embedded‖ | 24 |
| $M_{412}$ state¶ | 10 |
| Sodium borohydride reduced** | ~0 |
| Sodium borohydride reduced, extensively white light radiated** | 38 |

*In the presence of hydroxylamine.
‡Gibson and Cassin (1989*b*).
§Draheim et al. (1988*a*).
‖Gibson and Cassim (1988*c*).
¶Draheim and Cassim (1985*a*).
**Gibson (1988).
‡‡Calculated from the relationship $\Theta_\alpha = \sin^{-1}(2R_\alpha/3R_0)^{1/2}$, where $R_\alpha$, $R_0$ are the magnitudes of the 206–207 nm rotatory band at any tilt angle $\Theta_\alpha$ and the tilt angle when the segments are randomly oriented, respectively. $R_0$ has been obtained from CD measurements of ethanol-treated PM films.

involvement in the $bR_{568}$ to $M_{412}$ transition (Draheim and Cassim, 1985*a*).

Although the oriented CD revealed tilting phenomenon stands on firm theoretical grounds, substantiated by experimental verification; to add further credence, the bleaching studies summarized in Table 1 were repeated using mid-IR linear dichroic spectral analysis. In contrast to the CD method, analysis by this method cannot yield a unique tilt angle but a range of tilt angles dependent on the experimentally determined dichroic ratios and the physical characteristics of the helices assumed. It is shown that excellent consistency is achieved between the two methods only when it is assumed that the structures of the α-helices contain equal $\alpha_I$ and $\alpha_{II}$ type characteristics. That is, the amide planes of bR helices are on average tilted significantly from the helix axis. Furthermore, such an assumption also results in selfconsistent calculated tilt angles which are independent of the three amide transition (amide A, amide I, and amide II) used for analysis. The net tilt angle of oriented segments completely random to the incident light should be theoretically 54.736° in view of the helix geometry. A value of 54.735° ± 0.001° was achieved by this method, demonstrating its potential for accuracy. It is concluded that there is now evidence, both theoretical and experimental, from two independent spectral methods which are only in accord with a structurally dynamic model for in situ bR in contrast to a more static one now in vogue.

## MATERIALS AND METHODS

### PM preparation

PM was isolated from the S9 strain of *Halobacterium halobium* according to the method of Becher and Cassim (1975*b*) with the omission of the sucrose gradient step. However, many washing steps were incorporated into the purification process. Although this modified procedure sharply reduced the PM yield, it produced preparations of equal or greater purity without exposing the PM to sucrose (Draheim and Cassim, 1985*b*). Centrifugations were accomplished at the relatively low speed of 22,000 *g* for 90–120 min. This produced preparations uncontaminated by red membrane.

Sodium azide (0.05%) was added whenever membrane suspensions were stored to prevent changes in membrane structure due to possible action of bacterial proteolytic enzymes. However, membrane suspensions were never stored for more than a few days before use.

### PM modifications

The cross-linking of the bR with dimethyl adipimidate, 10 mM in 0.2 M borate buffer at pH 10 (Pierce Chemical Co., Rockford, IL), was carried out by a method described by Konishi et al. (1979). Papain digestion of the PM was done by suspending PM at a concentration of 0.4 mg/ml in 20 mM phosphate buffer at pH 7 containing 5 mM cysteine and 1.5 mM ethylenediaminetetraacetic acid (EDTA). Papain (Sigma Chemical Co., St. Louis, MO; type III, 16–40 U/mg protein) was added to give a bR/papain ratio of 20:1. The mixture was incubated at 37°C for 1 wk. The samples were washed repeatedly in double distilled demineralized water after digestion. Bleaching with hydroxylamine HCl, 0.3 M in 20 mM phosphate buffer at pH 6.5 (Sigma Chemical Co.) was achieved by methods described by Muccio and Cassim (1979*a*). Ethanol-treated PM films were made by adding USP quality ethanol (IMC Chemical Group, Inc., Terre Haute, IN) dropwise to the film until the film color completely changed from purple to yellow. The ethanol was then allowed to evaporate from the film in a 90% relative humidity environment for at least 24 h before spectral measurements. Both acetone and diethylether treatments produced identical spectral changes in PM films as those produced by the ethanol treatment.

### Film preparation

The PM was suspended in double-distilled water to an $OD_{568}$ of ~5. In the case of bleached PM, which lacks appreciable absorbance at 568

nm, the suspensions were made to match the $OD_{280}$ of the native ones. The suspensions were filtered using model GA-1 5-μm filters (Gelman Sciences Inc., Ann Arbor, MI) and degassed. Approximately 400 μL aliquots of the membrane suspensions were then layered onto a 25-mm diam calcium fluoride crystal IR flats (Spectra-Tech, Inc., Stamford, CT) and allowed to dry overnight in a sealed chamber at 30% relative humidity. Films were incubated for at least 24 h at 90% relative humidity before spectral measurements.

## FTIR linear dichroism spectroscopy

IR spectra were collected using a 60SX Fourier transform infrared spectrometer (Nicolet, Madison, WI). Purged air spectral references were collected on a daily basis and data collection parameters were set for 4 $cm^{-1}$ resolution using Happ-Genzel apodization. 500 scans were collected for both sample and background files. Linear polarization of the IR signal beam was accomplished using a Harrick PSD-JIR diamond crystal of Brewster angle design (Harrick Scientific, Ossining, NY). A specially designed holder oriented the film normals 45° with respect to the signal beam. Each sample file was carefully referenced to a background spectrum of the appropriate polarization.

## Data analysis

Linear dichroism measurements were made on PM films whose normals were oriented at an angle of 45° with the IR incidented beam. Therefore, the horizontally polarized radiation, $E_H$, was polarized in the plane of the film whereas the vertically polarized one, $E_V$, was polarized 45° out-of-the plane of the film.

The orientation of the bR helices were determined by the following equation, which was derived in a manner analogous to the derivation published by Bazzi and Woody (1985):

$$D = \frac{A_V}{A_H} = 1 + \frac{3\sin^2\theta_f}{n^2}\left[\frac{f_I S_I + f_{II} S_{II}}{f_I(1 - S_I) + f_{II}(1 - S_{II}) + f_u}\right].$$

Where $D$ is the observed dichroic ratio at a given frequency, $A_V$ is the absorbance of $E_V$, $A_H$ is the absorbance of $E_H$, $\theta_f$ is the film tilt angle (45° in this study), $n$ is the refractive index of PM film (estimated to be 1.5, see Papadopoulos and Cassim, 1981*b*), $f_I$, $f_{II}$, and $f_u$ are the fraction of residues in $\alpha_I$-helix, $\alpha_{II}$-helix, and aperiodic secondary structure, respectively, $S_I$ and $S_{II}$ are the total order parameters for the $\alpha_I$-helix and the $\alpha_{II}$-helix, respectively.

$S_I$ and $S_{II}$ were determined from the following relationships

$$S_I = S_m S_a S_{MI}$$
$$S_{II} = S_m S_a S_{MII},$$

where $S_m$, $S_a$, $S_{MI}$, and $S_{MII}$ are the mosaic spread, helix axis, $\alpha_I$ amide transition dipole moment and $\alpha_{II}$ amide transition dipole moment order parameters, respectively, which describe four nested axially symmetric cones as illustrated in Fig. 1. The angles of these cones can be calculated from an equation of the type:

$$S = \frac{3\cos^2\Theta - 1}{2},$$

where $S$ is $S_m$, $S_a$, $S_{MI}$, or $S_{MII}$ and $\Theta$ is $\Theta_m$, $\Theta_a$, $\Theta_{MI}$, or $\Theta_{MII}$, respectively. $\Theta_m$ is the mosaic spread tilt angle (the angle between the film normal and the membrane normal). In previous calculations, $\Theta_m$ values have been assumed to be in the range of 0–15° (Rothschild and Clark, 1979; Nabedryk and Breton, 1981; Aldashev, 1985; Bazzi and Woody, 1985). In the present calculations, $\Theta_m$ was assumed to be 10°. $\Theta_a$ is the α-helical axis tilt angle (the angle between the helix axis and the membrane normal). $\Theta_{MI}$ and $\Theta_{MII}$ are the amide transition dipole moment angles for $\alpha_I$ and $\alpha_{II}$ helices, respectively (the angle between the transition moment and the helix axis). They were determined from the following relationship:

$$\cos\Theta_M = \cos\gamma\cos(\epsilon + \varsigma),$$

where $\gamma$ is the angle between the amide plane and the helix axis, $\epsilon$ is the angle between C = O bond and the projection of the helix axis on the amide plane, and $\varsigma$ is the angle between the amide transition dipole moments and the C = O bond direction in the amide plane.

For $\alpha_I$-helix $\varsigma$ is 10°, 15°, and 30° for the amide A (N – H stretching), amide I (C = O stretching), and amide II (N – H deformation) transition dipole moment orientations, respectively (Ambrose and Elliot, 1951; Sandeman, 1955; Tsuboi, 1962). The amide plane of the $\alpha_I$-helix is nearly parallel to the helix axis so that $\gamma$ and $\epsilon$ normally lie well within the range $0 < \gamma < 10°$ and $7 < \epsilon < 13°$ (Tsuboi, 1962; Fraser and MacRae, 1973). Using these ranges, $\Theta_{MI}$ becomes 17–25° for the amide A, 22–29° for the amide I, and 82–88° for the amide II transition dipole moment orientations (Rothschild and Clark, 1979). The $\alpha_{II}$-helix differs structurally from the $\alpha_I$-helix only in dihedral angles ($\alpha_I$, $\phi$ 127°, $\Psi$ 128°; and $\alpha_{II}$, $\phi$ 87°, $\Psi$ 162°). This results in the amide plane in the $\alpha_{II}$-helix being significantly tilted in respect to the helix axis (Némethy et al., 1967). With molecular modeling (based on previous theoretical calculations [Leach et al., 1968] which have indicated that while the peptide NHO and COH bond angles are 157° and 164°, respectively, for the $\alpha_I$-helix, they are 109° and 118°, respectively, for the $\alpha_{II}$-helix), it can be shown that for this helix, $\gamma$ and $\epsilon$, conservatively lie within the range $35 < \gamma < 45°$ and $10° < \epsilon < 16°$. Therefore, $\Theta_{MII}$ becomes 40–51° for amide A, 42–53° for amide I, and 86–91° for amide II transition dipole moment orientations.

## RESULTS AND DISCUSSION

The polarized IR spectra of oriented PM films in the 940–4,000-$cm^{-1}$ region are shown in Fig. 2. The films were formed from unperturbed and chemically perturbed PM's (cross-linked with dimethyl adipimidate, 1 wk papain-digested or ethanol-treated) both unbleached and light-bleached in the presence of hydroxylamine. Amide A, amide I, and amide II transition frequencies of the bR's for the various PM film samples are given in Table 2. The frequencies for both vertical and horizontal polarization are very similar for the unperturbed and perturbed PM's before bleaching except for the ethanol-treated case. Upon bleaching, there is a significant reduction in the frequencies of the amide A and amide I transitions but not in that of the amide II transition. This change in frequencies due to bleaching is also very similar for the unperturbed and the perturbed PM samples. Comparison of the unperturbed PM amide frequencies with those of ethanol-treated PM indicates even a much greater reduction in the amide A and amide I frequencies upon ethanol treatment as well as a significant reduction in the amide II frequency in this case.

Previous observations of spectral changes of a somewhat similar nature during the light bleaching of PM

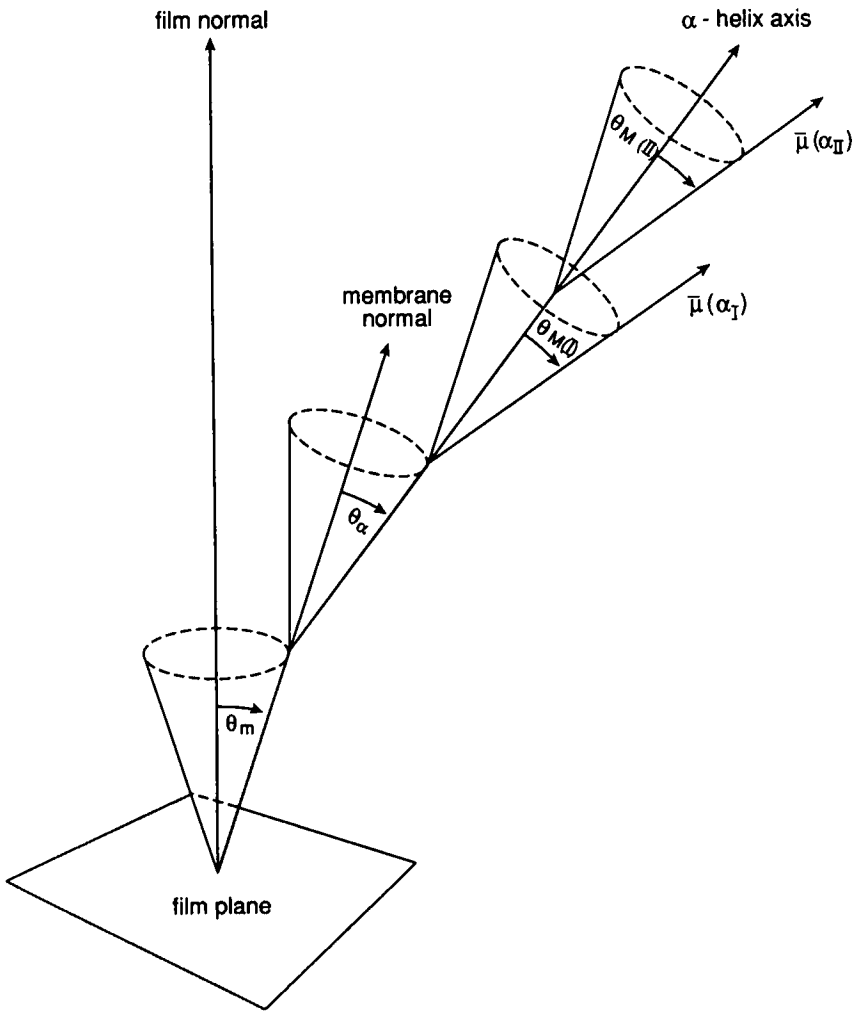


FIGURE 1 The geometry of the four nested axially symmetric cones utilized for data analysis of the polarized IR spectra of oriented PM films in the mid-IR region. For details, see Data Analysis.

with hydroxylamine were interpreted as indicative of a decrease in the $\alpha_{II}$-helix content in favor of the $\alpha_{I}$-helix content of the bR (Duñach et al., 1989). However, this interpretation is inconsistent with the results of oriented film far-UV CD studies in which the incident light is parallel to the membrane normal and helix axis. Bleaching of the PM results in no observable changes in the CD band attributed to the $n$-$\pi^*$ transition of the bR amide group (Gibson and Cassim, 1989*b*). The dipole moment of this transition is directed along the C=O bond of the amide group and is, therefore, oriented nearly parallel to the helix axis in the $\alpha_{I}$-helix, but significantly away from the helix axis in the $\alpha_{II}$-helix (Leach et al., 1968). Any appreciable change of α-helix types of the kind suggested is expected to decrease the magnitude of the $n$-$\pi^*$ CD band observably. Also, it is noteworthy that while the frequencies of the amide transitions of the unperturbed PM are consistent with significant amounts of $\alpha_{II}$-helix, those of the ethanol-treated PM are essentially consistent with $\alpha_{I}$-helix only (Miyazawa, 1960; Krimm and Dwivedi, 1982). If ethanol treatment causes most, if not all, of the $\alpha_{II}$-helix types to be transformed to the $\alpha_{I}$-helix type, then the far-UV CD of the PM should demonstrate a very large increase in elliptices as a result of such perturbation due to the change of the amide planes of the $\alpha_{II}$-helices toward the helix axes (Parrish and Blout, 1972). CD studies have demonstrated no such changes (Gibson, 1988; Gibson and Cassim, 1989*a*). However, these frequency changes may best be correlated with the large changes in the near-UV CD of the PM observed previously during the bleaching process which were attributed to changes in the environments and interactions of the aromatic amino acid side chains of the bR during this process (Muccio and Cassim,

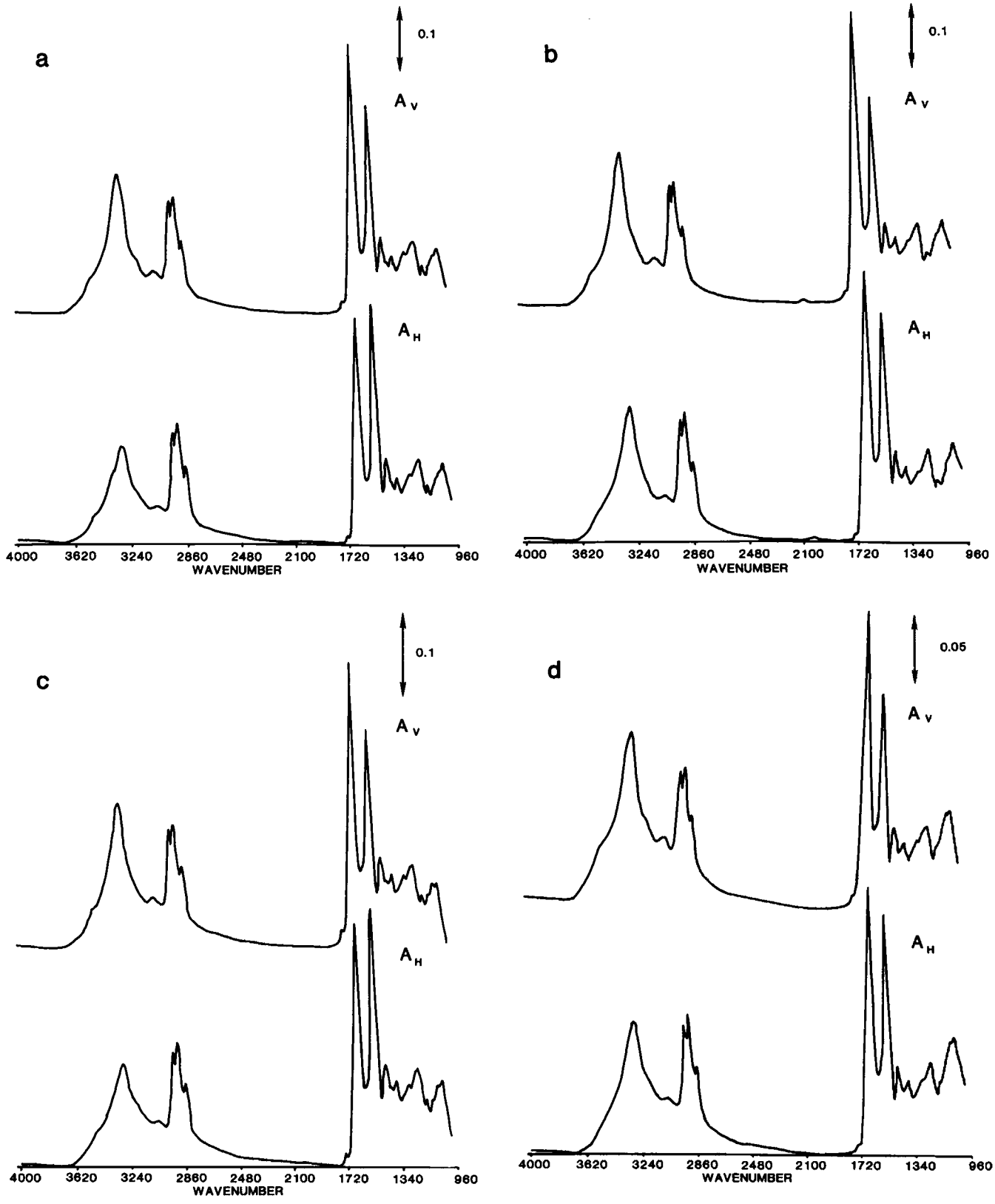


FIGURE 2 Dichroic mid-IR absorption of PM films, $A_V$, $A_H$ vertical and horizontal polarization, respectively. Spectra and perturbations of PM: (*a*) none; (*b*) light-bleached; (*c*)cross-linked; (*d*) cross-linked light-bleached;

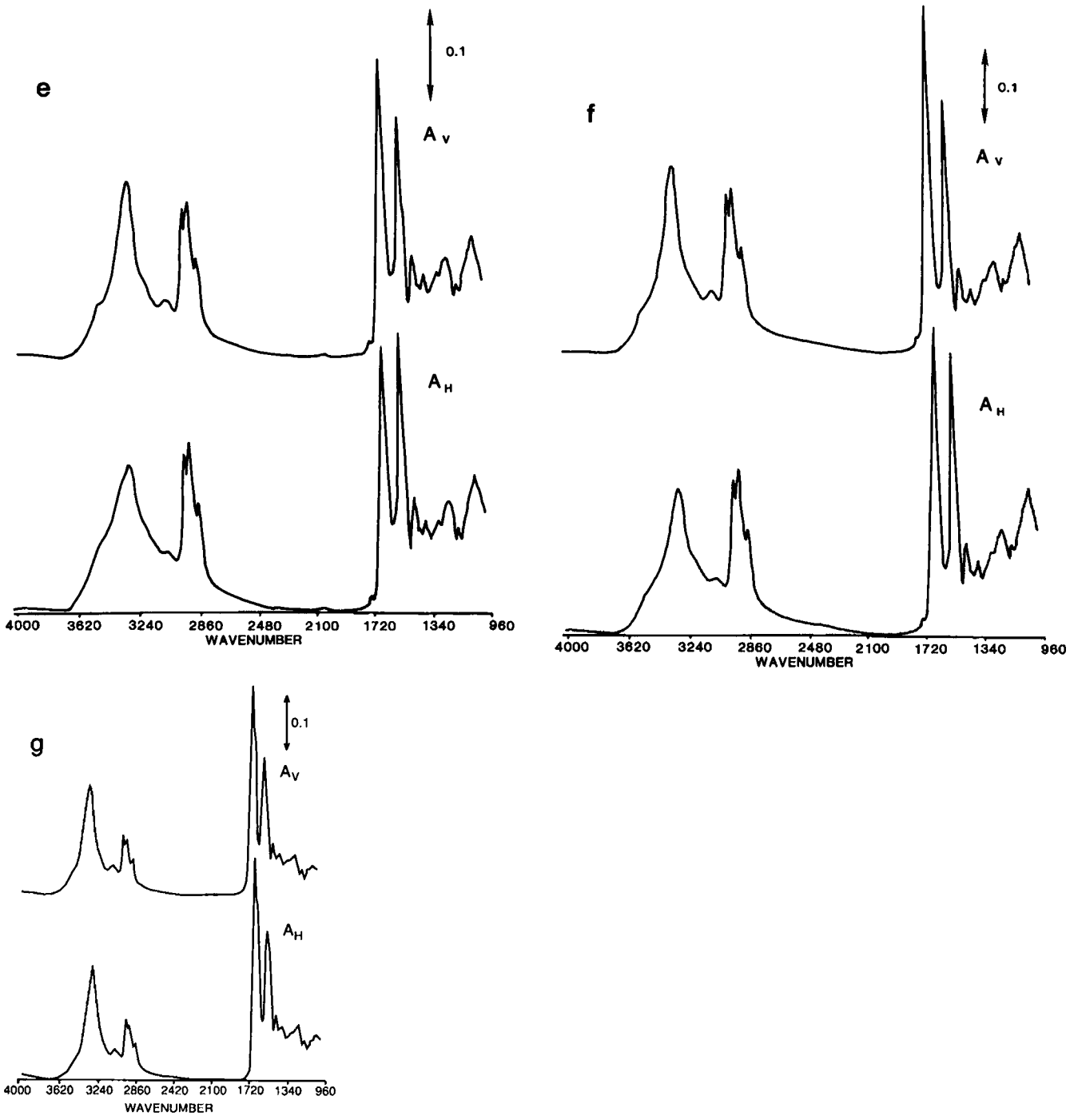


FIGURE 2 (continued) (*e*) moderately papain-digested; (*f*) moderately papain-digested light-bleached; and (*g*) ethanol-treated. All light bleaching in the presence of hydroxylamine. The arrows indicate absorbance scales.

TABLE 2 **Effect of various perturbations on the frequencies of the IR amide bands of bacteriorhodopsin**

| Perturbation | Amide band | Frequency ($cm^{-1}$) $A_V$ | $A_H$ |
|---|---|---|---|
| None | A | 3306.5 | 3303.4 |
| | I | 1664.5 | 1659.3 |
| | II | 1544.2 | 1543.9 |
| Light bleached* | A | 3303.1 | 3300.3 |
| | I | 1662.0 | 1658.5 |
| | II | 1544.7 | 1543.9 |
| Cross-linked | A | 3305.2 | 3301.0 |
| | I | 1664.6 | 1658.7 |
| | II | 1544.4 | 1544.2 |
| Cross-linked light bleached* | A | 3303.0 | 3300.2 |
| | I | 1661.7 | 1658.3 |
| | II | 1544.9 | 1544.9 |
| Moderately papain digested | A | 3306.9 | 3303.0 |
| | I | 1664.0 | 1659.1 |
| | II | 1545.0 | 1544.9 |
| Moderately papain digested light bleached* | A | 3304.3 | 3300.4 |
| | I | 1661.7 | 1658.6 |
| | II | 1545.3 | 1545.0 |
| Ethanol treated | A | 3292.0 | 3291.0 |
| | I | 1656.7 | 1655.8 |
| | II | 1541.8 | 1539.6 |

*In the presence of hydroxylamine.

1979*a*; Gibson and Cassim, 1989*b*). All vibrational frequencies are sensitive to molecular environments and interactions (Hallam, 1963). In view of this, the reason for these frequency shifts may lie in part in the findings from oriented far-UV CD studies summarized in Table 1. For PM samples identical to those used in these IR studies, bleaching causes an appreciable tilting of the helical segments of ~24–35° away from the membrane normal, whereas cross-linkage and papain digestion perturbations solely cause no changes. Changes of this magnitude in the orientations of the helical segments of the protein should cause dynamic changes in the environments and interactions of the protein amide groups in which these amide IR transitions originate. To add further support to this argument, CD studies of ethanol-treated PM film have provided evidence that this treatment not only bleaches the bR of the PM but results in the helical segments of the bR becoming randomized in respect to the membrane normal without altering the secondary structure (Gibson and Cassim, 1989*a*, *b*). In view of the helix symmetry, the net tilt angle for the completely randomized case is given by $\Theta_\alpha = \sin^{-1} \cdot (\frac{2}{3})^{1/2} = 54.736°$. Such a large change in segmental orientation is expected to cause dynamic changes in the environments and interactions of many more amide groups than the bleaching process and may account for the greater reduction in the amide frequencies noted in the IR spectra of ethanol-treated PM as compared with those of bleached PM. It is also clear that the bR segments involved in the frequency reduction must be those in the membrane bilayer because the moderate papain digestion removes both terminal tails of the bR polypeptide which are located outside the bilayer (Ovchinnikov et al., 1979) and yet does not alter the amide frequencies as compared with the unperturbed PM sample.

The $\Theta_\alpha$ values calculated from the dichroic ratios at the three amide frequencies for the various kinds of PM films according to the equations given in Data Analysis are presented in Table 3. In contrast to the far-UV CD method, this method yields a range of $\Theta_\alpha$ values rather than a single value for a given dichroic value due to the differences in the geometric formalism involved in the two methods. The values given for each kind of PM film

TABLE 3 **Analysis of infrared linear dichroic spectra of purple membrane: calculated* bacteriorhodopsin segmental tilt angles, $\Theta_\alpha$ for various perturbations**

| Perturbation | $\Theta_\alpha$ range in degrees Amide A | Amide I | Amide II | # PM films studied |
|---|---|---|---|---|
| None | 0–1.4 | 0.6–21.5 | 0.4–1.3 | 8 |
| Light-bleached‖ | 2.7–20.3 | 10.8–32.6 | 21.5–23.7 | 8 |
| Cross-linked‡ | 0–14.4 | 4.8–28.1 | 0–90 | 3 |
| Cross-linked light-bleached‡‖ | 13.3–28.6 | 25.8–38.1 | 4.0–90 | 7 |
| Moderately papain-digested§ | 0–4.5 | 1.3–13.9 | 3.5–5.8 | 8 |
| Moderately papain-digested light-bleached§‖ | 4.0–36.8 | 12.6–34.3 | 38.7–39.4 | 12 |
| Ethanol-treated‡ | 54.734–54.736 | 54.734–54.736 | 54.734–54.735 | 4 |

*See Data Analysis.
‡Calculations based on $f_\perp, f_\parallel$, and $f_u$ values of 0.4, 0.4, and 0.2, respectively.
§Calculations based on $f_\perp, f_\parallel$, and $f_u$ values of 0.5, 0.5, and 0.0, respectively
‖In the presence of hydroxylamine.

in Table 3 are average values from data obtained from three to twelve films. The given calculated values are based on $f_I = 0.4, f_{II} = 0.4$, and $f_u = 0.2$ for bleached and unbleached unperturbed and cross-linked PM as well as for ethanol-treated PM and $f_I = 0.5, f_{II} = 0.5$, and $f_u = 0$ for unbleached and bleached papain-digested PM's. It was assumed that the molar extinction coefficient per residue is the same for the three secondary structures at the frequency of the amide band in question. It is apparent that this is probably a reasonable approximation in this case because the calculations based on the data from the three amide band frequencies are fairly consistent for this kind of calculation and this consistency is the same for PM samples containing bR's with aperiodic secondary structures and those without as in the papain-digested PM's.

It is clear that for a given perturbation in Table 3, a $\Theta_\alpha$ value can be found which is common or very nearly common to all three ranges of $\Theta_\alpha$ values calculated from the data at the three amide transition frequencies, and which is identical within experimental uncertainty to the value obtained from the CD method for the same PM perturbation given in Table 1. For example, for the unperturbed and unbleached PM, a common IR value is 0.6° which is in excellent agreement with the CD estimated value of 0°.

Upon bleaching, the common IR value becomes 20.3°, for which the far-UV CD determined value is 24°. Similar excellent agreement between common IR values and CD determined values for the rest of the perturbed PM samples is also evident (cross-linked, 4.8°, CD-0°, cross-linked bleached 28.6°, CD-32°; papain-digested, 3.5°, CD-0°, papain-digested bleached 34.3°, CD-35°). Of particular interest is the results for ethanol-treated PM. For this sample, the common IR value is 54.735° and the $\Theta_\alpha$ ranges are very narrow. Because the theoretically expected value is 54.736°, this provides unambiguous evidence for the helical segments of the bR being completely randomized in this perturbed PM film and provides a demonstration of the accuracy potential of the IR method.

The $\Theta_\alpha$ values calculated from the mid-IR spectra will depend on the relative magnitudes used for $f_I$ and $f_{II}$. If the magnitudes of $f$ values deviate significantly from those indicated, the consistency of the $\Theta_\alpha$ values calculated from the data at the three amide transition frequencies become increasingly poor. Furthermore, the values of $\Theta_\alpha$ calculated from the mid-IR spectra become increasingly less consistent with those calculated from far-UV CD. For example, if $f_I, f_{II}$, and $f_u$ are assumed to be 0.8, 0.0, and 0.2 as was done in previous calculations of $\Theta_\alpha$ from mid-IR spectra, then $\Theta_\alpha$ becomes ~30° and 47° for the unbleached and bleached unperturbed PM, respectively, with poor internal consistency (Rothschild and Clark, 1979; Aldashev, 1985). It is evident that the only feature of the protein helix that the $\Theta_\alpha$ calculation is sensitive to is the orientations of the amide planes of the protein relative to its helix axis. Any single type of helix or a number of different types of helices on the average with amide planes oriented midway between the values given for the $\alpha_I$- and $\alpha_{II}$-helix in Data Analysis will give identical results as those shown in Table 3.

These results provide additional strong evidence for the amide plane of the bR helices on the average being tilted significantly from the helix axis in view of the excellent consistency of $\Theta_\alpha$ determinations based on the mid-IR and the far-UV CD film studies. Previously, far-UV CD studies had demonstrated that (*a*) while the magnitude of the $n$-$\pi^*$-induced CD were reduced by only 15% when the orientations of the protein helical segments were changed from random to parallel to the direction of the incident light in the case of the bR of the PM (Gibson and Cassim, 1989*a*), but ~50% in the case of polypeptides and proteins known to have structural characteristics of the pure $\alpha_I$-helix (Hofrichter, 1971; Olah and Huang, 1988); and (*b*) the magnitudes of the ellipticies of the far-UV suspension CD of PM were about two-thirds of that of myoglobin (whose secondary structure is similar to that of bR and whose helix is almost entirely of the $\alpha_I$ type) independent of wavelengths (Gibson and Cassim, 1989*a*). Also, Raman spectroscopic analyses have indicated that only half of the bR helices are of the $\alpha_I$ type (Vogel and Gärtner, 1987). It is clear that the magnitude of $f_{II}$ can be reduced from 0.4 and $f_I$ increased appropriately from 0.4 so that the calculated $\Theta_\alpha$ for the unperturbed PM would be identical to the value estimated from the results of the electron imaging and diffraction studies of Henderson and co-workers (Henderson and Unwin , 1975; Henderson et al., 1990). However, such changes in $f_I$ and $f_{II}$ would not be consistent with all the experimental evidence discussed.

## CONCLUSIONS

It has been shown that the two independent spectral methods, far-UV oriented CD and mid-IR-linear dichroism, are in excellent agreement in regards to a number of important points concerning the in situ molecular structure and structural dynamics of the bR (1). The helix axes of the bR in the native unperturbed PM are all oriented parallel to the PM normal. This point presents a view of the orientation of the bR helix axes in the native state which is quite different from the one proposed by Henderson and co-workers based on their three-dimensional structural model of the bR obtained by high-resolution electron cryo-microscopy (Hender-

son et al., 1990). In Fig. 15 of their recent publication three of the seven bR segments are shown to be relatively straight and tilted at various degrees from the membrane normal. The rest are shown to be kinked and bent resulting in different extents of distortion of the segmental parallelism with the membrane normal. The reasons for this significant difference most probably lie not so much in the difference of the techniques used per se but in the nature of the conditioning of the PM specimens required before study. Oriented CD and IR of native PM were studied in hydrated films of freshly prepared PM specimens at pH 7.0 and 25°C. In contrast, high-resolution electron cryo-microscopic studies required among other things actyl-glucoside treatment, specimen aging, glucose embedding, high vacuum, −120 to −268° temperatures and relatively high radiation energies (Henderson et al., 1990). The low-temperature and glucose-embedding techniques are intended to protect the PM specimens from radiation and vacuum-induced dehydration damages, respectively. Ironically, glucose embedding can cause as much damage as dehydration to the PM structure as exemplified by the tilt angle changes given in Table 1. Therefore, in view of the self-consistent evidence present here the structural model of the bR of Henderson and co-workers cannot reasonably be considered to be that of the native state but that of a structurally altered state of the PM (2). The amide planes of the bR helices are, on the average, tilted ~20–30° away from the helix axis. This point presents the first solid evidence for the existence of a natural protein with such structural characteristics. Extensive research of protein structure for over a quarter of a century has failed to uncover such characteristics. The bR is an intrinsic protein studied in its native bilayer lipid environment, whereas most proteins studied structurally have been extrinsic proteins, which have mainly been studied in aqueous environments. Poly-L-alanine, a synthetic homopolypeptide, assumes an α- helical conformation with dihedral angles intermediate between those of $\alpha_I$ and $\alpha_{II}$ types in an organic solvent hexafluoro-2-propanol (Parrish and Blout, 1972). However, on the addition of water to the organic solvent (1:3 by vol), the polypeptide transforms to a classical $\alpha_I$ type. This can suggest the possibility that the amide plane orientation of the bR in the PM may not be unique to bR but could be a more general feature of intrinsic helical proteins in situ in hydrophobic environments (3). Ethanol treatment of PM films completely randomizes the bR helix axis relative to the PM normal without observably altering the secondary structure of the bR. The significance of this point is twofold in respect to the goals of this paper: (*a*) it establishes the ethanol-treated PM film as an ideal random state standard for determining $R_0$ in the relationship $\Theta_\alpha = \sin^{-1}(2R_\alpha/3R_0)^{1/2}$ and (*b*) it demonstrates the accuracy potential of the mid-IR linear dichroic spectral method for determining $\Theta_\alpha$ (4). Light-bleaching of the PM in the presence of hydroxylamine results in the reversible net tilting of the helix axis of the bR by 20–24° away from the PM normal without altering the secondary structure of the bR (5). Chemical perturbation of the PM, with dimethyl-adipimediate-mediated intramolecular cross-linkage or papain-mediated peptide digestion, which do not observably change the orientation of the bR helix axis nor the secondary structure of the bR enhance reversibly the bleach-induced net tilting up to 34 –35° again without altering the secondary structure of the bR. The last two points demonstrate the in situ dramatic reversible conformation dynamical capabilities of the bR. These results pose an intriguing question yet to be answered: is this dramatic capability a unique characteristic solely of the bR or an inherent characteristic of transmembrane transport proteins in general?

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