Evidence for an α_{II}-Type Helical Conformation for Bacteriorhodopsin in the Purple Membrane

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ABSTRACT: Bacteriorhodopsin in native purple membrane is generally thought to be approximately 80% α-helical. However, all published far-UV circular dichroism spectra for purple membrane suspensions have been found to differ, both in shape and in magnitude, from published spectra of soluble proteins such as myoglobin, whose structure has been established as 80% α-helical by X-ray diffraction techniques. This has been interpreted as evidence that (1) bacteriorhodopsin has considerable β-sheet content or (2) optical artifacts are significant in the circular dichroism spectrum. It is proposed that this discrepancy is in fact more consistent with a substantial α_{II}-type conformational character for the protein α-helices. Although α-helical proteins are generally envisioned as having a classical α_I-type geometry in which the amide planes are all nearly parallel to the helical axis, there is no steric barrier to changing the dihedral angles so that the amide planes become significantly tilted with respect to the helical axis. The hydrogen-bonding character of the helices, however, will be affected, so this transition may require energy input. If the amide planes tilt far enough to eliminate hydrogen bonding along the helix, the structure is considered to be α_{II}. The conclusion that bacteriorhodopsin has significant α_{II} character is strongly supported by oriented film circular dichroism studies presented here. In addition, assuming a substantial α_{II}-type rather than an exclusive α_I-type conformation for the bacteriorhodopsin can result in excellent agreement between circular dichroism and infrared linear dichroism techniques as regards estimated helix tilt angles both before and after bleaching of the purple membrane with hydroxylamine in the presence of light.

Bacteriorhodopsin (bR),[1] the sole protein of the purple membrane (PM)[1] of *Halobacterium halobium*, is one of the best characterized of all membrane proteins. Its projected structure is now known to a resolution of 3.5 Å as a result of improvements in electron diffraction techniques (Henderson et al., 1986). Although the electron diffraction map clearly indicates that bR is predominantly α-helical, the lack of high-resolution, three-dimensional data has prevented the establishment of a definite secondary structure. Attempts to reduce the uncertainty via spectroscopic means have proven to be either inconclusive (Lewis et al., 1985) or contradictory [compare Jap et al. (1983), Nabedryk et al. (1985), and Mao and Wallace (1984)].

At the present time, circular dichroism (CD) spectroscopy offers the most promise for determining protein secondary structure. However, interpretation of CD spectra, especially for proteins in situ, is often complicated by the presence of numerous artifacts which remain incompletely understood. If CD spectroscopy is to attain its potential as a noninvasive probe of secondary structure, these artifacts must be better characterized and ways found to eliminate them.

The debate over interpretation of the CD spectra of bR in the purple membrane has come to a focus in the pages of this journal (Mao & Wallace, 1984; Glaeser & Jap, 1985; Wallace & Teeters, 1987). Although a good many points are raised in these papers, the central argument concerns the mean residue molar ellipticity of bR ($[\theta]$) and the implications for determination of the secondary structure of bR. The values for $[\theta]$ for bR, at all wavelengths from 250 to 190 nm, are only about two-thirds of those for myoglobin, a protein which has been shown by X-ray diffraction (Kendrew et al., 1960) to be 80% α-helix. Jap et al. (1983) interpret this discrepancy to mean that bR is at most 50% α-helix, with perhaps 18% being antiparallel β-sheet. Yet there is a large body of evidence which suggests that bR is indeed 80% α-helix [see, for example, Henderson and Unwin (1975), Nabedryk et al. (1985), and Tsygannik and Baldwin (1987)]. Wallace and co-workers, on the other hand, attribute the low $[\theta]$ to absorption flattening effects and state that the discrepancy is eliminated in small unilamellar vesicles (SUV's) in which bR exists as monomers (Mao & Wallace, 1984; Wallace & Teeters, 1987). We suggest that this discrepancy results neither from the presence of β-sheet nor from absorption flattening. Rather, we propose that bR in native PM disks exists in an α-helical conformation which, on average, is more α_{II}-like than α_I-like. It has been shown that a large number of protein α-helices, ranging from the α_I form to the α_{II}, are theoretically possible with the same rotation and axial rise per residue but with different dihedral angles and different orientations of the amide planes with respect to the helical axis (Némethy et al., 1967). The α_{II} interpretation of the bR CD spectra is consistent with all experimental solution CD results previously published. Furthermore, it is supported by oriented film CD experiments presented here and by previous infrared (IR) spectroscopy studies of Krimm and Dwivedi (1982), Aldashev (1985), and Draheim et al. (1988a) and by Raman spectroscopy studies of Vogel and Gärtner (1987).

Materials and Methods

PM was isolated according to the methods of Becher and Cassim (1975) with the exception that the sucrose step-gradient purification step was eliminated. However, many washing steps were incorporated into the purification process

[1] Abbreviations: bR, bacteriorhodopsin; PM, purple membrane; CD, circular dichroism; SUV, small unilamellar vesicle; PLA, poly(L-alanine); HFIP, hexafluoro-2-propanol; IR, infrared; UV, ultraviolet; T_c, lipid phase transition temperature; DMPC, dimyristoylphosphatidylcholine.

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which produced preparations of equal or greater purity as judged by the ratio of absorbances at 280 and 568 nm (1.55:1), without exposing the membrane to sucrose. Also, 0.05% sodium azide was added whenever membrane suspensions were stored in order to prevent changes in membrane structure due to the possible action of bacterial proteolytic enzymes. However, membrane suspensions were never stored for more than a few days before use.

Membrane films were prepared as follows: PM preparations were suspended in double-distilled water to an OD_{568} of 0.2–0.3. The solutions were passed through 5-μm filters (Gelman GA-1) and degassed. Approximately 0.75 mL of a given solution was then placed on a 25-mm-diameter optical flat of Suprasil-S quartz (Precision Cells, Inc., Hicksville, NY) and allowed to dry in a desiccator containing either a beaker of Drierite or a saturated $LiCl_2$ solution. After formation, films were incubated at high humidity in a desiccator with a beaker of saturated K_2SO_4 solution (95% relative humidity) for at least 24 h prior to measurement. They were hydrated in specially designed airtight film holders without their cover plates in place (Oriel Corp., Stamford, CT). This arrangement made it possible to quickly seal them without having to remove them from the hydration chamber, minimizing possible drying effects of ambient humidity. It was also observed that these films were sufficiently thin to lack noticeable light-scattering effects resulting from hydration state (Draheim et al., 1988b). For solution studies, membrane preparations were suspended in double-distilled water, filtered, and degassed prior to spectral measurements.

CD spectra were recorded on a Cary 60 spectropolarimeter with a 6003 CD attachment (Varian Associates, Inc., Instrument Group, Palo Alto, CA) or on a Jasco J-500A spectropolarimeter (JASCO, Inc., Easton, MD). Absorption spectra were recorded on a Cary 118C spectrophotometer with far-ultraviolet modification and a scattered transmission accessory (Varian Associates, Inc., Instrument Group, Palo Alto, CA). Details of the instrumentation techniques used are as previously published [see Muccio and Cassim (1979)]. For solution CD and absorption measurements, 1-mm path-length cylindrical cells with fused-silica windows (Precision Cells, Inc., Hicksville, NY) and 1-mm path-length rectangular far-UV quartz cells (Markson Science, Inc., Del Mar, CA) were used, respectively. Precision machined aluminum blocks were used to align the films in airtight film holders for CD and absorption measurements. Solutions were adjusted so as to give an OD_{193} of 0.2–0.3 in 1-mm path-length cuvettes and in dried films.

The films were observed to dry across the optical flats in a linear manner due to the presences of a slight tilt in the desiccator platform. Therefore, the films were uniform in optical density across the center of the flat in a direction parallel to the drying front. Films were marked and oriented so that this uniform region was perpendicular to the narrow measuring light beam in all instruments. This approach avoided potential linear dichroic contributions to the CD spectra due to uneven sample thickness.

In order to monitor any CD artifacts due to light scattering, samples run on the Jasco spectropolarimeter were measured both on the optical bench (16 cm from the photomultiplier, 11° acceptance angle) and close to the photomultiplier (approximately 2 cm, 90° acceptance angle). The films used for this study showed no difference at the two positions, indicating the absence of measurable scattering artifacts. The films whose spectra are presented here were measured on the Jasco spectropolarimeter, approximately 5 cm from the photomultiplier (35° acceptance angle) at a sensitivity range of 0.5 mdeg/cm. The Cary 60 spectropolarimeter, used to check the Jasco results, allows very little adjustment of sample to photomultiplier distance. The value for this spectropolarimeter was approximately 2 cm.

It cannot be overemphasized that problems such as differential light scattering, absorption flattening, and mosaic spread reported by other laboratories are in large part due to the fact that the films used were relatively thick. Because of the high sensitivity and good signal to noise ratio offered by the Jasco spectropolarimeter, it was possible to use extremely thin films which did not present the above-mentioned problems. A linear relationship between OD_{193} and θ_{222} was evident for these films, clearly indicating that they were sufficiently thin to avoid these difficulties.

Treatment of the membrane films with acetone, diethyl ether, or 95% ethanol was done by carefully adding drop amounts of these compounds to the films and then allowing them to completely evaporate prior to spectral recording. The compounds are all of reagent quality and were used as received. Absorbance studies (800–190 nm) showed that such treatments did not alter the thickness of the films. Therefore, the effects of this treatment on a given film spectrum could be compared directly with the same film before treatment. Helices oriented at random in three-dimensional space would give a spectrum identical with a mixture of three equal groups of helices oriented along orthogonal axes. It can be shown that such an assembly would exhibit an apparent tilt angle of 54.7°. Calculations based on IR linear dichroism data for the solvent-treated films gave an average helix tilt angle of 53 ± 2° from the membrane normal, indicating that the helices were indeed oriented at random with respect to the measuring light (Draheim et al., 1988a). [For a discussion of the linear dichroic technique, see Rothschild and Clark (1979) and Papadopoulos and Cassim (1981).]

Solvent addition was found to have no effect on the bR secondary structure as demonstrated by comparison of far-UV CD spectra for a PM suspension and a randomized film (Figure 1). As a further check, a PM suspension, to which sufficient ethanol had been added to cause decolorization, was then washed to remove the ethanol and compared with a native PM suspension. The far-UV CD spectra were identical (data not shown), which again indicates that solvent addition has no effect on bR secondary structure.

Films were screened for the presence of mosaic spread using electron microscopy and linear dichroism techniques as detailed in Rothschild and Clark (1979) and Nabedryk and Breton (1981). Electron microscopic examination of PM films deposited on carbon-coated formvar grids showed the disks to be free of aggregation problems and oriented parallel to the substrate surface. Linear dichroism studies of these films indicated that the orientations of the transition dipole moments of the retinals were 21.2 ± 2° from the membrane plane. This value is in close agreement with the one published by Heyn et al. (1977), who indicated that they used neutron diffraction techniques to show that mosaic spread was negligible in their PM films. A further check for mosaic spread was conducted by gradually building up layers of PM on a quartz plate until sufficiently thick to allow investigation of the visible CD spectrum. There was found to be no evidence of the characteristic bilobed exciton band which would have been present if there were any appreciable tilt of the membrane disks with respect to the measuring light (Muccio & Cassim, 1979).

Film spectra shown in Figure 2 were normalized to an OD_{193} of 1.0. The protein concentrations (per unit path lengths) of the PM films were determined by measuring the solution

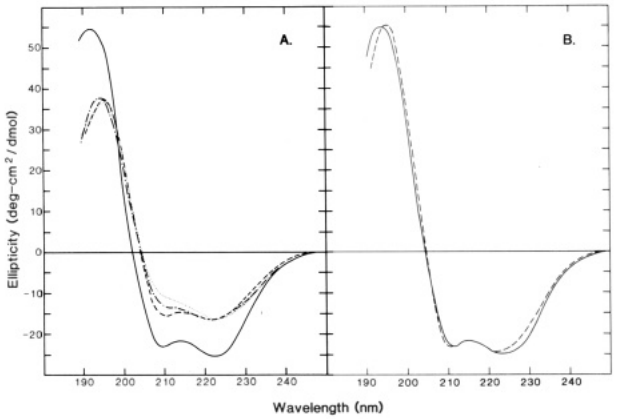

FIGURE 1: (A) Comparison of far-UV CD spectra for myoglobin in solution (—), a PM suspension (···), and PM films randomized by a drop of solvent (–·– and - - -). The myoglobin curve is from data published by Chen et al. (1974) but was confirmed in this laboratory. For the (–·–) film, the OD_{193} was 0.3; for the (- - -) film, it was 0.15. (B) Comparison of two of the curves shown in (A), with the following modifications: the myoglobin curve (—) has been shifted 2 nm to the right; the randomized film (– –) curve has been normalized to match the myoglobin curve at 222 nm. (Note: the mean residue molar ellipticities given in units of degrees centimeter squared per decimole have been multiplied by a factor of 10^{-3}.)

absorbance of the light-adapted PM at 568 nm, pH 7.0, and 25 °C in an optical cell with a known optical path length using the conventional extinction coefficient of 63 000 M^{-1} cm^{-1}. The far-UV CD spectrum of films treated with organic compounds was normalized at 222 nm with the far-UV CD spectrum of a precision hundredfold dilution of this solution. Since the protein concentration in an untreated film is identical with the one in the same treated film as determined by absorbance spectra, the concentration can be readily obtained for the untreated film as well.

Results and Discussion

Figure 1A compares a bR suspension, randomized PM films, and a myoglobin solution spectrum published by Chen et al. (1974). The myoglobin curve was independently confirmed in this laboratory. It will be noticed that the bR samples are quite similar but that they are significantly lower in $[\theta]$ at all wavelengths as compared to myoglobin. In addition, it is apparent that the myoglobin curve is blue shifted by about 2 nm relative to the PM curves. This small displacement is much less than the one previously observed in the spectra of intact and solubilized erythrocyte ghosts (Gordon & Holzwarth, 1971) and is probably due to effects of the different solvent systems to which the proteins are exposed (i.e., water for myoglobin vs lipids for bR). In Figure 1B, two of the curves are shown with changes made for purposes of comparison. First of all, the myoglobin curve has been shifted 2 nm to the right to align it with the bR curves. Second, the bR curve has been multiplied by a factor of 1.5 to normalize its $[\theta]_{222}$ to that of myoglobin. It is readily apparent that, within experimental error, there is excellent agreement in curve shape between the randomized thin bR film and myoglobin at all wavelengths. Stated another way, the ratios of the 193- and 225-nm bands are virtually identical in bR and myoglobin. The reduced ellipticity at 208–215 nm for the thicker film and the bR suspension is explained by the fact that myoglobin is dissolved in water while bR is a complex suspension of protein embedded in lipid which is in turn surrounded by water. The different refractive indices involved in the native PM suspension therefore give rise to light scattering. As refraction is a dispersive phenomenon, this effect will be minimal at the protein's absorption max (193 nm), maximal at nearby wavelengths longer and shorter than 193 nm, and minimal again at more distant wavelengths. Therefore, artifacts due to light scattering are expected to be most prominent in the 205–215-nm region, where refractive index differences are greatest. It is apparent that the randomized films exhibit this effect to a lesser degree than does the PM suspension, probably due to the absence of bulk water in the former. As would be expected, addition of glycerol has also been shown to eliminate this depression at 208–215 nm (Draheim et al., 1988b). These results are at variance with the conclusions of a number of laboratories (Jap et al., 1983; Mao & Wallace, 1984; Nabedryk et al., 1985) that light scattering is insignificant at all wavelengths from 240 to 190 nm if large acceptance angles are used during CD measurements. With this one exception, the far-UV CD spectrum of bR can be said to be virtually identical in shape with that of myoglobin, though the two differ by a multiplicative factor and are offset by 2 nm relative to each other.

It should be obvious from the above discussion that the myoglobin minus bR difference spectrum, when first corrected for the wavelength offset, will once again resemble the two curves, being equal to the PM spectrum multiplied by 0.5. It is equally obvious that such a curve can best be fit by assuming it to be 80% α-helix and 20% aperiodic in secondary structure, the proportions found in myoglobin. Yet Jap et al. (1983) were able to achieve a fit using 18% β-structure in their curve-fitting procedure. There are two likely explanations for this fact. First of all, the depression of the band at 208–215 nm by light scattering would tend to mask some of the α-helical character of the spectrum. As β-sheet is the only other conformation characterized by a long-wavelength negative CD band, it would be selected by the curve-fitting routine. Second, Jap et al. (1983) made no mention of correcting the wavelength discrepancy between their soluble protein reference data and the bR suspension curve before attempting a fit. To complicate matters, it is not known whether the spectra of each conformational type (α, β, or aperiodic) would be shifted by the same or different amounts due to the solvent effect.

The close similarity in CD band shapes for bR and myoglobin can be interpreted to mean that the secondary structure of bR is most likely 80% α-helical and 20% aperiodic. This conclusion is supported by a compelling amount of data. Tsygannik and Baldwin (1987) have obtained the three-dimensional structure of bR to 6 Å and interpret their data to mean that bR consists of seven transmembrane α-helices. Furthermore, Nabedryk et al. (1985), using far-UV CD spectroscopy and IR absorption and linear dichroism techniques, obtained a value of 69 ± 5% helix for sonicated bR. Their curve-fitting procedure was apparently based solely on band shapes and not molar ellipticities, because the resultant spectra were reported in terms of arbitrary units. The difference between curve-fitting results for these authors and for Jap et al. (1983) might be explained by the fact that the former group first shifted their experimental spectra to correct for the wavelength difference between soluble and membrane protein CD spectra. There are numerous additional arguments against the presence of β-sheet structure in bR. As these are discussed at length in the paper by Wallace and Teeters (1987), there seems little point in repeating them here.

Wallace and co-workers were quite correct in maintaining that absorption flattening effects were present in their native PM CD spectra, at least at the shorter wavelengths. A good indicator of absorption flattening effects is the ratio of the positve and negative extrema. Since the protein absorbance is much greater at 193 nm than at 225 nm, absorption flat-

tening effects are also much greater at 193 nm. In our hands, native PM suspensions have a CD ratio of 2.4–2.6. Mao and Wallace (1984) showed a native PM ratio of 1.4 and an SUV ratio of 2.5, while Wallace and Teeters (1987) obtained a ratio of 1.9 for native PM, 2.1 for sonicated PM, and 2.3 for SUV's. Similarly, Nabedryk et al. (1985) obtained ratios of 1.9, 2.4, and 2.4 for the native, sonicated, and vesicle preparations. Jap et al. (1983) obtained a ratio of 2.4 using native membranes, and Glaeser and Jap (1985) have presented extensive evidence that absorption flattening effects are negligible in their preparations. The variation in ratios was due to the 193-nm band, as all of the groups which reported their results in terms of molar ellipticity were in close agreement at 222–225 nm. Thus, it appears that Wallace and co-workers had some experimental difficulties with their native membranes. Possible causes include membrane aggregation, reduced machine performance at short wavelengths due to deteriorating optics or insufficient purging with nitrogen, or insufficiently diluted samples. Nevertheless, incorporation into SUV's corrected the problem, demonstrating the importance of the technique for examining membrane proteins in which aggregation causes a problem. Thus, in terms of band shape, there is substantial agreement between the native PM spectra of Jap et al. (1983) and this laboratory and the SUV spectra of Wallace and co-workers with the exception of the 208–215-nm region discussed earlier.

There remains, however, a major inconsistency to be resolved. Although all agree that native PM has a $[\theta]_{222}$ of ca. $-16\,200$ deg cm^2 dmol^{-1}, Wallace and Teeters (1987) obtain a calculated mean residue molar ellipticity at 222 nm by normalization of ca. $-22\,000$ deg cm^2 dmol^{-1} for PM in SUV's and ascribe the difference in magnitudes to absorption flattening in native preparations. However, as just explained, neither the curves of Jap et al. (1983) nor those of this laboratory show the flattening artifacts present in the native spectra of Wallace and co-workers. The similarity of curve shapes for the native spectra of Jap et al. (1983) and the SUV spectra of Wallace and co-workers suggests that both are correct in terms of shape. Although we are not in a position to evaluate or verify the normalization procedure used by Wallace and Teeters (1987), they have confirmed their concentration determinations using direct amino acid analysis, as did Jap et al. (1983). This should minimize the effects of incorrect normalization as a cause for disagreement. A possible explanation for this difference in calculated $[\theta]_{222}$ may be an α_{II} to α_I transition for bR on going from native membranes to SUV's, in which protein–protein interaction is minimized. Such a transition would not involve changes in conformational energy due to steric barriers but would involve changes in hydrogen-bonding energies.

An α_{II}-type α-helical conformation for bR has been previously proposed on the basis of IR studies by Krimm and Dwivedi (1982). It was also mentioned as a possibility by Lewis et al. (1985) and has recently gained strong support from the work of Vogel and Gärtner (1987) in which it was estimated that only half of the helices are of the α_I type. In an α_{II}-helix, the planes of the amide groups are significantly tilted with respect to the helix axis so that the carbonyl groups point away from the helix. Némethy et al. (1967) suggested the occurrence of very small amounts of α_{II}-type conformation at the ends of the myoglobin helices and also referred to more extensive regions of α_{II}-type conformation in lysozyme. Parrish and Blout (1972) have shown that poly(L-alanine) (PLA) in hexafluoro-2-propanol (HFIP) assumes an α-helical conformation, with dihedral angles intermediate between those of α_I and α_{II} types, which they called the "doubly bonded helix". On addition of a small amount of water (H_2O:HFIP ratio of 1:3), PLA converts to a classical α_I-helix. Furthermore, Parrish and Blout (1972) demonstrated that whereas the α_I form had a characteristic helical–homopolypeptide far-UV CD solution spectrum ($[\theta]_{222} = -34\,100$ deg cm^2 dmol^{-1}), the intermediate form gave a sharply reduced spectrum ($[\theta]_{219} = -13\,300$ deg cm^2 dmol^{-1}). That is, the ellipticity of the α_I form was about 2.56 times that of the intermediate one at ca. 222 nm. According to this result, if one considers a case in which the structure is composed of an equal mixture of these two forms of the α-helix, a simple calculation shows that the ellipticity of the α_I form should be about 1.44 times that of this mixed structure. It is noteworthy that the ellipticity of myoglobin as shown in Figure 1B is 1.5 times that of bR. The authors also demonstrated that the carbonyl oxygens were "doubly bonded" both to the fourth adjacent NH hydrogen along the helix and to hydroxyl groups of HFIP. In a true α_{II}-helix, bonding does not occur with intrahelix NH hydrogens, due to a greater tilting of the amide planes. Additionally, it has been demonstrated that any α-helical conformation in the range from α_I to α_{II} would have about the same energy (Ramachandran & Sasisekharan, 1968), so there would be no barrier to interconversion as the solvent system changed other than that imposed by the hydrogen-bonding character of the helix. At any rate, the major lesson to be gained from the PLA experiment is that molar ellipticity is not a strict predictor of helix content, being strongly affected by variations in the dihedral angles between amide groups. These angles, in turn, are determined by the characteristics of the medium surrounding an α-helix. Furthermore, Manning and Woody (1987) have calculated that the far-UV CD of antiparallel β-sheets will also be highly variable, depending on the separation and relative orientation of the strands.

Further support for a "non-α_I" conformation in bR comes from work in this laboratory with the far-UV CD of oriented PM films. PM disks dried on quartz optical flats form extremely thin multilayers in which the helical segments are aligned essentially perpendicular to the surface of the flats. When these films are mounted in a spectropolarimeter so that the polarized light is incident normal to the flats, only helical transitions perpendicular to the helix axes can occur (Muccio & Cassim, 1979). Figure 2A shows the CD spectrum for such a film, as well as the spectrum which results when the helices are randomized following addition of a solvent to the film. Great care was taken to check the absorption spectra of the films before and after solvent addition to ensure that the product of sample path length and concentration did not change. The resulting difference spectrum (Figure 2B) shows the appearance of what is essentially a single Gaussian-like band centered at ca. 207 nm and strictly polarized along the helix axis. The existence of a CD band with such properties is central to the predictions of the excitonic paradigm of the polypeptide α-helix (Woody & Tinoco, 1967). Although there have been numerous attempts in the past to verify experimentally this predicted band (Mandel & Holzwarth, 1972), the results shown in Figure 2B provide the most convincing evidence ever presented. There is also a slight increase in the n–π^* band at 225 nm following randomization.

According to the predictions of quantum theories of amide spectra, the transition which gives rise to the 225-nm n–π^* band will be polarized along the C=O bond of the amide carbonyls (Schellman & Oriel, 1962). In an α_I-helix, these C=O bonds are aligned nearly parallel with the helix axis, and, therefore, the n–π^* band must vanish or be very sharply

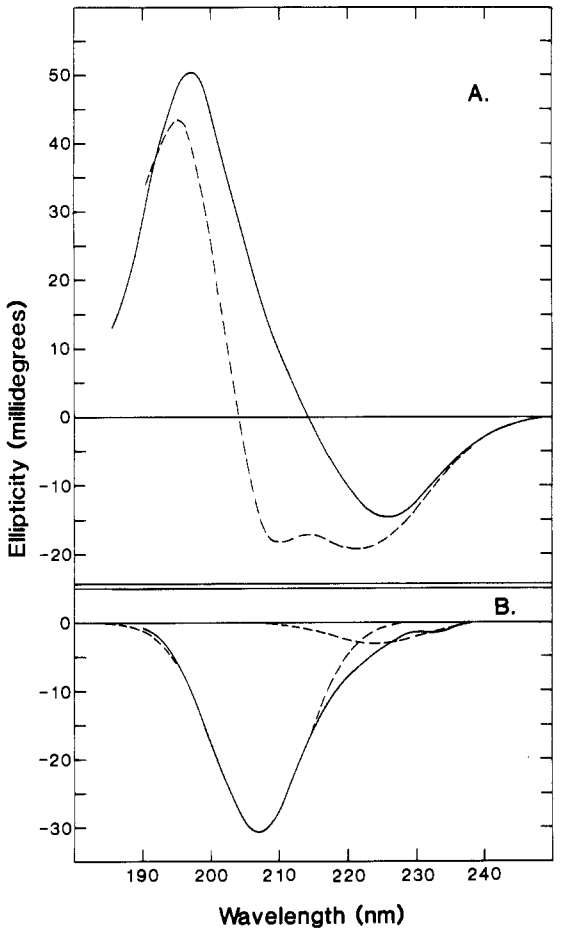

FIGURE 2: (A) Far-UV CD spectra for a PM film before (—) and after (--) addition of a drop of solvent. (B) Difference spectrum for the above (—) and Gaussians fitted to it (--).

reduced in magnitude (70–90% by theoretical calculations) in the oriented films (Woody & Tinoco, 1967; Woody, 1968). Previous experiments with polyamino acids which have been partially oriented by electric fields or by shear have provided support for this conclusion (Mandel & Holzwarth, 1972; Hofrichter, 1971). We find, however, that the oriented n–π^* band is 85% of the randomized band, indicating only a 15% reduction in magnitude. For an α_I-helix to show an n–π^* band of this magnitude would require an average helix tilt of 49° from the normal. Such a tilt cannot be present, however, as it would also introduce a band at 207 nm which was 85% of the one shown in the difference spectrum. For the sake of illustration, it can also be calculated that if inherent intramembrane helix tilt and mosaic spread combined to give an average helix tilt of 30°, almost 3 times as much as has been suggested by electron diffraction studies, the "oriented" n–π^* band for an α_I-helix would only be 37% of the randomized band. Once again, the only way out of this dilemma is by assuming that the bR α-helices are not α_I in form but are instead more α_{II}-like. The C=O bonds in such a helix would not be aligned parallel to the helix axis and thus would exhibit a large n–π^* band even in oriented films.

It is important to emphasize the fact that the 207-nm band in a far-UV CD film spectrum is entirely independent of the angle of the amide planes to the helix axis. Because the 207-nm band represents the component of the excitonic π–π^* interactions polarized along the helix axis, it is an accurate indication of helix orientation. Thus, the complete absence of a 207-nm band in oriented film spectra indicates orientation of the helices parallel to the incident light. Furthermore, we have recently duplicated the IR linear dichroism work first done by Rothschild and Clark (1979) and more recently by Aldashev (1985). We obtained dichroic ratios for the amide I, II, and A bands which were in agreement with their results but have found that by assuming an average α-helical conformation intermediate between α_I and α_{II}, we obtain a value for the average helix tilt which is in much better agreement with electron diffraction and CD results (Draheim et al., 1988a). We have also shown in the same work that, on the basis of IR linear dichroism data, the average helix tilt can be calculated to change from 31° to 40° on bleaching if an α_I conformation is assumed but from 11° to 31° if a more α_{II}-type conformation is assumed. As increasing α_{II} character is used in the calculation, the angles approach the 0–24° change calculated from oriented film CD results (Gibson & Cassim, 1986).

In view of the evidence discussed above, the bR probably exists as a complex mixture of helix types ranging from pure α_I to pure α_{II}. Furthermore, a single helix could show this variation. For example, the amide groups adjacent to lipids would be expected to have an α_I conformation, as the lipids would not hydrogen bond with the amide carbonyls. On the other hand, the sides of a helix which were adjacent to other helices or to hydrophilic regions of the core might exhibit varying outward tilts of the amide planes, depending on the ability of the neighboring regions to hydrogen bond. It appears entirely possible that the classic pure α_I-helix will prove to be a rarity in membrane proteins.

The bR in DMPC vesicles is thought to exist as monomers above the lipid phase transition temperature (T_c) of 23 °C (Cherry et al., 1978). It is quite conceivable that loss of interaction between helix backbone carbonyl oxygens and side group hydroxyls or amino groups of neighboring helices could convert helices from an α_{II} or intermediate form to an α_I form. This would be analogous to the effect of adding water to the HFIP in the 1972 experiment of Parrish and Blout. If the larger calculated molar ellipticity for bR in SUV's is indeed due to an α_{II} to α_I transition following monomerization and not some error in concentration determination, caution will be needed in interpreting the results of vesicle work. For example, Lewis et al. (1985) used NMR of DMPC vesicles above T_c to investigate the secondary structure of bR. They interpreted their data as indicating either 100% α_I-helices tilted 20° from the membrane normal or 60% untilted α_{II}-helices and 40% β-sheet tilted at 10–20° from the membrane normal. Although their results were inconclusive, the true secondary structure might also have been affected by the vesicle system used. Similarly, α_{II} to α_I conversion might have effects on other parameters measured in vesicles such as permeability to ions.

It should be clear from the above discussion that the use of soluble protein basis functions, derived from dilute solution studies, to estimate secondary structure is at best quite uncertain when dealing with membrane proteins in situ. It now appears that experimental CD curves for a given protein can be both shifted in terms of wavelength and expanded or compressed in terms of molar ellipticity as the protein's environment changes. As interprotein and interhelix distance, as well as lipid composition and temperature, may all play important roles in causing these changes, a great deal of work is needed to characterize the many interactions which contribute to the in situ far-UV CD spectra of bR and membrane proteins in general. In addition, this study stresses the naivete of assuming all α-helices in biological structures to be of the classic α_I type. In reality, the structures of α-helices in situ in biological

structures may consist of a complex mixture of geometries varying from the α_I to the α_{II}. This possibility can be of significant importance in the understanding of in situ protein structure and function.

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