FURTHER CHARACTERIZATION OF PROTEIN SECONDARY STRUCTURES IN PURPLE MEMBRANE BY CIRCULAR DICHROISM AND POLARIZED INFRARED SPECTROSCOPIES

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ABSTRACT The conformation and the orientation of the protein secondary structures in purple membrane was analyzed by infrared absorption and linear dichroism of oriented membranes as well as by UV circular dichroism of bacteriorhodopsin in intact purple membrane and in lipid vesicles. A large amount (74 ± 5%) of transmembrane α-helices is detected with no significant contribution of β-sheet strands running perpendicular to the membrane plane. Thus, these data do not support the recent structural model proposed by Jap et al. (*Biophys. J.* 1983, 43:81–89).

INTRODUCTION

Low-dose electron microscopy and image reconstruction techniques as well as x-ray diffraction analysis of purple membrane (PM)[1] have led to a three-dimensional model of bacteriorhodopsin (BR) consisting of seven close-packed transmembrane α-helices (1–4). These α-helices make up 70 to 80% of the polypeptide (1). On the other hand, estimations of α-helical content by ultraviolet circular dichroism (UVCD) vary from 45 to 83% (5–8) depending on the state of the BR (intact PM, solubilized BR, BR reconstituted in lipid vesicles) and/or the magnitude of distortion of the membrane protein UVCD spectra by optical effects such as differential light scattering and absorption flattening (6, 8). Recently, Jap et al. (7) reconsidering high resolution electron microscopy data (9) suggested a new structural model consisting of only five α-helical rods and four β-sheet strands of 11 amino acids. These structural predictions were corroborated by the analysis of their CD and infrared (IR) data, which indicate that as much as 20% of the BR is in a β-sheet conformation with no more than 50% α-helix (7).

In our laboratory, we have been applying both CD and polarized IR spectroscopy techniques to study the conformation and the orientation of intrinsic membrane proteins in the various protein-pigment complexes such as reaction centers (10–12) and antennae (13–14) in photosynthetic systems. In these complexes, it appears that the organization of the pigments is governed by the presence of transmembrane α-helices (50%) while the small amount of β-sheets does not play a major role in the binding of chlorophylls in vivo (14). These conclusions, however, rely in part upon the conformation and orientation of the protein secondary structures in PM because this system was used as a standard to calibrate our measurements on photosynthetic membranes (15).

In a previous paper (15), we have already noticed that a weak shoulder around 1,685 cm^{-1} in the polarized IR absorption spectra of PM could be due to the presence of a small amount of β-structures in the BR. The proposal of Jap et al. (7) thus prompted us to reinvestigate the secondary structures of the protein in PM. Furthermore, the possibility that in PM, several oriented polypeptide domains are in a β-sheet conformation can be tested by analyzing IR dichroism spectra on oriented membrane multilayers. More specifically, with our conventions (13, 15), α-helices perpendicular to the membrane plane would lead to a positive dichroism signal whereas strands of β-sheets perpendicular to this plane would exhibit a negative one.

Here we report further data on the conformation and orientation of protein secondary structures in PM by analyzing IR linear dichroism spectra of oriented PM and by reinvestigating the CD spectra of BR in intact PM and in lipid vesicles. Our CD data are in good agreement with those recently reported by Mao and Wallace (8) and favor the generally accepted models derived from diffraction experiments (1–3). Furthermore, our IR data do not support the existence of a large contribution of oriented β-sheets in this membrane.

MATERIALS AND METHODS

Purple membrane was a generous gift of Drs. J. L. Rigaud and S. Buschlen. Bacteriorhodopsin was reconstituted in dimyristoylphospha-

[1] *Abbreviations used in this paper:* PM, purple membrane; BR, bacteriorhodopsin; CD, circular dichroism; IR, infrared; DMPC, dimyristoylphosphatidylcholine.

tidylcholine (DMPC; Sigma Chemical Co., St. Louis, MO) vesicles according to Mao and Wallace (8). Analysis of IR dichroism spectra of air-dried multilayers has been reported elsewhere (13, 15). Estimation of the protein secondary structure composition was obtained by UVCD, using a Jobin-Yvon (Les Ulis, France) Mark V dichrograph linked to a Micral (Longjumeau, France) 8031B computer. Circular dichroism spectra (40 runs average) were analyzed between 195 and 260 nm at 2 nm intervals by a linear square curve-fitting procedure (12–14). The least-square fit constraints require the sum of the α-helical (α), β-sheet (β), and aperiodic (γ) structure percentages to be 100% but did not require the percentage of each structure to be positive. Negative coefficients would indicate that the analysis has failed. The reference data set used was obtained from Chen et al. (16), using α-helical segments of 10 or 20 residues. To take into account the red shift in the UVCD spectra compared to soluble protein data used as reference, experimental spectra can be shifted towards shorter wavelengths (usually by 1–4 nm) before analyzing by curve-fitting procedures (12–14). The quality of the fit between experimental and calculated spectra was expressed as a root mean square deviation $\bar{\sigma}$ and by the difference curve between calculated and experimental spectra.

RESULTS AND DISCUSSION

The UVCD spectrum of intact PM (Fig. 1 *A*) exhibits a characteristic shape in the 205–230 nm region which is also noticed in other PM spectra (5–8, 17). After fragmentation of PM sheets by sonication, the UVCD spectrum (Fig. 1 *B*) appears less distorted in this spectral range. Furthermore, the ratio of the amplitudes at 195 and 230 nm increases. These observations are probably related to optical effects such as light scattering and optical flattening. Indeed, it has been recently demonstrated that the main significant optical artifacts contributing to the CD spectra of membranes is differential flattening (18). Differences in the spectra reported by several authors (5–8, 17) might also be due to experimental conditions such as differences in the state of aggregation of the sample and/or in the instrument used. Upon reconstitution of BR in DMPC vesicles, the UVCD spectrum (Fig. 1 *C*) shows no major distortion (compared to soluble protein spectra) as described in (8). A similar observation has also been reported for the photosystem I (from higher plants) reconstituted in lipid vesicles (12). This confirms that the optical effects are minimized after reconstitution in lipid vesicles (8, 12, 19).

The protein secondary structure composition of the three types of samples are given in Table I. Only small variations ($\pm 3\%$) in the secondary structures content were observed using 10 or 20 residues per α-helical segment. It is clear that UVCD spectra of intact PM are difficult to adjust, as reflected by a high $\bar{\sigma}$ value and a high amplitude of the difference curve. In these conditions, the calculated α-helical content is 56%, which is slightly higher than the 45 and 50% reported in (8) and (7) respectively. For the sonicated membrane, the quality of the fit is significantly better, leading to 69% α-helix. Even better adjustments are found for BR reconstituted in DMPC vesicles, leading to 74% α-helical structure with the lowest $\bar{\sigma}$ value (see also the featureless difference curve in Fig. 1 *C*). The quality of the fit obtained in reconstituted BR (Fig. 1 *C*), and also to a lesser extent with the sonicated PM sample (Fig. 1 *B*), indicates that the α-helical content of BR is close to $74 \pm 5\%$. On the other hand, the low values (5–8, Table I) determined from the intact membrane spectra are most probably affected by the various effects described above and do not reflect the actual α-helical content of BR in vivo. Furthermore, Table I indicates that for the three types of samples, the β-sheet content is $<10\%$. Thus, our CD data appear difficult to reconcile with a structural model consisting of 50% α-helix and 18% β-sheet as recently proposed by Jap et al. (7). On the other hand, the $74 \pm 5\%$ determined here is in good agreement with the values reported in Mao and Wallace (8) and Wallace and Mao (18). These data are consistent with Henderson and Unwin's model (1). They also compare well with the 70–72% value, which can be derived from a recent struc-

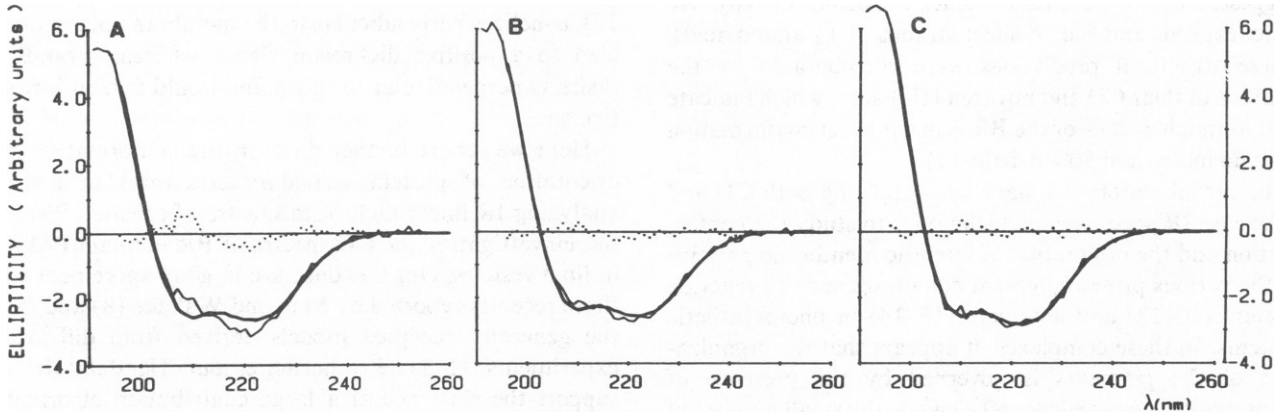

FIGURE 1 UV circular dichroism spectra (40 runs average) of (*A*) intact purple membrane, (*B*) sonicated purple membrane, and (*C*) bacteriorhodopsin reconstituted in dimyristoylphosphatidylcholine vesicles at a protein/lipid ratio of 1:4. Experimental (———), calculated (▬▬) spectra representing the best fit with Chen et al. reference curves (16) and difference (· · ·) between experimental and calculated spectra. CD spectra were recorded in 0.1 mm path-length cuvette at a protein concentration of 0.2 mg/ml (ϵ_M [570 nm] = 54,000 $M^{-1} \cdot cm^{-1}$) and at a sensitivity of $5 \cdot 10^{-6}$ OD · mm^{-1}.

TABLE I
PROTEIN SECONDARY STRUCTURES CONTENT (%) IN PURPLE MEMBRANE

	α	β	γ	$\bar{\sigma}$
Intact PM	56	9	35	0.108
Sonicated PM	69	6	25	0.067
BR-DMPC	74	9	17	0.051

Estimation of α-helix (α), β-sheet (β), and aperiodic (γ) structures from UV circular dichroism spectra for intact purple membrane (PM), sonicated purple membrane (PM) and bacteriorhodopsin reconstituted in dimyristoyl-phosphatidylcholine vesicles (BR-DMPC). $\bar{\sigma}$ is the root mean square deviation between calculated and experimental data.

tural model fitting best the known amino acid sequence of BR to the structural map (20).

The IR absorption spectrum of air-dried multilayers of PM is presented in Fig. 2 *A*. This spectrum, calculated from the absorptions $A_\parallel$ and $A_\perp$ as described in (15), shows the amide I band (80% C═O stretching) at 1,663 cm^{-1} and the amide II band (60% N-H bending) at 1,543 cm^{-1}. The amide I peak is asymmetric with two weak shoulders at ~1,685 and 1,630–1,640 cm^{-1}. The signal at 1,515 cm^{-1} on the amide II peak is ascribed to tyrosine residues. This spectrum is in good agreement with the spectra previously reported for air-dried films of PM (15, 21). In reference 15, we have previously assigned the ~1,685 cm^{-1} shoulder on the amide I peak to some anti-parallel β-sheets and/or β-turns (22, 23). In contrast, Jap et al. (7) assigned the 1,640 cm^{-1} shoulder in their spectra of air-dried membranes to some β-sheet conformation. However, caution should be exercised in interpreting this absorption feature since the presence of some residual tightly bound water (OH deformation at 1,640 cm^{-1}) in air-dried membranes could contribute to this signal. Furthermore, the amide I curve analysis described by Jap et al. (7) does not take into consideration the orientation of the amide transitions in air-dried oriented films. From the ratio of close to unity for the amide I to amide II absorbances, which is observed in Jap et al. (7), it can be concluded that the spectrum was recorded with the IR beam perpendicular to the plane of the film (15, 21). Under these conditions, the amplitude of the amide I band is significantly reduced compared with a band originating from a nondichroic transition.

The IR dichroism spectrum ($A_\parallel - A_\perp$) of air-dried PM is presented in Fig. 2 *B*. It shows a sharp positive signal for the amide I at 1,666 cm^{-1} and a negative one for the amide II at 1,545 cm^{-1}. IR dichroism spectra of PM thus indicate that the α-helical segments are preferentially oriented perpendicularly to the membrane plane, as previously demonstrated (15, 21). The difference in the shape of the absorption (Fig. 2 *A*) and linear dichroism (Fig. 2 *B*) spectra for the amide I band reflects the composite nature of this band even in the case of a predominantly α-helical structure. No significant contribution at 1,630–1,640 cm^{-1} can be detected, thus indicating that the corresponding

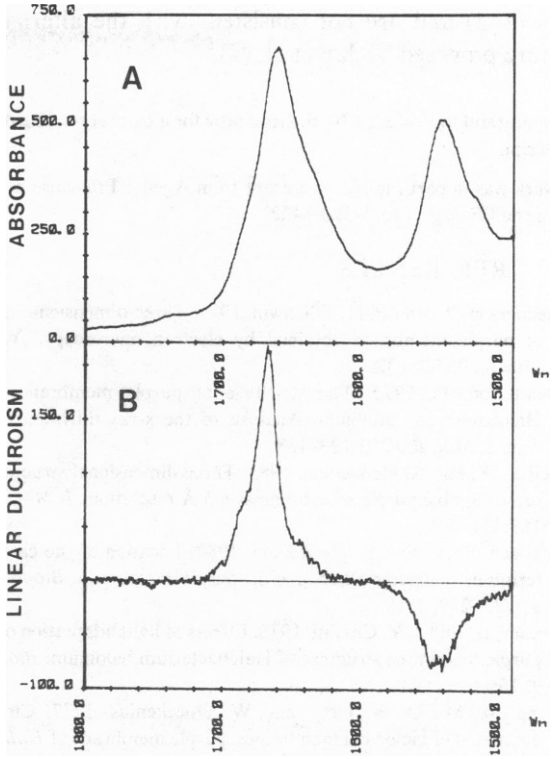

FIGURE 2 (*A*) Absorption and (*B*) dichroism ($A_\parallel - A_\perp$) spectra in the infrared of air-dried purple membrane. $A_\parallel$ and $A_\perp$ refer to the absorption measured with light polarized parallel and perpendicular to the incidence plane, respectively (15).

signal in the absorption spectrum reflects random structures which we tentatively assign to bound water molecules. On the other hand, the small positive dichroism signal around 1,685 cm^{-1} corresponds to a shoulder at the same position in the absorption spectrum. This could indicate that some C═O peptide groups (from antiparallel β-sheets and/or β-turns) lie preferentially perpendicular to the membrane plane. In the case of β-sheet strands, the polypeptide chains would be oriented parallel to the membrane, in contrast to the proposal in (7). Looking at the arrangement of the polypeptide chain of BR across the membrane (24), it is noted that 8 out of the 11 proline residues are localized outside or at the interface of the bilayer. In a polypeptide chain, Pro can promote the formation of β-turns (25). Furthermore, the Chou and Fasman method applied to BR predicted six potential β-turns between the seven ordered α-helical segments (26). The dichroism of the 1,685 cm^{-1} signal might thus be related to some β-turns joining the α-helical rods.

In summary, the conformation and orientation of the bacteriorhodopsin protein secondary structures estimated from CD as well as our IR absorption and dichroism spectra imply a large amount (74 ± 5%) of transmembrane α-helices and no significant contribution from β-sheet strands running perpendicular to the purple membrane plane. Thus, our data are in good agreement with the generally accepted models derived from diffraction experi-

ments (1–3) and are not consistent with the alternative structure proposed by Jap et al. (7).

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