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ORIENTATION OF INTRINSIC PROTEINS IN PHOTOSYNTHETIC MEMBRANES

POLARIZED INFRARED SPECTROSCOPY OF CHLOROPLASTS AND CHROMATOPHORES

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Summary

In order to estimate the degree of orientation of the α-helices of intrinsic proteins in photosynthetic membranes, polarized infrared spectroscopy has been used to measure the dichroism of the amide I and amide II absorption bands of air-dried oriented samples of purple membranes, chloroplasts and chromatophores from *Rhodopseudomonas sphaeroides*. Using purple membrane, in which the orientation of the α-helices is precisely known (Henderson, R. (1977) Annu. Rev. Biophys. Bioeng. 6, 87—109), as a standard to calibrate our measurements and estimating the mosaic spread (extent of orientation) of the membranes from linear dichroism measurements performed in the visible spectral range, it is concluded that in photosynthetic membranes, the α-helices of intrinsic proteins are tilted at less than 40° with respect to the normal to the plane of the membrane.

Introduction

Linear dichroism studies of oriented photosynthetic membranes have demonstrated an extensive orientation of most of the pigments absorbing in the visible spectral range, both in green plants and in photosynthetic bacteria

Abbreviation: LD, linear dichroism.

[1—3]. Owing to the specific binding of these pigments to intrinsic proteins in the native membrane, one may question if the proteins themselves have a defined orientation with respect to the membrane plane. These proteins contain a large percentage (40 to 50%) of α-helices; furthermore, peptide groups in an α-helix present characteristic infrared transitions which are polarized predominantly parallel (amide A and amide I bands) or perpendicular (amide II band) to the helix axis [4]. Accordingly, by measuring the infrared dichroism of the amide absorption bands, it is possible to estimate the degree of orientation of the α-helices of intrinsic proteins relative to the membrane plane. Recently, polarized infrared spectroscopy of oriented purple membranes from *Halobacterium halobium* [5] and oriented rod outer segments [6] has been used to determine such an orientation for the α-helices of bacteriorhodopsin and rhodopsin, respectively.

In the present work, polarized infrared spectroscopy has been successfully applied to air-dried samples of purple membranes, spinach chloroplasts and chromatophores from *Rhodopseudomonas sphaeroides.* Using purple membrane (in which the orientation of the α-helices is precisely known [7]) as a standard to calibrate our measurements on photosynthetic membranes, we present direct evidence that in photosynthetic membranes, the α-helices of intrinsic proteins are preferentially oriented along the normal to the membrane plane.

Materials and methods

(1) Preparation and orientation of samples. Photosynthetic membranes deprived of extrinsic proteins by EDTA treatment have been isolated from spinach chloroplasts [2]. Usual procedures were used to obtain purple membranes [8] and purified chromatophores from *Rhodopseudomonas sphaeroides* strain 2.4.1 [9].

Calcium fluoride discs were cleaned with sulfochromic acid, thoroughly rinsed in distilled water and immediately layered with the suspension of membranes (1 mg protein/ml in distilled water). Orientation of the membranes parallel to the CaF_2 disc was achieved by slow evaporation in darkness at 5° C. Air-dried photosynthetic membranes have been shown to be structurally intact and photochemically active [2,3].

(2) Spectroscopic measurements. Infrared spectra were recorded on a Perkin-Elmer 180 double beam spectrometer equipped with a common beam Perkin-Elmer wire grid polarizer. The infrared spectrometer was flushed with dry nitrogen and the spectral width normally used was 1.5—2 cm^{-1}. Polarized infrared absorption spectra were measured by tilting the sample at 60° with respect to the direction of the beam. As shown on Fig. 1, infrared light was polarized either perpendicular to the normal to the disc plane (to record $A_\perp$) or parallel to the plane of incidence (to record $A_\parallel$). When recording $A_\perp$, the transitions parallel to the disc surface will absorb strongly, while the transitions oriented along the normal N will not absorb. A blank CaF_2 disc was mounted with an identical geometry in the reference beam.

The extent of orientation (mosaic spread) of the membranes in the air-dried specimens was monitored by measuring the linear dichroism of the retinal

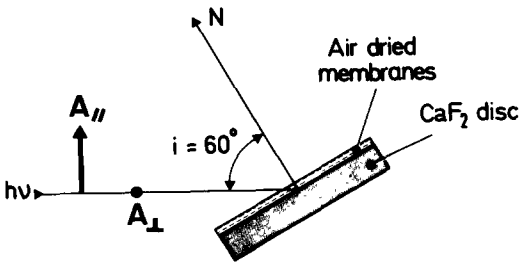

Fig. 1. Schematic representation of the polarized light measurement on membranes oriented parallely to the CaF_2 disc surface.

in purple membranes (at 560 nm), of the Q_X transitions of bacteriochlorophyll *a* in the chromatophores (at 590 nm) and of the Q_Y transitions of chlorophyll *a* in the chloroplasts (at 681 nm), and comparing the results with published values [1—3]. For each type of membrane, the linear dichroism, absorbance and polarized infrared spectra were recorded on the same air-dried sample. Linear dichroism and absorbance measurements were performed on a previously described instrument [10] and a Cary 17, respectively.

Results and Discussion

(1) Qualitative analysis

Fig. 2 shows the polarized infrared spectra of air-dried purple membrane, chloroplasts and chromatophores, from 4000 to 1100 cm^{-1}. The amide A (NH stretching at about 3300 cm^{-1}), amide I (C=O stretching at about 1660 cm^{-1}) and amide II, (NH bending at 1545 cm^{-1}) bands are easily identified, as well as the lipid ester carbonyl stretching vibration at 1740 cm^{-1} and the various CH stretching modes around 2930 cm^{-1}. The shoulder at 1515 cm^{-1} on the amide II band is probably due to tyrosine residues [11]. In proteins, the frequency of the amide I band depends on the peptide backbone conformation [12]. The amide I maxima at 1666 cm^{-1} (purple membrane), 1658 cm^{-1} (chloroplasts) and 1659 cm^{-1} (chromatophores) indicate the presence of some α-helical structure [13]. For purple membrane, a weak shoulder around 1685 cm^{-1} is assigned to antiparallel β-structure and/or β-turns [12,14]: it is thus likely that there exists a small amount of β-conformation in bacteriorhodopsin, as has been recently reported for rhodopsin [15].

It can be noted that the relative amplitudes of the amide bands in the polarized spectra of purple membrane (Fig. 2A) are in good agreement with the data of Rothschild and Clark [5]. Furthermore, for the three types of membranes (Fig. 2) the amide A and amide I bands are more intense in the $A_{//}$ spectra (when the light is preferentially polarized along the membrane normal), than in the $A_{\perp}$ spectra; an opposite situation is observed for the amide II band. In α-helices, the NH (amide A) and C=O (amide I) stretching vibrations of the polypeptide chain are polarized preferentially parallel to the helix axis while the NH deformation vibration (amide II) is mostly polarized perpendicular to this axis [4]. Therefore, the observed dichroism for the amide

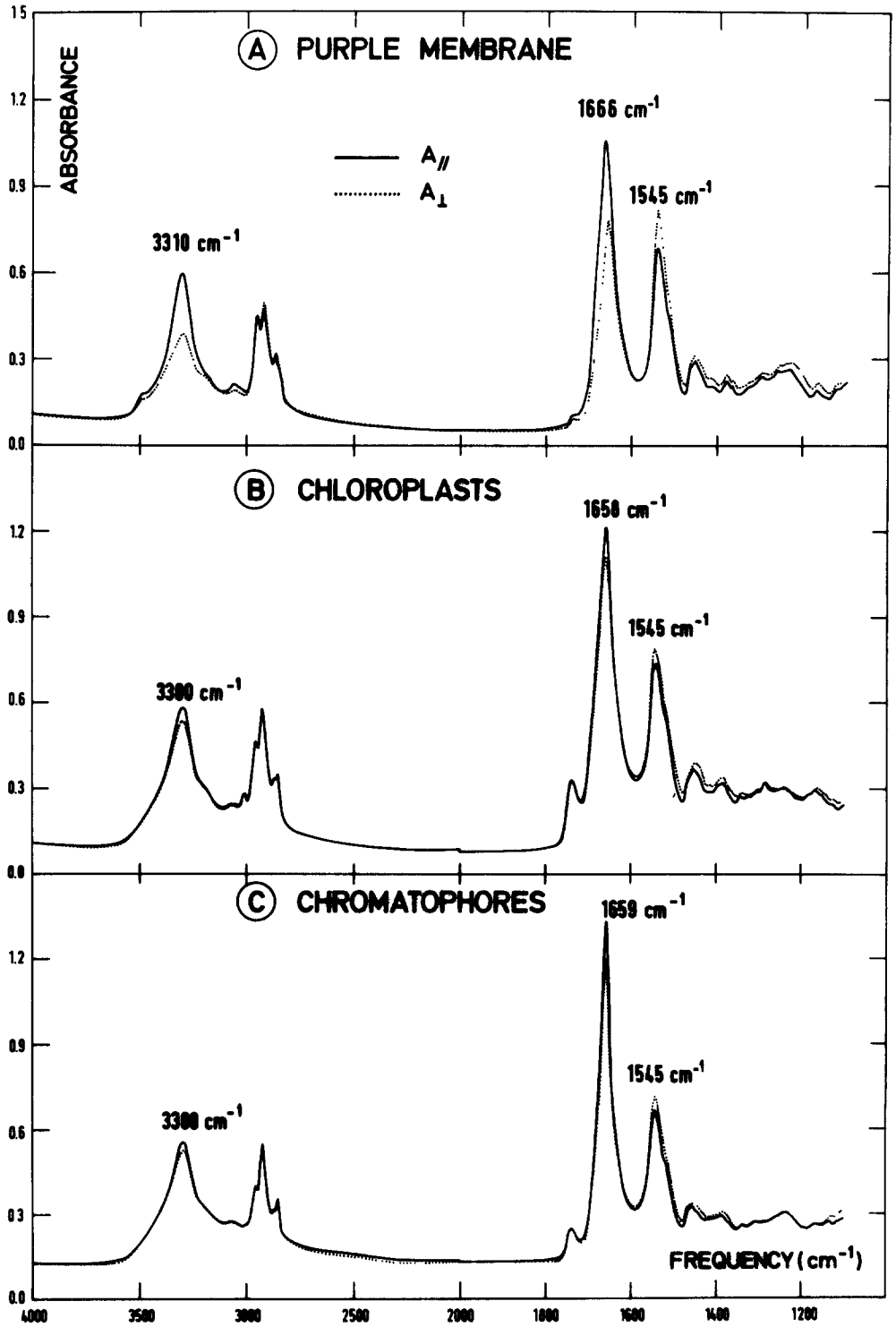

Fig. 2. Polarized infrared spectra of air-dried purple membrane (A), spinach chloroplasts (B) and chromatophores (C), for $i = 60°$ and with the polarisation direction preferentially parallel (———) and perpendicular (· · · · · ·) to the normal to the disc.

bands qualitatively indicates that in photosynthetic membranes and in purple membranes, α-helix axes are preferentially oriented along the normal to the membrane plane.

The dichroism which can be detected for some of the less intense bands shown in Fig. 2 is presently under investigation and will not be discussed in this work. Furthermore, when comparing the dichroism of the different amide bands on several oriented specimens, we noticed a larger variation for the amide A band than for either the amide I or amide II bands. The presence of some trace amounts of tightly bound water in our samples could explain this observation as the extinction coefficient of water is larger around 3300 cm^{-1} than in the 1650—1540 spectral region (Ceccaldi, M., personal communication). Accordingly, a procedure which minimizes the contribution of water has been selected to estimate the amplitude of the amide I and amide II bands (see below) and only the dichroism of these bands has been used for the quantitative analysis of the orientation of the α-helices.

(2) Quantitative analysis

If we consider a set of transition moments M (corresponding to one absorption band) oriented with respect to the normal N to the disc, such a distribution of orientation can be characterized by an order parameter S. By appropriate measurements of the absorption of plane polarized light (visible or infrared) by this set of transition moments, S can be determined [1,2]. Furthermore, the equation $S = (3\cos^2\varphi - 1)/2$ allows to calculate an angle corresponding to a specific distribution of orientation in which all the transition moments make the same angle φ with N (Fig. 3a). A model depicted in Fig. 3b is used to describe the orientation of the α-helices in the air-dried sample of membranes. Due to the mosaic spread, there is a distribution (S_{ms}) of the membrane normals with respect to N. There is also a distribution (S_α) of the α-helix axes with respect to the membrane normals. Finally, the transition moments for an infrared amide band are at an angle φ_M with respect to the α-helix axis and can be characterized by an order parameter S_M. Provided all the other parameters

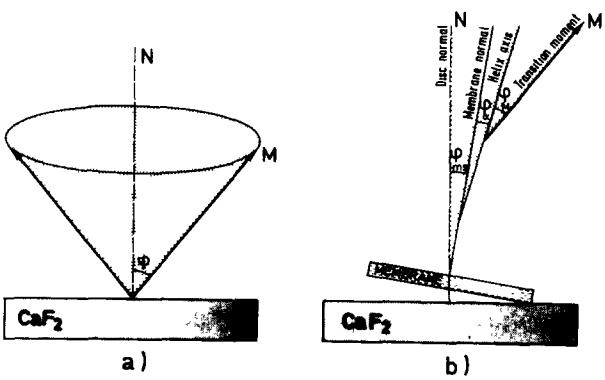

Fig. 3. Models for the orientation of the transition moments M making an angle φ with the disc normal N. The transition moment M is inclined at φM to the α-helix axis which is tilted by φ_α with respect to the membrane normal. The mosaic spread is defined by φ_{ms}.

have been estimated, S_α can be calculated from the equation [5,6]:

$$S = (S_{ms})\,(S_\alpha)\,(S_M) \tag{1}$$

S is estimated from the amide bands absorbances for polarized light and the α-helical content (α). The amide I and amide II bands are treated independantly, thus leading to two S values. For each of these bands, the amplitude of the maximum is measured with respect to the trough at 1590 cm^{-1}, leading to the values $A_{/\!/}$ and $A_\perp$. A correction is then applied to take into account the contribution of the non dichroic random coil component, assuming an identical extinction coefficient at the absorption maximum for the α-helical and random coil components structures. The respective contributions of these structures to the measured absorbances can thus be calculated by using the following equations:

$$A_{/\!/} = A_{/\!/\alpha} + (1-\alpha)\,(A_{/\!/} + 2A_\perp)/3 \tag{2}$$

$$A_\perp = A_{\perp\alpha} + (1-\alpha)\,(A_{/\!/} + 2A_\perp)/3 \tag{3}$$

where $A_{/\!/\alpha}$ and $A_{\perp\alpha}$ are the polarized components of the absorbance of the α-helices only, while $(A_{/\!/} + 2A_\perp)/3$ represents the total absorbance of the sample [1,2]. From these values, the dichroic ratio $D = A_{/\!/\alpha}/A_{\perp\alpha}$ for the α-helices can be calculated. The general formula [1,2] which relates the dichroic ratio measured at a tilt angle i, to the order parameter S is:

$$D = \left(\frac{3S}{1-S}\right)\left(\frac{\sin^2 i}{n^2}\right) + 1 \tag{4}$$

where n is the refractive index of the layer of air-dried membranes. For $i = 60°$ and n = 1.5 (this choice of n will be discussed later), Eqn. 4 can be written as:

$$S = (D-1)/D \tag{5}$$

S_{ms} has been estimated by monitoring, on the same oriented samples, the linear dichroism of the chromophores in the visible region. By comparing the order parameter obtained for these chromophores to previously published values, we have determined that in our samples the orientation of the membranes was similar to the best ones reported to date [2,3].

S_M has been calculated from the data of Tsuboi [16]: according to this author and to others [17], the transition moments of the amide I and amide II vibrations make an angle of 39° (φ_{MI}, amide I) and 75° (φ_{MII}, amide II) with the helix axis, leading to $S_{MI} = +0.41$ and $S_{MII} = -0.40$ respectively. Although a 27° value has been described for φ_{MI} [18], the values of Tsuboi [16] have been used for the most recent infrared dichroism studies of oriented membranes [5,6,19]. Furthermore, an extensive discussion of the φ_M limits can be found in [5].

The orientation of the α-helices of bacteriorhodopsin with respect to the normal to the plane of purple membrane has been precisely determined by electron microscopy and diffraction experiments [7]. In this study, we have used air-dried samples of purple membranes as a standard to calibrate our infrared dichroism measurements. For the retinal chromophore in purple membranes air-dried on a CaF_2 disc, the ratio of the linear dichroism to the absorbance

TABLE I

ESTIMATION OF D AND φ_α FROM INFRARED DICHROISM DATA

Average obtained from three different air-dried samples for each type of membrane.

	Purple membrane		Chloroplasts		Chromatophores	
	Amide I	Amide II	Amide I	Amide II	Amide I	Amide II
D	1.64	0.73	1.42	0.82	1.36	0.83
φ_α	9°	13°	25°	33°	29°	35°

(LD/A), both measured at 560 nm, leads to $S_{560} = +0.27$, taking $n = 1.5$ [20]. This S_{560} value is in good agreement with that determined by Heyn et al. [20] on oriented purple membrane and therefore indicates a negligible mosaic spread ($S_{ms} = 1$). Using a α-helical content of 80% ($\alpha = 0.8$) [21], the amide I and amide II dichroism measured on the same oriented sample leads to the results given in Table I. The dichroic ratio of the amide I band leads to $\varphi_\alpha = 9°$, while from the dichroic ratio of the amide II band, a value $\varphi_\alpha = 13°$ is obtained. Indeed, these φ_α values compare fairly well with the 11° value found by Henderson [7,21] indicating that polarized infrared spectroscopy is quite suitable for elucidating the average orientation of α-helices in a membrane. This good agreement also suggests that the assumptions made to calculate φ_α are valid. This notably applies to the choice of the refractive index in the visible and infrared region ($n = 1.5$), to the assumption that the extinction coefficient of the amide bands for α-helix and random coil conformations are similar and to the choice of the angle φ_M between the transition moment and the helix axis [16]. However, it must be borne in mind that the determination of φ_α in purple membrane relies on a large number of independent measurements and that a cancellation of errors could lead to a fortuitous agreement.

In the case of photosynthetic membranes, the LD/A values at 681 nm (chloroplasts) and at 590 nm (chromatophores) lead to $S_{681} = -0.10$ and $S_{590} = +0.50$, respectively, using $n = 1.5$. These S values are in agreement with previous determinations [1—3] and indicate a high degree of orientation of the membranes in the plane of the disc. Assuming $S_{ms} = 1$, a refractive index in the infrared of 1.5 and taking an α-helical content of 40% ($\alpha = 0.4$) for chloroplasts and 50% ($\alpha = 0.5$) for chromatophores [22], the infrared dichroism results calculated from the spectra shown in Fig. 2 are presented in Table I. It can be noted that the dichroism measurements of the amide I and amide II peaks lead to similar estimations of φ_α for both the chloroplasts (25 and 33°) and the chromatophores (29 and 35°). When the calculations are made on the amide I band, the φ_α angles are slightly smaller than when using the amide II band. This small discrepancy, which is also observed in the case of purple membrane (Table I), may indicate some imperfection in the estimation of the dichroic ratio of the two amide bands (choice of the base line, overlap of these two bands . . .) and/or in the choice of at least one of the φ_M angles (a change of less than 1° on φ_{MI} would lead to identical φ_α values for purple membrane).

In contrast to the case of purple membrane [20], the quantitative determina-

TABLE II

EFFECT OF VARYING THE DIFFERENT PARAMETERS (S_{ms}, n IN THE INFRARED AND α-HELIX CONTENT) ON φ_α, FOR CHLOROPLASTS AND CHROMATOPHORES

Chloroplasts

$\alpha = 0.4$, $n = 1.5$	φ_α (°)		$\alpha = 0.4$, $S_{ms} = 1$	φ_α (°)		$n = 1.5$, $S_{ms} = 1$	φ_α (°)	
	Amide I	Amide II		Amide I	Amide II		Amide I	Amide II
$S_{ms} = 1$	25	33	$n = 1.33$	30	39	$\alpha = 0.5$	31	38
$S_{ms} = 0.8$	14	27	$n = 1.50$	25	33	$\alpha = 0.4$	25	33
$S_{ms} = 0.7$	—	23	$n = 1.70$	17	24	$\alpha = 0.3$	14	24

Chromatophores

$\alpha = 0.5$, $n = 1.5$	φ_α (°)		$\alpha = 0.5$, $S_{ms} = 1$	φ_α (°)		$n = 1.5$, $S_{ms} = 1$	φ_α (°)	
	Amide I	Amide II		Amide I	Amide II		Amide I	Amide II
$S_{ms} = 1$	29	35	$n = 1.33$	34	40	$\alpha = 0.6$	33	39
$S_{ms} = 0.8$	21	30	$n = 1.50$	29	35	$\alpha = 0.5$	29	35
$S_{ms} = 0.7$	13	26	$n = 1.70$	23	28	$\alpha = 0.4$	23	30
$S_{ms} = 0.6$	—	20	$n = 2$	9	—	$\alpha = 0.3$	8	16

tion of the order parameter for the chromophores and of the mosaic spread has never been done on the same oriented sample for photosynthetic membranes, and so, a possible decrease of the mosaic spread has to be considered in the calculations. Furthermore, some uncertainties must be taken into account regarding the membrane refractive index in the infrared region and the α-helical content of the intrinsic proteins. The effect of varying these different parameters on the φ_α values for chloroplasts and chromatophores is summarized in Table II. For each parameter, there is a bound value for which φ_α can no longer be caculated. Table II shows that an upper limit of φ_α (38—40°) is only obtained under extreme conditions, i.e. when $S_{ms} = 1$ and $n = 1.33$, or when the percentage of α-helix is increased with respect to the published values; on the contrary, a decrease of S_{ms} or of α leads to a decrease of φ_α.

After due consideration to the most probable causes of uncertainties, it can be concluded from Tables I and II that on the average the α-helices of the intrinsic proteins are tilted at less than 40° with respect to the normal to the plane of the photosynthetic membrane. This angle represents an average value and the possibility that a few α-helices are lying parallel to the membrane plane while a larger number are at a small angle to the normal cannot be excluded. However, this result indicates a dominant transmembrane orientation of the α-helical segments of the intrinsic proteins in the presently investigated photosynthetic membranes.

It therefore appears that such transmembrane orientation of the α-helices is not a special feature of bacteriorhodopsin [5] and rhodopsin [6,19] but can be also detected in the more heterogenous proteins of photosynthetic membranes. In view of this heterogeneity, it would be of interest to apply polarized infrared spectroscopy to the structural study of oriented samples containing purified chlorophyll-protein complexes. It has been proposed [23] that the diamagnetic anisotropy of some constituents oriented with respect to the normal to the membrane plane is responsible for the orientation of purple membrane [24], rod outer segments [25], chloroplasts [26], and some photosynthetic bacteria [27] in an externally applied magnetic field. In the case of bacteriorhodopsin and rhodopsin, it has been shown that the preferential transmembrane orientation of the α-helices was responsible for this effect [28,29]. In photosynthetic membranes, the macrocycles of the chlorophyll or bacteriochlorophyll molecules are preferentially oriented perpendicularly to the membrane plane [1,2, 27] and such an orientation could qualitatively explain the observed behaviour of these systems in magnetic fields. The infrared dichroism study presented here demonstrates that the orientation of the α-helical segments of the intrinsic proteins is also adding to the net diamagnetic anisotropy of photosynthetic membranes.

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