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CONFORMATION AND ORIENTATION OF THE PROTEIN IN THE BACTERIAL PHOTOSYNTHETIC REACTION CENTER

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The protein structure of the *Rhodospseudomonas sphaeroides* reaction center reconstituted in lipid vesicles was investigated by circular dichroism and polarized infrared spectroscopy. By measuring the infrared dichroism of the amide absorption bands of air-dried oriented membranes (native chromatophores and reconstituted reaction centers), it is possible to estimate the degree of orientation of the polypeptide chains with respect to the bilayer plane. Linear dichroism spectra were investigated from the ultraviolet to the infrared region; measurements of the large linear dichroism of the bacteriochlorophyll and bacteriopheophytin chromophores were used to check the extent of orientation of the air-dried membranes. The major conclusions are: (1) there is a net orientation of the tryptophan heterocycles preferentially in the plane of the membrane. (2) An orientation of the lipids is detected with the polar groups (C=O ester and PO₂⁻) rather parallel to the bilayer plane. (3) The protein of the reaction center is composed to a large extent of α -helix ($50 \pm 10\%$) compared to $60 \pm 10\%$ in chromatophore membranes. (4) Both the reaction center and the chromatophore membrane contain a small amount of oriented β -structure. (5) The α -helices tend to be aligned along the normal to the membrane. The α -helix axes are tilted at less than 35° in reaction centers and 40° in chromatophores.

Introduction

The primary events in photosynthesis occur in membrane-bound chlorophyll-protein complexes whose specific position within the chromatophore membrane ensures high efficiency of energy transfer from antenna to reaction center and membrane-bound electron carriers. The reaction center of photosynthetic bacteria has been fairly well characterized [1,2]. As isolated from *Rhodospseudomonas sphaeroides*, it is composed of three protein subunits and contains four bacterio-

chlorophylls (BChl), two bacteriopheophytins (BPh), one iron (Fe²⁺) and two ubiquinones. The spatial positions of pigment molecules within the reaction center have been partially characterized by electron paramagnetic resonance (EPR) and by optical linear dichroism (LD) studies [3–6]. However, very little is known about the organization of the protein itself. The overall shape has been suggested from X-ray [7] and neutron diffraction [8] studies. The amino acid composition of the subunits has been determined but not the complete sequence [1,9]. Currently, there is no information on the secondary and tertiary protein structures within the complex. The understanding of the protein structure of the reaction center is important both with respect to its function as a

Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin.

highly efficient photosynthetic protein complex as well as its localization as an intrinsic membrane protein.

Polarized light spectroscopy has been extensively used to analyze the organization of photosynthetic membranes [10] and recently an average transmembrane orientation of the α -helices of the intrinsic proteins in bacterial chromatophores and in chloroplast membranes has been detected by polarized infrared spectroscopy [11]. However, photosynthetic membranes are heterogeneous and contain several chlorophyll-protein complexes (at least one type of antenna and one type of reaction center). A more critical evaluation of the structure of the photosynthetic membrane requires an estimation of the contribution of each protein complex to the overall structure of the membrane. This notably involves the isolation of the various chlorophyll-protein complexes, their purification and their reconstitution in lipid vesicles.

In this paper, the protein structure of *Rps. sphaeroides* reaction centers reconstituted in lipid vesicles was investigated by ultraviolet circular dichroism (CD) and polarized infrared spectroscopies. By measuring the infrared dichroism of the amide absorption bands, it is possible to estimate the degree of orientation of the polypeptide chains with respect to the bilayer plane [11,12]. We present the first LD investigation from the ultraviolet to the infrared region for both the native chromatophore membranes of *Rps. sphaeroides* and the isolated reaction center reconstituted in phospholipid vesicles. Membranes were oriented by air drying. Conformational analysis of the reaction center indicates that a large proportion of the polypeptide is in α -helix (50%). Polarized infrared spectra of oriented reaction centers demonstrate an orientation of the α -helix segments preferentially perpendicular to the bilayer [13], as previously determined for the antenna in the chromatophore membrane [11].

Materials and Methods

Preparation and orientation of samples

The photosynthetic bacteria *Rps. sphaeroides* (carotenoid-less mutant R26) was grown as described earlier [14]. The methods used for preparing purified chromatophores [15] and reaction

centers [16] have also been described. Reaction centers were isolated with lauryldimethylamine *N*-oxide detergent and their purity checked by polyacrylamide gel electrophoresis. The reconstitution of reaction centers into phospholipid vesicles [4] was accomplished from purified reaction centers in which the lauryldimethylamine *N*-oxide had been exchanged for the detergent octyl- β -D-glucopyranoside. Egg yolk phosphatidylcholine [17] vesicles were formed by sonicating a suspension of lipids (300 μ M) in Tris-HCl buffer (10 mM, pH 8) for three 20-s periods. The reaction centers in 30 mM octyl- β -D-glucopyranoside, 10 mM Tris-HCl pH 8, were diluted to a concentration of 3 μ M in the vesicle suspension and sonicated at room temperature again for three 20-s periods. The solution was then dialyzed against 10 mM Tris-HCl pH 8, 0.1% sodium ascorbate for 24 h to remove the detergent. Finally, the reconstituted reaction centers were suspended in distilled water. Orientation of the sample was achieved by air drying on to CaF₂ [11,12], so that the stacking of the vesicles was mainly parallel to the disc.

Spectroscopic measurements

The LD, absorbance and polarized infrared spectra were recorded on the same air-dried sample as described in Ref. 11. The extent of orientation (mosaic spread of the membranes in the air-dried specimens) was monitored by measuring the linear dichroism of the Q_x transition of BChl in the chromatophores (at 590 nm) and of the Q_y transition of BPh in the reaction centers (at 760 nm) and comparing the results with the published values. Infrared spectra were recorded on a Perkin-Elmer 180 double-beam spectrometer equipped with a common-beam Perkin-Elmer wire-grid polarizer and linked to a Hewlett-Packard 9825 A computer. Infrared dichroism was measured with an angle of incidence (*i*) of 60°. Infrared light was linearly polarized either perpendicular to the normal to the disc (to record A_{\perp}) or parallel to the plane of incidence (to record A_{\parallel}) as described earlier [11,12]. When recording A_{\perp} , the transitions parallel to the disc surface will absorb strongly while the transitions oriented along the normal will not absorb. A blank CaF₂ disc was mounted with an identical geometry in the reference beam.

Ultraviolet spectra were measured at ambient

temperature with a Jobin Yvon Mark III dichrograph linked to a Micral computer. Data were recorded from 260 to 190 nm in 0.1 mm path length quartz cells (Hellma). Blank runs of lipid vesicles without protein were subtracted from the spectra of reconstituted reaction center. For each sample, spectra were digitized with averaging (usually 10 times).

Data analysis

Determination of the secondary structure of the reaction center protein from CD spectra. Ultraviolet CD spectra of proteins from 190 to 260 nm reflect the secondary structure adopted by the polypeptide chain in solution. CD spectra can be seen as linear combinations of conformationally related subspectra [18]. The fractions of α -helix, β -sheet and aperiodic segments in protein of reaction centers and chromatophores were estimated by fitting experimental CD spectra to sums of published reference spectra [19,20], using a general least-squares program kindly supplied by Dr. K. Lintner (C.E.N. Saclay). Reference data from Chen et al. [19,20] were used.

Determination of pigment and α -helix orientation in reaction center. The model to estimate the orientation of the transition moments in our air-dried samples of reaction centers has been described in a previous paper [11]. The distribution of transition moments corresponding to a given absorption band can be characterized by an order parameter, $S = (3\cos^2\phi - 1)/2$, where ϕ is the angle between the transition moment and the normal to the disc. S can be estimated from the absorbance for parallel and perpendicular polarized light [11]. With the amide bands, a correction must be applied to take into account the contribution of the nondichroic random coil component: calculation of the dichroic ratio $D = A_{\parallel}/A_{\perp}$ for the α -helices only has been described in an earlier paper [11]. D measured at a tilt angle i is related to S by:

$$D = \frac{3S}{1-S} \frac{\sin^2 i}{n^2} + 1$$

where n is the refractive index of the layer of air-dried membranes. For $i = 60^\circ$ and $n = 1.5$, $S = (D - 1)/D$.

In order to calculate the tilt angle ϕ_α of the

helix axis relative to the bilayer normal, two additional parameters have to be taken into account: (1) the angle ϕ_M between the transition moments of the amide vibrations with the helix axis, and (2) the mosaic spread of the samples characterized by an order parameters S_{ms} . According to Tsuboi [21], the transition moment of the amide I and II bands makes 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. S_α , the distribution of the α -helix axes with respect to the membrane normal, and then ϕ_α can be calculated from the equation:

$$S = S_\alpha \cdot S_{ms} \cdot S_M$$

Results

Ultraviolet CD spectra of chromatophores and reaction centers

A quantitative analysis of protein orientation by polarized infrared spectroscopy requires a determination of the extent of the various secondary protein configurations. The extent of α -helix, β - and aperiodic structures can be evaluated from peptide ultraviolet CD spectra [19,20].

The 190–260 nm region of the CD spectra of chromatophores and reconstituted reaction centers in vesicles is presented in Fig. 1. These are the difference spectra taken with the buffer as the blank in the case of chromatophores, and with a blank of egg phosphatidylcholine vesicles, having a matched lipid concentration, in the case of the reconstituted reaction centers. Both reaction center and chromatophore spectra show an α -helical pattern having two negative bands located around 210 and 223 nm and a positive band at about 194 nm. A comparison of these spectra with those of soluble proteins [19,20] suggests that these spectra are not severely distorted by light-scattering artifacts. This point is further emphasized by the close similarity between the spectra of the reaction center solubilized in either the detergent or the lipids. As a result, it has been assumed that the reference spectra obtained with soluble proteins [19,20] can also be used as the basis for the conformation analysis of the intrinsic reaction center protein.

The percentage of α -helix, β - and aperiodic

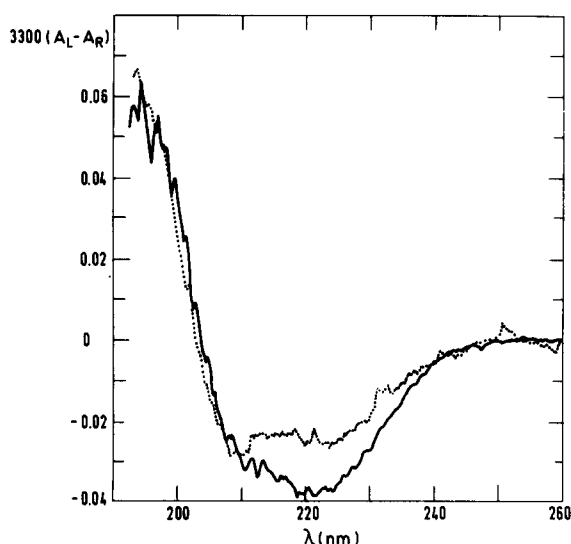


Fig. 1. Far-ultraviolet CD spectra of *Rps. sphaeroides* chromatophores (—) and reaction centers reconstituted in phosphatidylcholine vesicles (·····). Membranes were suspended in Tris-HCl buffer. Each spectrum is the average of ten runs.

protein structures in *Rps. sphaeroides* R-26 chromatophore membranes and reaction centers was calculated based upon fits to the experimental spectra using the reference spectra as described in Materials and Methods. The results are compared in Table I. The α -helix content of native chromatophores (61%) is in reasonable agreement with the results of Menke [22] who estimated a 50% α -helix extent in chromatophores of *Rps. sphaeroides*. Table I also indicates that a large proportion (46%) of the reaction center polypeptide chains are in the α -helical structure; a

TABLE I

ESTIMATION OF THE PERCENTAGE ($\pm 10\%$) OF α -HELIX (α) β - (β) AND APERIODIC (γ) STRUCTURES FROM ULTRAVIOLET CD DATA

Structure	Chromatophores	Reaction centers (in lipid vesicles)
α	61	46
β	13	5
γ	26	49

similar α -helix content (50%) was observed for isolated reaction centers in detergent solution. Accordingly, for the analysis of the infrared spectra described below, an α -helical content of $50 \pm 10\%$ was used for reaction centers. This range of values takes into account slight variations between different samples and allows for the fact that no corrections for light scattering have been applied.

Ultraviolet and visible LD spectra

Figs. 2 and 3 show LD and absorption spectra in the ultraviolet, visible and infrared region from 250 to 10000 nm for air-dried preparations of chromatophores and reaction centers reconstituted in phospholipid vesicles.

The dichroism in the ultraviolet at 280 nm can be identified with aromatic amino acid residues. This, together with the signal at 290 nm, indicates that on average, tryptophan heterocycles in chromatophores and in reaction centers tend to be preferentially aligned along the membrane plane. The near-infrared region of reconstituted reaction centers shows the usual dichroism pattern characteristic of BChl (800 and 860 nm transitions lying in the membrane plane) and BPh (760 nm transitions lying nearly perpendicular to the membrane plane) [5]. Measurement of the large LD of the BChl and BPh chromophores was used to check the mosaic spread, i.e., the extent of orientation of our air-dried membranes. The ratio of the linear dichroism to the absorbance (LD/A) at 760 nm (reaction centers) and at 590 nm (chromatophores) leads to $\phi_{760} = 44^\circ$ and $\phi_{590} = 38^\circ$. These values are in good agreement with previous determinations ($\phi_{760} = 45^\circ$ [5] and $\phi_{590} = 37^\circ$ [11]) and indicate that our vesicles are well oriented in the plane of the disc.

Infrared spectra

Absorption infrared spectra of air-dried chromatophores and reconstituted reaction centers display marked similarities in the protein and lipid absorption bands in the 4000–1000 cm^{-1} region (Figs. 2 and 3). The amide A (NH stretching at about 3300 cm^{-1}), amide I (C=O stretching at 1656 cm^{-1}) and amide II (NH bending at 1543 cm^{-1}) bands are easily identified [23,24] as well as the lipid ester carbonyl stretching at 1738 cm^{-1} , the PO_2^- stretching transition at 1250 cm^{-1} and

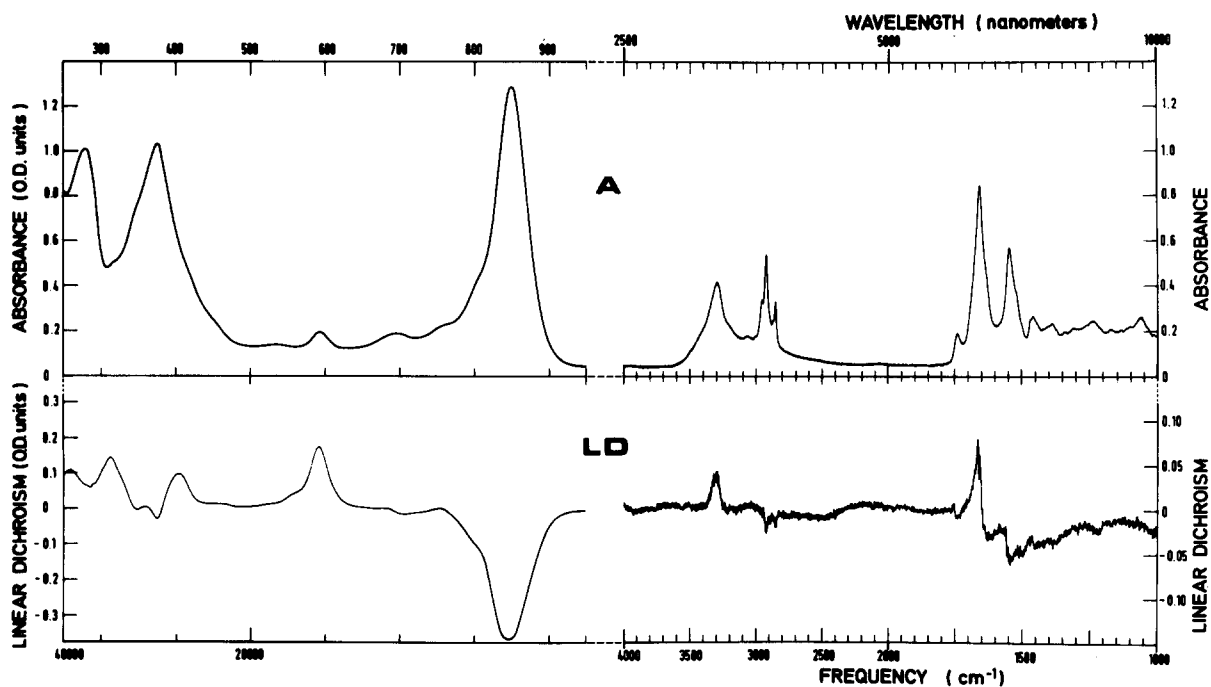


Fig. 2. Ultraviolet, visible, infrared absorption (A) and linear dichroism (LD) spectra of air-dried chromatophores for $i = 60^\circ$.

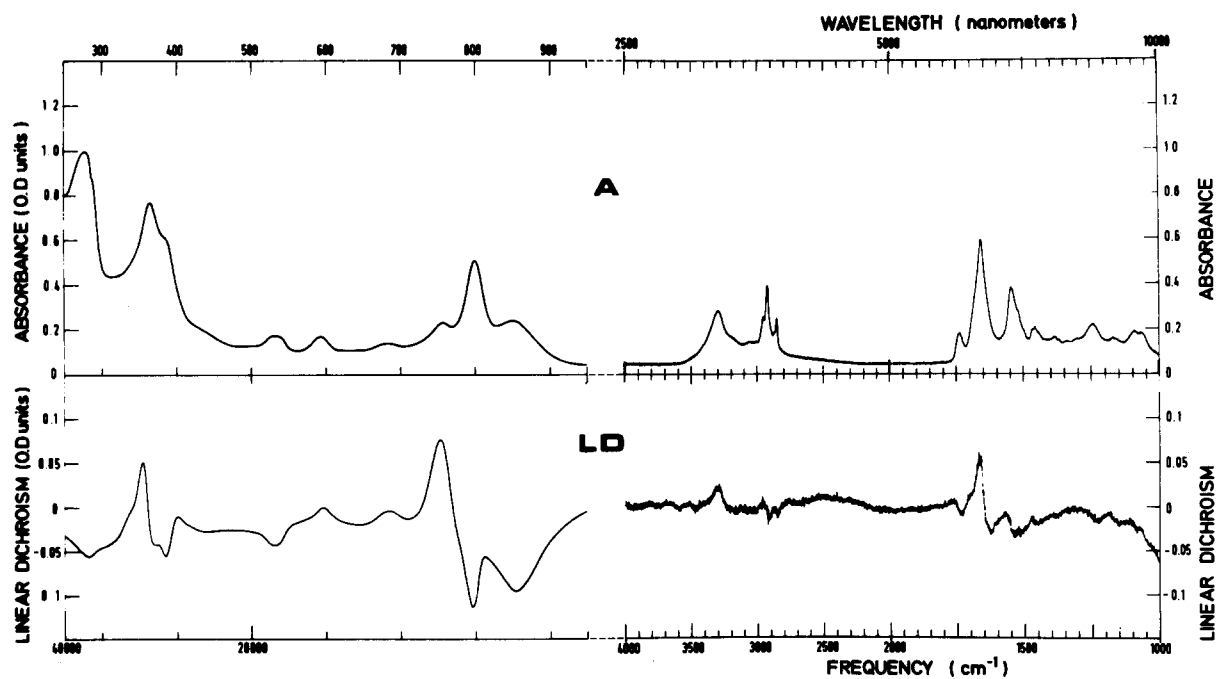


Fig. 3. Ultraviolet, visible, infrared absorption (A) and linear dichroism (LD) spectra of air-dried reaction centers reconstituted in phosphatidylcholine vesicles

the various CH_2 stretching modes around 2900 cm^{-1} [25]. The shoulder at 1515 cm^{-1} on the amide II band is attributed to the aromatic ring stretching modes of the tyrosine residues [26]. In proteins, the frequency of the amide bands depends on the backbone conformation [23,24]. In reaction centers, as well as in chromatophores, the amide I maximum at 1656 cm^{-1} indicates the presence of α -helical structure. An asymmetry in the shape of the amide I absorption is tentatively assigned to the presence of some β -structure with a peak absorption at about 1630 cm^{-1} [23,24]. The existence of this band becomes more obvious in the LD spectra described below.

Polarized infrared difference spectra ($A_{\parallel} - A_{\perp}$) for both native chromatophore membranes and reconstituted reaction centers show a positive signal for the amide A (3300 cm^{-1}) and amide I (1656 cm^{-1}) bands, i.e., a preferential absorption with the light polarized parallel (A_{\parallel}) to the plane of incidence; for the amide II band, the infrared dichroism signal is negative and corresponds to a preferential absorption with the light polarized perpendicular to the plane of incidence (A_{\perp}). A small negative signal at 1630 cm^{-1} (shoulder on the amide I band) is consistently seen in these difference spectra, which corresponds to a preferential A_{\perp} absorption. This point will be discussed later. Several of the lipid bands exhibit a dichroism in which the absorption is seen to be more intense in the A_{\perp} spectrum (for example, $\text{C}=\text{O}$ ester at 1738 cm^{-1} , PO_2^- at 1250 cm^{-1} and CH_2 of hydrocarbon chains around 2900 cm^{-1}). This dichroism spectrum compares fairly well with previous infrared data reported on pure lipids and liposomes [12,25,27]. In an α -helix, both the peptide $\text{C}=\text{O}$ stretching mode (amide I) and the NH stretching mode (amide A) are preferentially polarized parallel to the helix axis while the NH deformation vibration (amide II) is largely polarized perpendicular to this axis [28]. Therefore, the observed dichroism behavior for the amide bands qualitatively indicates that in reconstituted reaction centers just as in native chromatophores the α -helix axes of the proteins tend to be preferentially oriented along the normal to the membrane.

The dichroic ratios for the amide I and II absorptions are summarized in Table II, along

TABLE II

ESTIMATION OF D AND ϕ_{α} FROM INFRARED DICHROISM DATA

The average was obtained from four different air-dried samples for each type of membrane

	Chromatophores		Reaction centers	
	Amide I	Amide II	Amide I	Amide II
D	1.22	0.85	1.32	0.79
$\phi_{\alpha} (^{\circ})$	37	38	31	29

with the values of the angles ϕ_{α} which are the average tilt angle for the collection of α -helices in chromatophores and reconstituted reaction centers. The ϕ_{α} values were calculated by taking a limiting case in which the membranes were assumed to be perfectly oriented ($S_{ms} = 1$), by using a refractive index in the infrared of 1.5 [11] and taking an α -helical content of 60% for the chromatophores and 50% for the reaction centers. The dichroic ratios of the amide I and II lead to almost identical ϕ_{α} values for the reaction centers (31 and 29°) and the chromatophore membranes (37 and 38°).

Discussion

For the first time and for both native chromatophore membranes and reaction centers reconstituted in vesicles, it has been possible to observe on the same air-dried sample the LD of each of the different components: aromatic amino acid residues, BChl and BPh molecules, lipids and ordered peptide segments in the protein backbone.

The subsequent calculation of the α -helix orientation relies on a series of approximations. These include the extent of membrane order, the membrane refractive index in the infrared ($n = 1.5$) and the assumption that the extinction coefficients of the amide bands for α -helix and random coil conformations are similar. In addition, precision in this calculation is also dependent upon the accuracy of the estimations of the extent of the α -helix content (α), and the angles, ϕ_M , between the infrared transition moment and the helix axis. In principle, the mosaic spread of oriented membranes can be estimated by EPR [4] and diffraction [7,8] techniques. As described in the previous section, the LD/A values measured in this study

indicate that our reconstituted reaction centers and chromatophore membranes are well oriented in the plane of the disc. Accordingly, we have assumed $S_{ms} = 1$ in the subsequent calculations of ϕ_α . The effect of varying these different parameters (S_{ms} , n , α) on the ϕ_α value has already been described for chromatophores [11]. A decrease in S_{ms} leads to a decrease in ϕ_α , i.e., a better orientation of α -helices with respect to the normal to the membrane. Furthermore, an upper limit to the angular inclination of the α -helices was only obtained under extreme conditions, when $S_{ms} = 1$ and $n = 1.33$. The effect of varying the α -helix percentage within its limits is shown in Table III for both chromatophores ($\alpha = 60 \pm 10\%$) and reaction centers ($\alpha = 50 \pm 10\%$): a decrease in α leads to a decrease in ϕ_α . From Table III it can be concluded that on average the α -helix axes are tilted at less than 40° in chromatophores and 35° in reaction centers. These results agree with our previous estimations on native chromatophores [11]. However, from the present data, we cannot conclude that the α -helices are better oriented in the reaction center than in the native chromatophore. The slight difference in the limiting values calculated for the two sets of membranes more probably reflects different contributions from the various causes of uncertainty described above.

The polarized infrared difference spectra of chromatophores and reaction centers (Figs. 2 and 3) reveal not only the dichroism of the protein bands as detected previously on polarized spectra of chromatophores [11], but, furthermore, show

some orientation of the phospholipid groups. The dichroism spectra qualitatively indicate that the polar lipid groups tend to lie preferentially in the membrane plane while the acyl chains tend to be perpendicular to the bilayer plane [12,25,27]. It must be noticed that it is the first time that an orientation of the lipids is detected in a photosynthetic membrane.

We have already mentioned that a shoulder on the amide I band near 1630 cm^{-1} appears dichroic on infrared spectra of both native and reconstituted membranes. However, some caution must be exercised in the interpretation of protein bands in this spectral range, since the pigments (BChl and BPh) contain carbonyl groups which could absorb in this region as has been observed by resonance Raman spectroscopy [29]. Each molecule of pigment contains two nonester carbonyl groups. A ratio of approx. 1 pigment/100 peptide bonds can be estimated for both reaction centers [1] and chromatophores. On the other hand, contribution from β -structure (mainly β -sheet *) is also expected in this infrared region [23,24]. The existence of a small amount of β -structure in the reaction center and chromatophore membrane proteins is also inferred from the analysis of the ultraviolet CD spectra (Table I). Assuming 10% of β -structure, we can calculate a ratio of 1 nonester carbonyl (from pigments) per 5 peptide carbonyls (from β -configuration). Although some orientation of the pigments cannot be excluded, we attribute most of the 1630 cm^{-1} dichroic band to oriented β -sheets. In a β -sheet, the internal hydrogen bonds and the peptide C=O groups are oriented approximately perpendicular to the long axis of the polypeptide chains (an opposite situation from that found in the α -helix). The polarized infrared spectra (Figs. 2 and 3) show these peptide C=O groups to absorb more strongly in the plane of the membrane. This suggests an orientation, on average for the β -configuration, in which the C=O groups of the β -sheets lie preferentially parallel to the plane of the membrane. From the present data, we cannot distinguish between two possible orientations of the β -sheets with the chains' axis either parallel to the membrane or perpendicular to it. In

TABLE III

EFFECT OF VARYING THE α -HELIX CONTENT ON ϕ_α FOR CHROMATOPHORES ($\alpha = 60 \pm 10\%$) AND REACTION CENTERS RECONSTITUTED IN VESICLES ($\alpha = 50 \pm 10\%$)

α -Helix content (%)	ϕ_α ($^\circ$)			
	Chromatophores		Reaction centers	
	Amide I	Amide II	Amide I	Amide II
70	40	40	—	—
60	37	38	35	35
50	35	34	31	29
40	—	—	26	24

* Characteristic amide I bands of β -turns are rather expected at higher frequencies, $1640\text{--}1690\text{ cm}^{-1}$ [31].

both cases peptide C=O stretching vibrations will be lying parallel to the membrane plane. However, an orientation of the chains parallel to the membrane with the peptide C=O groups lying perpendicular to this plane can be excluded.

From these data, a rough model for the protein reaction center can be proposed: the protein structure is largely constructed of a core of transmembrane α -helical segments tilted on average at less than 35° with respect to the membrane normal; this does not exclude the possibility of a few helices lying parallel to the membrane plane while a large number are at a small angle to the normal. This model should also include some oriented β -structure (for example, situated at the ends of the α -helix columns). The infrared spectra of chromatophores and reaction centers are very similar. Since the antenna-to-reaction center ratio in chromatophores is greater than 30 [30], it follows that amide absorption bands in infrared chromatophores are mainly due to proteins from antenna. Therefore, our data imply that some well oriented α - and β -structures must also be present in antenna proteins. The reaction center protein thus appears to have a similar composition and orientation as the antenna proteins in chromatophore membranes with α -helix segments aligned preferentially normal to the membrane plane. This should be verified by using antenna complexes reconstituted in lipid vesicles. Finally, the reaction center itself can be partitioned by removal of the H subunit which permits a strategy for further identification of the conformation and orientation of the substructures of the reaction center proteins.

Acknowledgements

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