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# CONFORMATION AND ORIENTATION OF CHLOROPHYLL-PROTEINS IN PHOTOSYSTEM I BY CIRCULAR DICHROISM AND POLARIZED INFRARED SPECTROSCOPIES

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**The protein conformation and orientation of Photosystem I (PS I) particles have been investigated by a combination of ultraviolet circular dichroism and polarized infrared spectroscopies. These PS I particles have been studied before and after reconstitution in phosphatidylcholine vesicles. The native state of the pigments of PS I was characterized by monitoring the low-temperature fluorescence emission spectra as well as the visible CD and linear dichroism spectra at room temperature. Computed analysis of the ultraviolet CD spectra of PS I complex indicates that the secondary structure of the protein is largely α-helical (52 ± 4%) with a very low amount of β-structure. Polarized infrared difference spectra of oriented PS I show a significant orientation of these α-helical segments with the α-helix axes tilted on the average at approx. 35° from the membrane normal.**

## Introduction

By using polarized infrared spectroscopy on oriented photosynthetic membranes it has been recently demonstrated that the α-helical segments of the intrinsic proteins are on the average oriented preferentially along the normal to the membrane plane [1]. These membranes contain several functionally differentiated chlorophyll-protein complexes which can be isolated in a native state. Most of these isolated complexes have now been reincorporated into lipid vesicles and studied separately in a membrane-like environment [2–6]. However, prior to the present investigation, the orientation of the α-helical segments in such reconstituted systems had been studied only in the bacterial reaction center [7] and in the light-harvesting complex (LHC) extracted from chloroplast thylakoids [8]. This LHC, which constitutes the main antenna system of green plants, comprises about half of the total chlorophyll *a*, the largest fraction of chlorophyll *b*, and about half of the protein in thylakoid membranes [9]. Most of the remaining chlorophylls and proteins are associated with Photosystem I (PS I), while Photosystem II (PS II) accounts for only about 10% of the total chlorophylls and proteins of the thylakoid [10–12]. PS I particles can be reliably prepared and have been well characterized [13,14]. Furthermore, it has been recently shown by using polarized light spectroscopy that the native orientation of the

Abbreviations: PS, Photosystem; LHC, light-harvesting complex; DMPC, dimyristoylphosphatidylcholine; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Chl, chlorophyll.

various pigments has been preserved in the isolated particles [15–17]. These observations have prompted us to investigate the protein conformation and orientation of PS I particles by a combination of ultraviolet circular dichroism and polarized infrared spectroscopy. We examined the particles both before and after the reconstitution into lipid vesicles. The native state of the pigments in both samples was characterized by monitoring the room temperature linear dichroism (LD) and CD spectra in the visible spectral range as well as the 77 K fluorescence emission spectra. In agreement with previous work done on the other intrinsic chlorophyll protein complexes we have observed a significant amount of $\alpha$-helices ($52 \pm 4\%$) and a preferential transmembrane orientation of the $\alpha$-helical segments tilted on the average at approx. 35° from the normal to the membrane plane.

## Materials and Methods

*Isolation of PS I.* PS I was isolated from greenhouse-grown dwarf peas by the method of Mullet et al. [13] with several modifications. The homogenization buffer comprised of 400 mM sorbitol/50 mM Tricine-NaOH (pH 7.8)/10 mM NaCl/5 mM $MgCl_2$. Chloroplasts were pelleted by centrifugation at $1000 \times g$ for 10 min and washed twice in a solution comprising 100 mM sorbitol/0.75 mM EDTA-NaOH (pH 7.8). They were resuspended in deionized water at 0.8 mg Chl/ml. The membranes were solubilized for 30 min at room temperature in 0.78–0.85% Triton X-100 (v/v). Unsolubilized fractions were removed by centrifugation at $53\,000 \times g_{max}$ for 35 min at 4°C. The supernatant was then loaded on a sucrose density gradient and centrifuged as in Ref. 13. PS I was collected from the gradient and used immediately, or diluted with 10 mM Tricine-NaOH (pH 7.8), and centrifuged at $110\,000 \times g_{max}$ for 1 h at 4°C. The pellet of aggregated PS I was resuspended in 10 mM Tricine-NaOH (pH 7.8) by repeatedly forcing the solution through a small bore syringe needle. Chlorophyll concentrations and $a/b$ ratios were determined by the method of Arnon [18].

*Reconstitution of PS I into lipid vesicles.* PS I was reconstituted by a modification of the procedure of Ryrie et al. [4,6]. The PS I was diluted to 1 mg Chl/ml in 10 mM Tricine-NaOH (pH 7.8) and stirred with Biobeads SM-2 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) for 2 h at 4°C (120 mg $H_2O$-washed Biobeads per mg chlorophyll). This treatment often increased the aggregation of the protein. The aggregation was then reduced by brief sonication (two 20-s bursts) in a probe tip sonicator.

Phospholipid vesicles were prepared by drying a chloroform solution of soybean phosphatidylcholine (Type III-S, Sigma Chemical Co., St. Louis, MO, U.S.A.) and resuspending the lipid in 10 mM Tricine-NaOH (pH 7.8) at 2 mg lipid/ml. The mixture was sonicated for 15 min at 0°C. In some experiments, vesicles were also prepared using purified dimyristoylphosphatidylcholine (DMPC, Sigma Chemical Co.), 2 mg/ml, and sonicating at 35°C for 10 min. Vesicles were added to PS I in the ratio of 2:1 (lipid-to-chlorophyll, w/w). The solution was then frozen rapidly in liquid nitrogen and allowed to thaw slowly at room temperature. This freeze-thaw step was repeated once again and the mixture was sonicated briefly (twice for 30 s). Freeze-thawed PS I controls were prepared using buffer in place of lipid.

Reconstituted PS I and control samples were layered on linear, 3–30% ficoll density gradients (50 mM sucrose/5 mM Tricine-NaOH (pH 7.8)/0.5 mM sodium-EDTA) and centrifuged at $150\,000 \times g_{max}$ for 16 h.

*Fluorescence spectroscopy.* Chlorophyll fluorescence spectra were measured on a SLM 4000 Spectrofluorimeter (SLM Instruments, Urbana, IL, U.S.A.). Samples were diluted to 10 $\mu$g Chl/ml in 66% glycerol, frozen in liquid nitrogen and the spectra measured immediately at 77 K. Excitation was at 440 nm and emission was measured between 650 and 800 nm. The spectra were not corrected for photomultiplier tube sensitivity.

*Circular dichroism and linear dichroism spectroscopies.* Ultraviolet CD as well as visible CD and LD spectra were measured at room temperature on a Jobin Yvon Mark V dichrograph linked to a Micral 8031B computer [8]. Ultraviolet CD spectra were measured from 190 to 260 nm in 0.1 mm pathlength quartz cuvettes (at a chlorophyll concentration of 0.30 mg/ml). The ultraviolet CD spectrum for each sample was averaged over 40

measurements. Analysis of the spectra and estimation of the $\alpha$-helix, $\beta$-sheet and aperiodic segments were performed as described previously [1,7,8]. For visible CD spectra, the sample was diluted in 10 mM Tricine-NaOH (pH 7.8) until its absorption in a 1 mm pathlength cuvette was 0.6–0.8 at 680 nm. Visible LD spectra were measured on samples which were air-dried on glass disks.

*Polarized infrared spectroscopy.* Infrared spectra were measured on a Perkin-Elmer 180 double-beam spectrometer equipped with a common-beam Perkin-Elmer wire-grid polarizer. Spectra were digitized and analyzed on a Hewlett-Packard 9825 A computer as described previously [7,8]. Samples were spread on the surface of $CaF_2$ disks and allowed to dry at room temperature in the dark. The dichroism was measured at an incident angle of 60° to the disk surface with a blank disk mounted at the same angle in the reference beam. To remove the major source of reflection at the membrane/air interface, the air-dried sample was covered with a spectroscopic grade paraffin oil (nujol) [8,19]. The quantitative analysis and calculation of $\alpha$-helix tilt angles (with respect to the membrane normal) from the infrared dichroism and the ultraviolet CD spectra have been described in previous papers [1,7,8].

## Results and Discussion

*Characterization of PS I before and after reconstitution in lipid vesicles*

The chlorophyll *a/b* ratio of isolated PS I was 5.0–6.0. SDS-polyacrylamide gel electrophoresis (data not shown) using the buffer system of Laemmli [20] showed good purification of PS I with minimal contamination by the major polypeptide of the light-harvesting complex (i.e., less than 5% stainable protein).

The incorporation of PS I in phospholipid vesicles was monitored using ficoll density gradient centrifugation. PS I taken directly from the sucrose gradient, PS I aggregated by the buffer wash, or PS I mixed with liposomes without a freeze-thaw treatment, sedimented through the 3–30% ficoll gradient. PS I reconstituted in lipid vesicles formed a more or less diffuse band at the low densities in the top region of the gradient. Reconstitution was successful with either DMPC or soybean phosphatidylcholine vesicles. No attempt was made to directly control the state of incorporation of PS I in vesicles.

Protein-to-lipid ratios of about 1 : 1 (w/w) are optimal for observation of the amide bands in infrared dichroism [1,7,8,19]. This ratio is significantly greater that the 1 : 4 protein-to-lipid ratio used by Ryrie et al. [4,6] to reconstitute LHC or PS I into lipid vesicles. Due to the small amount of lipid we used, it is possible that our PS I particles were not reconstituted in sealed vesicles but rather in planar sheets. The formation of intact vesicles, however, is not an important factor in obtaining good orientation of the specimen, as demonstrated by the infrared dichroism data on purple membrane and on intact photosynthetic membranes [1].

*Fluorescence emission spectra*

The 77 K fluorescence emission spectrum of isolated PS I (Fig. 1A) peaks at 733–735 nm, as

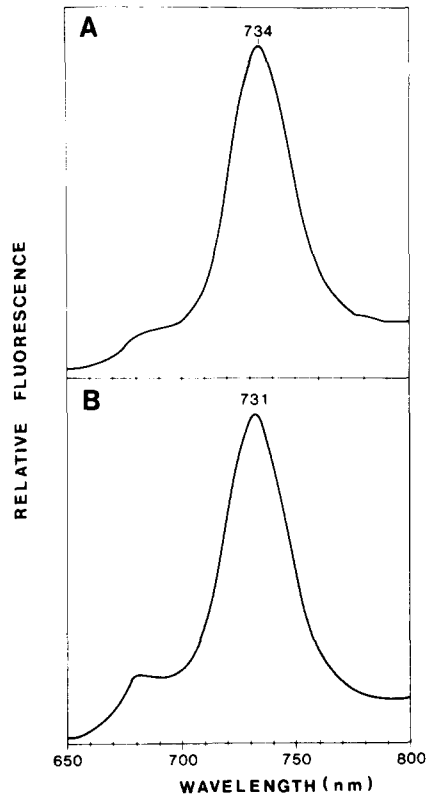


Fig. 1. 77 K fluorescence emission spectra of PS I before (A) and after (B) reconstitution in soybean phosphatidylcholine vesicles.

expected for a native PS I preparation [13]. Application of the reconstitution procedure to PS I particles in the absence of lipid did not change the fluorescence emission spectrum (data not shown). However, reconstitution of PS I with liposomes caused a small variable blue shift (3–5 nm) in the main fluorescence peak (Fig. 1B) as well as a slight increase in a fluorescence near 685 nm. A more complete fluorescence analysis of PS I in lipid vesicles will be described and discussed elsewhere.

*CD and LD spectra in the visible*

Fig. 2 shows visible CD spectra from 400 to 750 nm of PS I at several stages in our preparation. These spectra closely resemble those previously published for PS I [21], although we have observed that the magnitude of the shoulder at 692 ± 2 nm is sensitive to the aggregation state of the complex. More specifically, after centrifugation of PS I and resuspension in Tricine buffer, the 692 nm shoulder is much enhanced at this state (Fig. 2B). Biobeads and freeze-thaw control treatment of the PS I in the absence of lipid do not decrease this signal

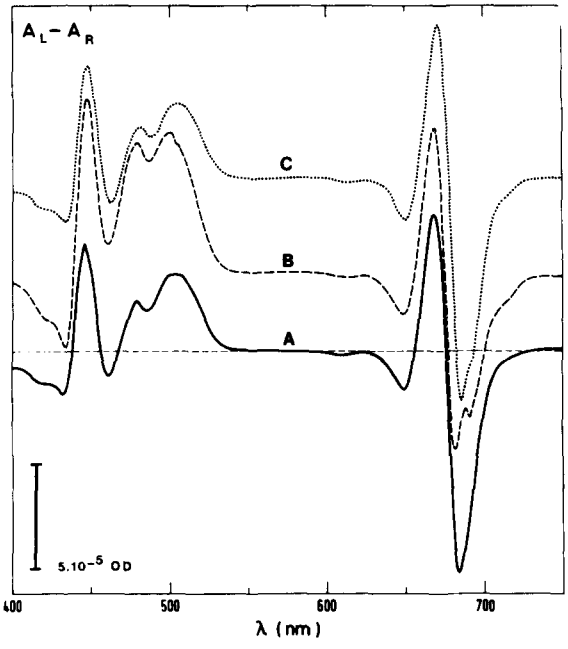


Fig. 2. Visible CD spectra of PS I: taken directly from the sucrose gradient (A), after recovery by centrifugation (B) and after reconstitution in soybean phosphatidylcholine vesicles (C).

(data not shown). However, reconstitution of the aggregated PS I significantly reduces the magnitude of the 692 nm shoulder (Fig. 2C). These results can be correlated with our ability to analyze the ultraviolet CD spectra in curve-fitting calculations (see below). Haworth et al. [21] reported a correlation between a similar shoulder (located at 686 nm in their spectra) and $LHC_I$, the light-harvesting antenna polypeptides of PS I. Upon further solubilization of PS I, the 686 nm optical component copurified with the $LHC_I$ fraction. In contrast, our observations seem to link the magnitude of this signal with the aggregation state of the PS I particles.

Visible LD spectra (data not shown) of PS I reconstituted into lipid vesicles closely resemble those previously described [16]. This indicates that no significant change in the orientation of the pigments during the isolation, reconstitution and air-drying procedures has occurred.

*Structural analysis*

*Estimation of PS I secondary structure from ultraviolet CD spectra.* Conformational analysis of PS I was investigated by using ultraviolet CD spectroscopy. The extent of each type of secondary structure present in the protein, i.e., α-helix, β-sheet and aperiodic structures, was evaluated by analyzing experimental CD spectra as a linear combination of reference spectra using a general least-squares program [7,8]. The reference CD spectra were obtained from soluble proteins of known structure [22]. The ultraviolet CD spectra of PS I taken immediately after the sucrose gradient step (Fig. 3A) are very similar to the spectra of PS I after reconstitution into lipid vesicles (Fig. 3C). Their analysis by curve-fitting procedures is summarized in Table I. Based on these analyses, we conclude that the secondary structure of the PS I complex is largely α-helical (52 ± 4%) with a very low amount of β-sheet.

An ultraviolet CD spectrum of resuspended, aggregated PS I is shown in Fig. 3B. This spectrum was more difficult to fit, which is reflected by the higher $\bar{\sigma}$ value in Table I. The difficulty of fit is most probably due to distortions induced by light-scattering effects [23] of the aggregated particles. Thus, the light-scattering effects in the

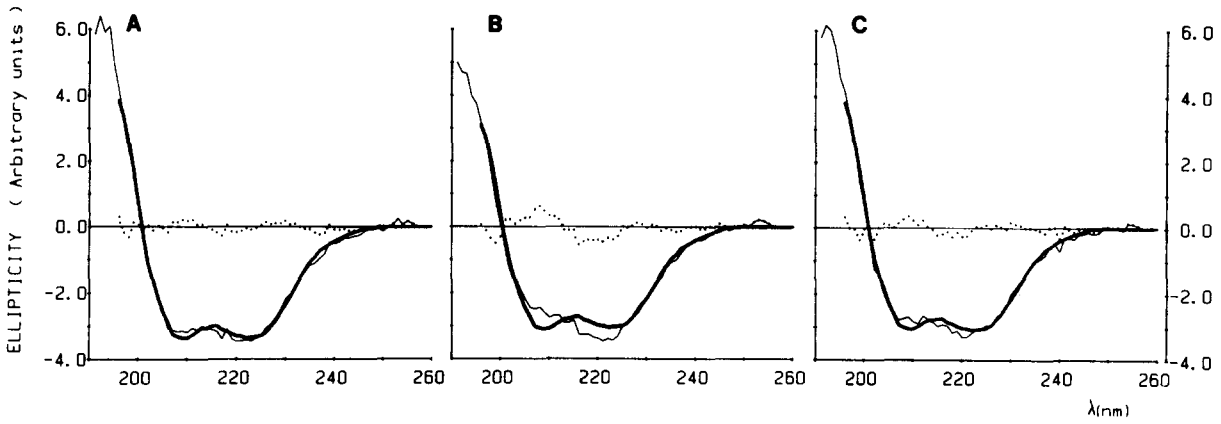


Fig. 3. Ultraviolet CD spectra of PS I: taken directly from the sucrose gradient (A), after recovery by centrifugation (B), and after reconstitution in soybean phosphatidylcholine vesicles (C). Experimental (———), calculated (▬) spectra and difference (· · · · · ·) between experimental and calculated spectra.

ultraviolet CD are strongest in the same sample that has the most pronounced shoulder at 692 nm in the visible CD (Fig. 2B). This strengthens our correlation between aggregation and the size of the 692 nm signal. In comparison, the other two samples (Fig. 3A and C) show no major distortion in the ultraviolet CD, suggesting that the optical effects are minimized in the presence of dispersed particles or vesicles as has been recently reported in other systems [24,25].

*Orientation of α-helices.* Absorption infrared spectra of isolated and reconstituted PS I are shown in Fig. 4. The frequencies of the amide bands (amide A at 3300 $cm^{-1}$, amide I at 1656 $cm^{-1}$ and amide II at 1547 $cm^{-1}$) are identical in both samples. This indicates the presence of α-helices and aperiodic structures [26] in the protein, in agreement with the ultraviolet CD data. The infrared spectrum of PS I before reconstitution into lipid vesicles (Fig. 4A) also shows some absorption at 1736 $cm^{-1}$ due to C=O ester groups which arise from chlorophyll and/or lipid molecules. Furthermore, the absorption at 1240 $cm^{-1}$ can be attributed to lipid phosphate groups. Therefore, it appears that some intrinsic lipids are still present even after isolation of the protein in detergent, as also noted for LHC (Nabedryk, E and Breton, J., unpublished data).

TABLE I

ESTIMATION OF THE PERCENTAGE OF α-HELIX (α), β-SHEET (β) AND APERIODIC (γ) STRUCTURE FROM ULTRAVIOLET CD SPECTRA

$\bar{\sigma}$ is the root mean square deviation between calculated and experimental spectra data.

| PS I | α | β | γ | $\bar{\sigma}$ |
|---|---|---|---|---|
| Isolated | 51 | 2 | 47 | 0.06 |
| Reconstituted | 53 | 3 | 44 | 0.07 |
| Aggregated | 47 | 3 | 50 | 0.10 |

Polarized infrared difference spectra of air-dried oriented reconstituted PS I (Fig. 5) show typical dichroism for the three main amide bands indicative of oriented peptide groups [7,8,19]. Qualitatively, a positive dichroism is associated with the alignment of the transition at less than 55° from the membrane normal [1]. The infrared dichroism spectrum of reconstituted PS I shows significant orientation of α-helix segments. Likewise, PS I without reconstitution shows similar orientation (data not shown). Apparently, the air-drying process allows the isolated protein to form planar aggregates effectively orienting itself even in the absence of a lipid membrane. This produces orientation of the unreconstituted sample similar to that found in the reconstituted one. It should be noted that in Fig. 5 the C=O ester signal at 1736 $cm^{-1}$ shows a negative dichroism indicating an orientation rather parallel to the bilayer plane, as in pure liposomes [27].

Quantitative determination of the α-helix orien-

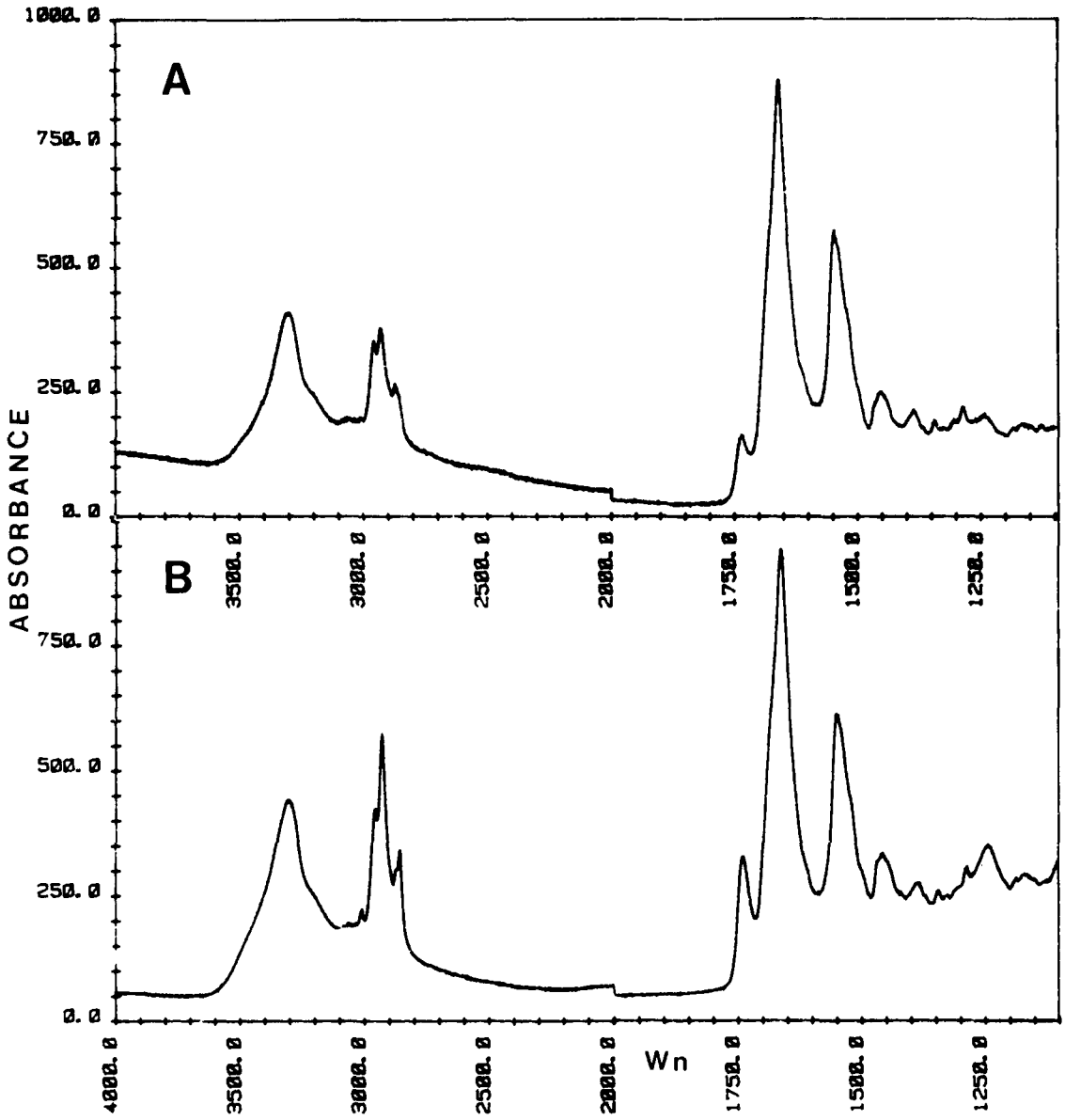


Fig. 4. Infrared absorption spectra of PS I before (A) and after reconstitution (B).

tation is given in Table II. Including the extremes of the range of possible $\alpha$-helix contents, and assuming perfect ordering of the air-dried samples, the upper limit for the $\alpha$-helix average tilt angle for PS I is placed at approx. 35° from the membrane normal.

We have previously reported similar measurements of protein conformation and $\alpha$-helix tilt angles for thylakoid membranes and reconstituted LHC [8]. The measurement of $52 \pm 4\%$ $\alpha$-helix in PS I reported in this study is comparable to the thylakoid ($56 \pm 4\%$) and the LHC ($44 \pm 7\%$) values, and leads to the general conclusion that $\alpha$-helices are roughly homogeneously distributed throughout the intrinsic proteins of the chloroplast thylakoid membrane. Furthermore, the tilt angle

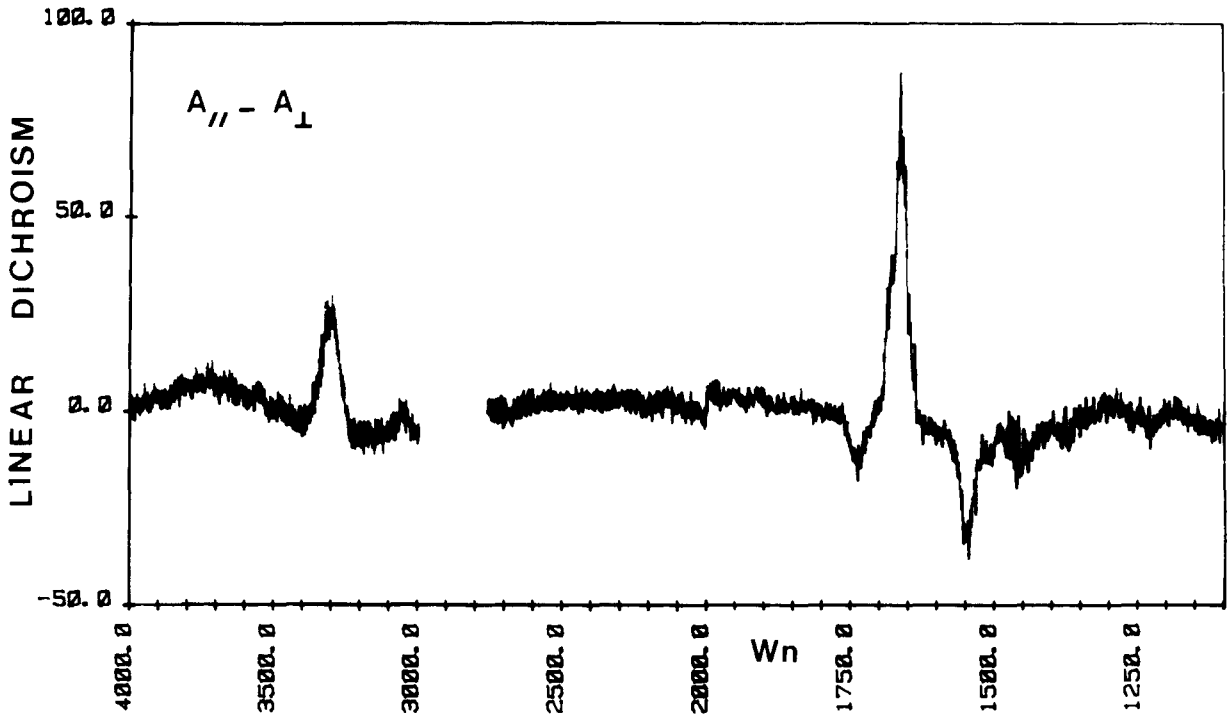


Fig. 5. Polarized infrared difference spectra ($A_{\parallel} - A_{\perp}$) of PS I reconstituted into lipid vesicles. The air-dried sample was covered with nujol.

calculated for the α-helices of PS I at about 35° from the membrane normal is similar to that reported for bacterial reaction center [2,19], LHC [8], chromatophores and thylakoid membranes [1]. Thus, the present data on PS I particles further illustrate the observation that the intrinsic pigment-protein complexes constituting the photosynthetic membrane possess a high content of α-helical segments exhibiting a preferential transmembrane orientation.

TABLE II

ORIENTATION OF α-HELICES IN PS I

Average obtained from seven different air-dried samples (four from isolated PS I, three from reconstituted PS I). $D$ is the experimental dichroic ratio $D = A_{\parallel}/A_{\perp}$. $D_{\alpha}$ is the corrected dichroic ratio for the α-helices (52%). $\phi_{\alpha}$ is the tilt angle of the α-helix axes with respect to the membrane normal.

| | Amide I | Amide II |
|---|---|---|
| $D$ | 1.15 | 0.90 |
| $D_{\alpha}$ | 1.30 | 0.82 |
| $\phi_{\alpha}$ | 32° | 33° |

**Note added in proof** (Received November 13th, 1984)

Fish, L.E., Kück, U. and Bogorad, L. (personal communication; J. Biol. Chem., in the press) have recently determined the amino acid sequence of the PS I core protein. From the hydropathy index plot, they suggest that out of the 750 amino acids, eleven α-helices cross the membrane. Our results, taking into account the contribution of $LHC_I$ in our PS I particles and the possible non-transmembrane α-helical segments in these proteins, could yield a more quantitative structural analysis.

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