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Fluorescence polarization spectra of granal and stromal membranes treated with linolenic acid. Orientation of the Photosystem I core complex within the membrane

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Polarized fluorescence spectra and fluorescence polarization ratios were compared in aligned isolated intact thylakoids and in granal and stromal membranes, without and after linolenic acid treatment at liquid N_2 temperature in squeezed polyacrylamide gel. Separation of granal membranes from stromal membranes allowed an improved alignment of the membranes as compared to isolated intact thylakoids. As a result, a higher anisotropy of fluorescence was measured with fragments than with chloroplasts. Incorporation of linolenic acid into the membranes affected the energy migration between the complexes, and induced changes in the orientation of the complexes within the membranes, as shown by a reduced fluorescence intensity and decreasing values (but still larger than 1) of fluorescence polarization ratios at longer wavelengths. In order to interpret these changes in the fluorescence polarization ratios, model calculations were carried out, the following parameters being taken into account: the direction of the absorption and emission dipoles in the complex, the orientation of the complex in the membrane, and the fluctuation of the orientation. Calculated values of the fluorescence polarization ratio changed in a similar manner as those observed experimentally. The character of the changes of the fluorescence polarization ratio suggests a picture of the orientation of the complexes within the membranes.

Introduction

The low-temperature emission spectra of green-plant chloroplasts exhibit fluorescence bands which originate from a number of chlorophyll-protein complexes [1] localized in the granal and stromal membranes [2,3]. Fluorescence polarization measurements show that the emitting species corresponding to the individual bands are oriented at various angles to the membrane plane [4–6]. The pigment-protein complexes are stabilized within the membranes by the lipid environment, which, similarly to all major membrane components, exhibits considerable lateral heterogeneity in distribution [7]. In the granal membranes the lipid-to-protein ratio has been shown to be lower than in the stromal membranes, and this allows a different freedom of movement for the pigment-protein complexes [8]. A specific arrangement of the components in the photosynthetic membranes is a prerequisite of normal functioning and can be regulated to a large extent by the lipid contents of the membranes [9,10].

We have demonstrated previously that the lipid enrichment of *Scenedesmus* thylakoids influences

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not only the energy migration, as indicated by the fluorescence spectra, but also the orientation pattern of the pigment-protein complexes [11,12].

Lipid enrichment by the addition of linolenic acid induced characteristic changes in the anisotropy of light absorption and in the polarization of fluorescence. These data provided information on the orientation of the chlorophyll-protein complexes within the membranes.

In measurements on isolated intact thylakoids, there is an overlap between the spectral characteristics of structurally and functionally different types of membranes. In order to separate the spectral and anisotropy properties of granal and stromal membranes, isolated membrane fragments have been studied with and without linolenic acid enrichment. Differences in anisotropy properties brought about by the isolation of different types of membranes, and changes induced by lipid enrichment, are interpreted through model calculations.

Materials and Methods

Seedlings of maize (Zea mays, convar MTC-255) were grown in the greenhouse for 8–12 days. Chloroplasts were isolated from the mesophyll of the first leaves in sucrose-phosphate buffer, pH 7.2 [13]. Granal and stromal membranes were obtained by differential centrifugation of homogenates prepared by breaking the chloroplasts in a French pressure cell according to Ref. [14]. Chlorophyll was determined spectrophotometrically in ethyl ether solution with the multiwavelength method [15]. The ratio chlorophyll-*a*/chlorophyll-*b* was 2.9 in isolated intact thylakoids, 2.1 in granum preparations, and 7.0 in stromal thylakoids. Chlorophyll/P-700 ratios, determined by light-induced absorbance changes [11] were: 450 for intact thylakoids, 650 for granal and 150 for stromal membranes, respectively.

Suspensions of intact thylakoids and thylakoid fragments adjusted to 10^{-3} M chlorophyll content were supplemented with $2 \cdot 10^{-2}$–$5 \cdot 10^{-2}$ M linolenic acid (Sigma Chemical Co.) and incubated for 30 min during continuous stirring under an N_2 atmosphere at room temperature in the dark. Aliquots were taken every 5 min and embedded in polyacrylamide gel according to Abdourakhmanov et al. [16]. The optical density of the samples was 0.15. Thylakoids were aligned by the previously described squeezing procedure [17], with a squeezing parameter of 1.9. Polarized fluorescence spectra were recorded at 77 K. Samples at temperatures higher than 77 K were carefully protected from light. The geometry of the measurements is outlined in Fig. 5A. Fluorescence was excited with the blue spectral band of an HBO 200 high-pressure mercury arc lamp. The excitation light was non-polarized and its direction was parallel to the direction of squeezing. The fluorescence was observed in a direction perpendicular to the direction of squeezing and to that of the excitation, and was recorded with polaroid sheets transmitting fluorescence intensity components emitted parallel ($F_{\parallel}$) and perpendicular ($F_{\perp}$) to the plane of the aligned membranes. The fluorescence polarization ratio was defined as $FP = F_{\parallel}/F_{\perp}$. Non-squeezed samples were used as a control to determine the conditions for $FP = 1$. No particular corrections were applied, and spectra were recorded on arbitrary scales.

Results

Experimental data

A common feature of all polarized fluorescence spectra recorded with isolated intact thylakoids or isolated granal and stromal membranes was that $F_{\parallel}$ was greater than $F_{\perp}$. Thus, the fluorescence polarization ratio $FP = F_{\parallel}/F_{\perp}$ was always higher than unity.

Differences between the spectra reflected the lateral heterogeneity of the membrane architecture due to the differential distribution of the photosystems (Figs. 1–3, 0 min). The spectra of the isolated intact thylakoids and of the granal membranes largely exhibited the same bands of similar types of orientation. Most of the emitting dipoles showed a tendency to lie close to the membrane plane. An exception was the dipoles emitting at 695 nm, which are known to be oriented at a wider angle than the others. The polarized fluorescence spectra of the stromal membranes demonstrated the enrichment of the Photosystem I pigment-protein complex emitting mainly at long wavelengths and with a very high degree of anisotropy. Both types of isolated fragments were characterized by a relative enhancement of the fluorescence intensity

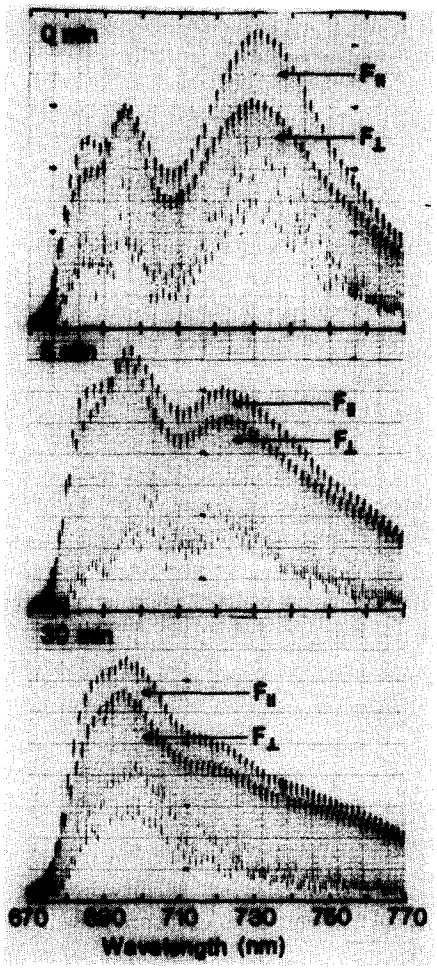

Fig. 1. Polarized fluorescence spectra of isolated intact thylakoids of maize, and the effects of incubation with linolenic acid. Recorded at 77 K. For other details, see Materials and Methods.

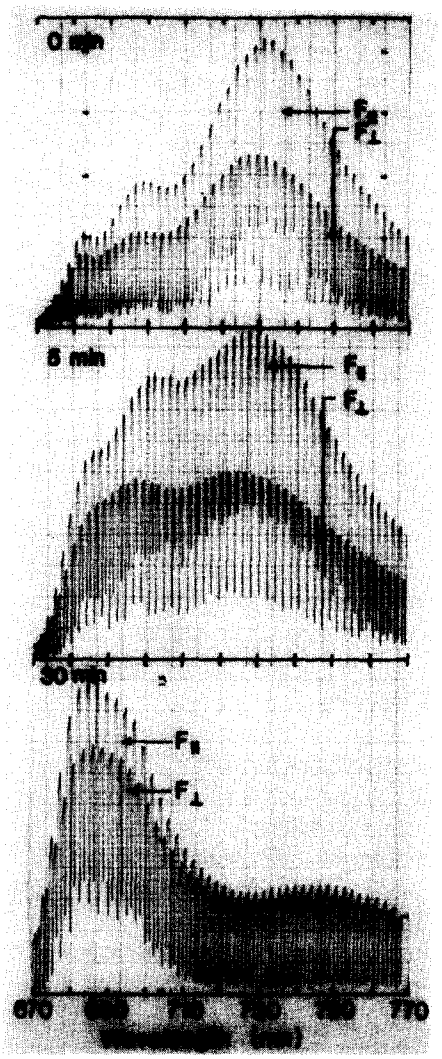

Fig. 2. Polarized fluorescence spectra of isolated grana, and the effects of incubation with linolenic acid. Recorded at 77 K. For other details, see Materials and Methods.

emitted parallel to the membrane plane. This was also shown by spectra of the fluorescence polarization ratios, which demonstrated a much higher anisotropy for either of the fragments than for intact thylakoids (Fig. 4, 0 min). This phenomenon was especially pronounced in the long-wavelength region.

Treatment with linolenic acid resulted in a short wavelength shift of the absorption maxima and changes in energy transfer, as shown by an intensified emission at the shorter wavelengths at the expense of the long-wavelength fluorescence (Figs. 1–3, 5–30 min).

At the beginning of the measurements, the extent of polarization was not much affected (Fig. 4, 5 min). Later, however, the fluorescence polarization ratio increased at shorter wavelengths, while at 730–760 nm was strongly diminished.

Model calculations

Polarized spectroscopy yields information on the orientations of absorbing and emitting dipoles with respect to the plane of the thylakoid membrane. As pointed out previously [12], the dipole orientation angle recorded for a particular membrane depends not only on the arrangement of

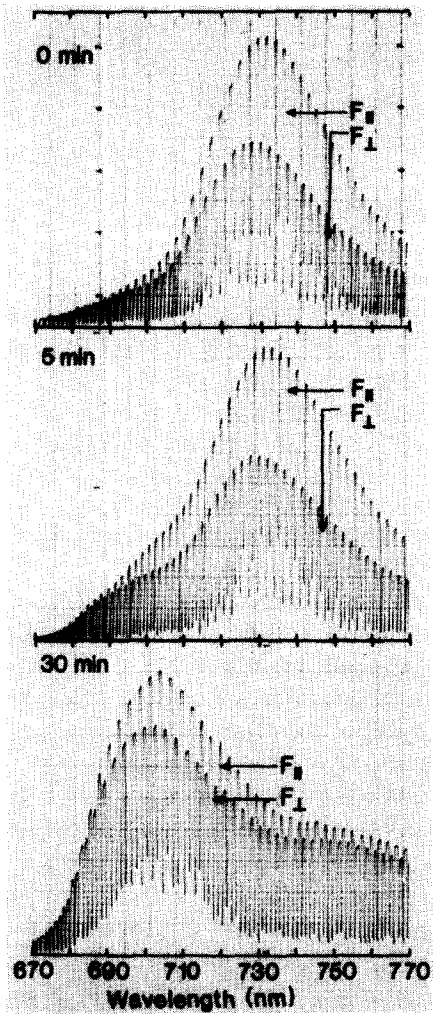

Fig. 3. Polarized fluorescence spectra of isolated stromal thylakoids, and the effects of incubation with linolenic acid. Recorded at 77 K. For other details, see Materials and Methods.

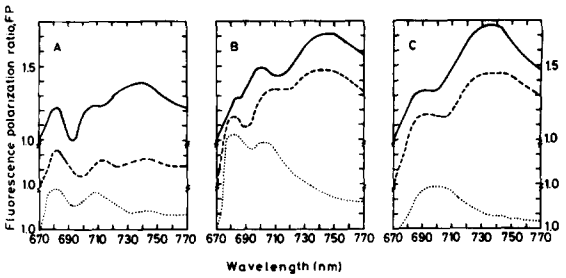

Fig. 4. Wavelength-dependence of the fluorescence polarization ratio (FP) calculated from the low-temperature polarized fluorescence spectra of isolated intact thylakoids (A), isolated granal (B) and stromal thylakoids (C), and the effects of linolenic acid on the FP spectra. After incubation for 0 min (———), 5 min (— — —) and 30 min (·····), respectively.

pigments within the pigment-protein complexes, but also on the orientation of the complexes within the membrane. We demonstrated that linolenic acid incorporated in the membrane induces an increased fluctuation of the complex orientation, i.e., it leads to a broadening of the angular interval which can be occupied by a particular pigment-protein complex. This effect was manifested in linear dichroism measurements by the change of the normally positive values to negative ones at shorter wavelengths, and by the decrease of linear dichroism at longer wavelengths. Linear dichroism and fluorescence polarization ratios have been treated routinely as analogous indications of the orientation of pigments [18] by assuming that a more intense absorption parallel to the membrane plane corresponds to a more intense fluorescence emitted in the same direction. This, however, is valid only in the case of individual molecules, and cannot be correct with membranes where in the absorption event all pigment molecules participate, but in the emission only the lowest excited states of interacting molecules appear. This can be the reason why our results do not show a close correspondence between linear dichroism and fluorescence polarization ratio, as samples with negative linear dichroism exhibited high fluorescence polarization ratios for the same pigment species.

In order to understand the behaviour of linear dichroism and fluorescence polarization ratios in linolenic acid treated thylakoids, model calculations were carried out using the following assumptions.

(1) The membranes are regarded as ideal planes that are perfectly aligned in the squeezed gel sample, i.e., they are parallel to one another and perpendicular to the direction of squeezing.

(2) We visualize the pigment-protein complexes as embedded into the membranes with their shortest axis ($\overline{Z}'$) oriented at an angle β to the normal ($\overline{Z}$) to the membrane plane (Fig. 5A). The shortest axis ($\overline{Z}'$) is defined by the orientation pattern of isolated pigment-protein complexes in squeezed gels as in Ref. 19.

(3) We consider an absorbing transition dipole

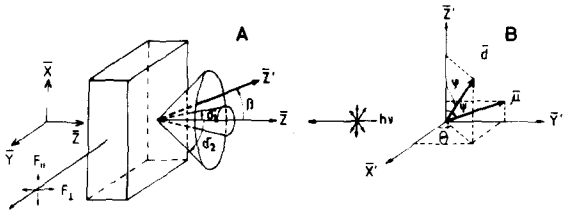

Fig. 5. Geometry of measurements (A) and of model calculations (A, B). Symbols for (A): $\bar{Z}$, normal to the plane of the aligned membranes, parallel to the direction of squeezing and excitation ($h\nu$); $F_{\parallel}$ and $F_{\perp}$, fluorescence intensity components emitted parallel and perpendicular, respectively, to the membrane plane; $\bar{Z}'$, shortest axis of the pigment-protein complex; β, the angle between $\bar{Z}$ and $\bar{Z}'$, defining the tilt of the pigment-protein complex to the membrane plane; $[\delta_1, \delta_2]$, fluctuation interval of β. Symbols for B: $\bar{X}'$, $\bar{Y}'$, $\bar{Z}'$, coordinates fixed in the pigment-protein complex: ϕ and θ, angles defining the position of the absorption dipole $\bar{d}$ within the complex; ψ, angle defining the position of the emission dipole $\bar{\mu}$ in the $\bar{Z}'\bar{Y}'$ plane with respect to $\bar{Z}'$.

($\bar{d}$), which can transfer the excitation to the emitting transition dipole ($\bar{\mu}$). Both dipoles correspond to chlorophyll-*a* molecules fixed in the same pigment-protein complex. The $\bar{X}'$ and $\bar{Y}'$ axes of the complex-fixed coordinate system are defined, so that the emission dipole ($\bar{\mu}$) lies in the $\bar{Y}'\bar{Z}'$ plane. The angles ϕ, θ and ψ determine the positions of the dipoles of pigments within the complex (Fig. 5B).

(4) The orientation angles ϕ, θ and ψ of different pigments are considered to be invariant upon linolenic acid treatment, whereas the orientation angle (β) of the pigment-protein complex is allowed to fluctuate in the angular interval $[\delta_1, \delta_2]$. (In the event of no fluctuation, $\beta = \delta_1 = \delta_2$.)

For excitation with non-polarized light propagating in the $\bar{Z}$ direction, the fluorescence intensity components polarized parallel and perpendicular to the plane of the aligned membranes are as follows:

$$F_{\parallel} = -\tfrac{1}{2}F_{\perp} + \tfrac{1}{8}C_3(3\cos^2\phi - 1)\langle\sin^2\beta\rangle + \tfrac{1}{4}C_3\sin^2\phi \qquad (1)$$

$$F_{\perp} = C_3\{f_1(\phi,\theta,\psi)\langle\sin^4\beta\rangle + f_2(\phi,\theta,\psi)\langle\sin^2\beta\rangle + \tfrac{1}{2}\sin^2\phi\cos^2\psi\} \qquad (2)$$

Here, $\langle\sin^2\beta\rangle$ and $\langle\sin^4\beta\rangle$ are the corresponding averages over the interval $\delta_1 \le \beta \le \delta_2$; the functions $f_1(\phi,\theta,\psi)$ and $f_2(\phi,\theta,\psi)$ are given in explicit form in Eqns. A-10 and A-11; C_3 is a proportionality constant (see Eqns. A-4, A-5, A-6 and A-9) which disappears in the expression of the fluorescence polarization ratio (FP). According to these formulae, FP is a rather complicated function of the variables ϕ, θ, ψ, δ_1 and δ_2. In order to identify the conditions which lead to a negative linear dichroism (LD) and $FP > 1$, it was reasonable to distinguish between the cases where $FP < 1$ and $FP > 1$ (Fig. 6). For the sake of simplicity, we chose equal values for ϕ and ψ, at 30°, 60° and 90°, and only several values for θ (0°, 30°, 60° and 90°). In this way we demonstrated the behaviour of the calculated FP as a function of the lower limit of the orientation angle (δ_1) of the pigment-protein complex and of the fluctuation interval ($\delta_2 - \delta_1$).

The most important fact revealed by Fig. 6 is that the area contours at $FP > 1$ do not coincide with the line representing $LD = 0$. When Q_Y dipoles of chlorophyll-*a* form a wide angle with the shortest dimension of the complex [19] (here, $\phi,\psi \ge 60°$), negative LD values correspond to $FP > 1.0$: the plots for $\phi = \psi = 60°$ and $\phi = \psi = 90°$ do not show any areas with $FP < 1$ when θ is 60° or 90°.

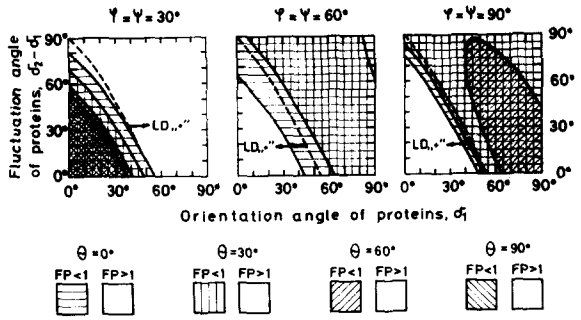

Fig. 6. Contour plots of areas where fluorescence polarization ratios, $FP < 1$ (dashed) and $FP > 1$ (open), are expected for pigment-protein complexes of different orientation angles (δ_1) and fluctuations of orientation ($\delta_2 - \delta_1$). Models for pigment-protein complexes with different types of pigment organization within the complex: $\phi = \psi = 30°$, a case where dipoles are lying close to the shortest axis ($\bar{Z}'$) of the complex; $\phi = \psi = 60°$, a case representing the emission of the light-harvesting complex of Photosystem II; $\phi = \psi = 90°$, a case representing the long-wavelength emission of the Photosystem I core and antenna complex. Dashed line: conditions for $LD = 0$, calculated from Eqn. 1 of Ref. 12.

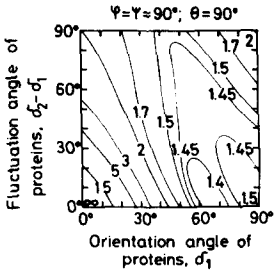

Fig. 7. Lines of constant values of the fluorescence polarization ratio, calculated for the geometry, $\phi,\psi,\theta = 90°$. The fluorescence polarization ratio changes as a function of δ_1 and $(\delta_2 - \delta_1)$.

An example of how fluorescence polarization measurements can be used to obtain information on the orientation of pigment-protein complexes within thylakoid membranes is shown for the Photosystem I core complex, which fluoresces at 735 nm. The isolated stromal membranes are suitable material for such studies, as they are fairly pure and the complex is left in situ.

In the Photosystem I core complex, Q_Y emitting dipoles in situ are known to lie very near to the membrane plane [18]. Thus, its geometry can be approximated by $\phi = \psi = 90°$, with $\beta = \delta_1$ most probably close to 0° [19]. In accordance with Eqns. 1 and 2 lines of constant values of the fluorescence polarization ratio were plotted for $\theta = 90°$ and various δ_1 and $(\delta_2 - \delta_1)$ (Fig. 7). At small δ_1 values, we see a very sharp decrease of the fluorescence polarization ratio when the fluctuation of orientation increases. With larger δ_1 values, the fluorescence polarization ratio is practically insensitive to changes in fluctuation.

From the set of experimental data obtained with linolenic acid, the Photosystem I core is represented most clearly by the isolated stromal membranes. From Fig. 3 and Fig. 4C it is evident that the fluorescence polarization ratio at 735 nm is sharply decreased as a result of linolenic acid treatment, which, by loosening the membrane structure [20], increases the freedom of movement of the protein within the membrane. These experimental data fit with Fig. 7 if it is supposed that the Photosystem I core is built up of a complex in which $\beta = \delta_1$ is indeed close to zero.

Discussion

A prerequisite for application of the fluorescence polarization technique used in our work is a near to perfect alignment of the membranes studied. For whole chloroplasts, this could not be attained, even if the equatorial planes of the chloroplasts were perfectly aligned. The reason is the complex internal organization of the thylakoids inside the chloroplasts. It is considered that only the granal membranes are more or less parallel to the equatorial planes. The stromal membranes, which run as helices around the granum piles, form a fret with an overall tilt of about 22° [21]. Resolution of the junction which fixes this angle, i.e., the separation of the stromal membranes from the granal allows a better alignment for each fragment than for the whole organelle. This explains why values of the fluorescence polarization ratio of thylakoid fragments were invariably found to be higher than those of the intact thylakoids.

The band compositions of the isolated intact thylakoids and granal membranes were similar; this is due partly to the high fluorescence efficiency of Photosystem II which is preferentially compartmented in the grana [1]. The stromal membranes seem to be very poor in pigment protein complexes emitting short-wavelength fluorescence. Relatively high intensities of F_{735} in the polarized fluorescence spectra of granal membranes do not necessarily mean a surplus of Photosystem I, but can be connected with the particular information content of the polarized fluorescence spectra, which is somewhat different from that of the non-polarized ones. This difference between the two kinds of fluorescence spectra is a complex problem, the analysis of which goes beyond our present subject. Nevertheless, we note that non-polarized fluorescence spectra of our preparations were similar to those previously described [30], i.e., intensity ratios of F_{685}/F_{735} were higher in granal fragments than in intact thylakoids.

The long-wavelength fluorescence with high fluorescence polarization ratio is a characteristic of the stromal membranes, but the granum preparations also contain pigments oriented as in Photosystem I. This shows the low but appreciable Photosystem I content of the grana [29], although the possibility of imperfect separation cannot be excluded.

The fragmentation of the chloroplasts induced marked changes in the anisotropy of the F_{695} band. The isolated intact thylakoids exhibited a very

intense $F_\perp$ component, which has been observed as a negative polarization by a number of authors [4,6,22] and has been claimed to originate from the pheophytin 'exciton trap' [23]. When the granal membranes were disconnected from the stromal membranes in our experiments, $F_\parallel$ increased considerably. A possible explanation is the low Photosystem I content of the isolated grana [2,3] in which excess energy from Photosystem II cannot migrate to Photosystem I. Thus, energy becomes accumulated in a pigment bed with Q_Y dipoles oriented in a manner similar to that in the Photosystem I reaction centers, a pigment core containing close-lying transition dipoles oriented parallel to one another and the membrane plane [18]. These excitons decay via fluorescence which originates from dipoles emitting mostly $F_\parallel$; by overlapping, they increase the fluorescence polarization ratio around 695 nm. This explanation implies that F_{695} is of a composite nature, in that it originates from the primary electron acceptor of Photosystem II [24] (main emitter in $F_\perp$) and the light-harvesting complex [25] (main emitter in $F_\parallel$).

Linolenic acid treatment of thylakoids has been used widely to investigate the effect of the lipid environment on the spatial organization and functioning of the photosynthetic membrane components [20,26–28]. We have recently reported that linolenic acid treatment changes the orientation pattern of pigments [11]. This effect does not seem to be attributable to a change in the internal organization of the complexes, since, even after harsher detergent treatment, the linear dichroism spectra of thylakoids could be reconstituted from the spectra of the separate subunits [19].

Through the analysis of linear dichroism data, we have concluded that the incorporation of linolenic acid changes the orientation and/or the fluctuation of orientation of the pigment-protein complexes within the membrane [12].

Data obtained from linear dichroism and polarized fluorescence measurements are mostly treated together [18,25] by assuming that absorbing and emitting dipoles are parallel not only in the individual molecules, but also in the collectives of chlorophyll-*a* molecules present in the membranes.

In fact, however, the fluorescence of the membranes cannot be regarded as being due to individual molecules: interaction between acceptors and donors should be considered, and photoselection effects are also displayed. Even our largely simplified model has shown that the fluorescence polarization ratio is more complex than linear dichroism, thus interpretation is more difficult than hitherto thought. Nevertheless, characteristic changes in the fluorescence polarization ratio as a consequence of linolenic acid treatment indicate that the Photosystem I core pigments emitting at 735 nm are incorporated in an anisotropic protein which has its shortest axis parallel to the normal to the membrane plane. Pigments emitting at shorter wavelengths are probably carried by complexes which have their shortest axis oriented at larger angles with respect to the normal to the membrane plane [12]. Detailed analyses of such cases could not be performed here because of the lack of pure in situ preparations of the light-harvesting complex serving Photosystem II.

Appendix

The components of the transition dipoles $\bar{d}$ and $\bar{\mu}$ in the protein-fixed coordinate system are as follows (Fig. 5B):

$$\bar{d}(d_{X'}, d_{Y'}, d_{Z'}) = (\sin\phi\cos\theta, \sin\phi\sin\theta, \cos\phi) \quad \text{(A-1)}$$

$$\bar{\mu}(\mu_{X'}, \mu_{Y'}, \mu_{Z'}) = (0, \sin\psi, \cos\psi) \quad \text{(A-2)}$$

The position of the protein-fixed $\bar{X}'\bar{Y}'\bar{Z}'$ coordinate system as compared to the laboratory-fixed axes is described by the Euler angles α, β and γ. The transformation of the coordinates of a vector in the $\bar{X}'\bar{Y}'\bar{Z}'$

system to the coordinates of the same vector in the $\bar{X}\bar{Y}\bar{Z}$ system is determined by the matrix:

$$\begin{pmatrix} (\cos\alpha\cos\gamma - \sin\alpha\cos\beta\sin\gamma) & -(\cos\alpha\sin\gamma + \sin\alpha\cos\beta\cos\gamma) & \sin\alpha\sin\beta \\ (\sin\alpha\cos\gamma + \cos\alpha\cos\beta\sin\gamma) & -(\sin\alpha\sin\gamma - \cos\alpha\cos\beta\cos\gamma) & -\cos\alpha\sin\beta \\ \sin\beta\sin\gamma & \sin\beta\cos\gamma & \cos\beta \end{pmatrix} \tag{A-3}$$

The emission intensity of the dipole $\bar{\mu}$ in the protein characterized by (α, β, γ) with polarization $\bar{X}$ and $\bar{Z}$ is proportional to $W\mu_X^2$ and $W\mu_Z^2$, respectively. Here, W stands for the probability of absorption of the exciting light by the dipole $\bar{d}$ in the same protein. The total emission with polarization $\bar{X}$ and $\bar{Z}$ is proportional to the average of $W\mu_X^2$ and $W\mu_Z^2$ over $\alpha[0, 2\pi]$, $\gamma[0, 2\pi]$ and $\beta[\delta_1, \delta_2]$, where $[\delta_1, \delta_2]$ is the allowed fluctuation interval of the orientation of the protein in the membrane.

The probability W is proportional to the average of d_Y^2 (or d_X^2) over $\alpha[0, 2\pi]$, since the excitation light is not polarized:

$$W = C_1\langle d_Y^2\rangle_\alpha = C_1\left\{\tfrac{1}{2}\sin^2\phi\left[1 - (\sin^2\theta\cos^2\gamma + 2\sin\theta\cos\theta\sin\gamma\cos\gamma + \cos^2\theta\sin^2\gamma)\sin^2\beta\right]\right.$$
$$\left. + \tfrac{1}{2}\cos^2\phi\sin^2\beta - \sin\phi\cos\phi(\cos\theta\sin\gamma + \sin\theta\cos\gamma)\sin\beta\cos\beta\right\} \tag{A-4}$$

where C_1 is the proportionality constant.

The emission intensities are as follows:

$$F_\parallel = F_X = C_2\langle W\mu_X^2\rangle_{\alpha,\beta,\gamma} \tag{A-5}$$

$$F_\perp = F_Z = C_2\langle W\mu_Z^2\rangle_{\alpha,\beta,\gamma} \tag{A-6}$$

with C_2 being the corresponding proportionality constant.

The average of μ_X^2 and μ_Z^2 over $\alpha[0, 2\pi]$ can be calculated alone, since W does not depend on α. Then

$$\langle\mu_X^2\rangle_\alpha = \tfrac{1}{2}\sin^2\psi(1 - \cos^2\gamma\sin^2\beta) + \tfrac{1}{2}\cos^2\psi\sin^2\beta - \sin\psi\cos\psi\cos\gamma\sin\beta\cos\beta \tag{A-7}$$

$$\langle\mu_Z^2\rangle_\alpha = \sin^2\psi\cos^2\gamma\sin^2\beta + \cos^2\psi\cos^2\beta + 2\sin\psi\cos\psi\cos\gamma\sin\beta\cos\beta \tag{A-8}$$

After averaging over $\gamma[0, 2\pi]$ and $\beta[\delta_1, \delta_2]$, the fluorescence intensity components polarized parallel and perpendicular to the membrane plane can be written as in Eqns. 1 and 2, where:

$$C_3 = C_1C_2 \tag{A-9}$$

$$f_1(\phi,\theta,\psi) = \tfrac{1}{4}\left[1 - \tfrac{1}{4}\sin^2\phi(2\sin^2\theta + 5)\sin^2\psi - 3\cos^2\phi\cos^2\psi + \sin 2\phi\sin\theta\sin 2\psi\right] \tag{A-10}$$

$$f_2(\phi,\theta,\psi) = \tfrac{1}{4}\left[\sin^2\phi\sin^2\psi + 5\cos^2\phi\cos^2\psi - 3\cos^2\psi - \sin 2\phi\sin\theta\sin 2\psi\right] \tag{A-11}$$

$$\langle\sin^4\beta\rangle_{\delta_1,\delta_2} = 1 + \frac{\cos^5\delta_1 - \cos^5\delta_2}{5(\cos\delta_1 - \cos\delta_2)} - \frac{2(\cos^3\delta_1 - \cos^3\delta_2)}{3(\cos\delta_1 - \cos\delta_2)} \tag{A-12}$$

$$\langle\sin^2\beta\rangle_{\delta_1,\delta_2} = 1 - \frac{\cos^3\delta_1 - \cos^3\delta_2}{3(\cos\delta_1 - \cos\delta_2)} \tag{A-13}$$

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