Biochimica et Biophysica Acta, 640 (1981) 628—639

BBA 79090

LINEAR DICHROISM OF BIMOLECULAR CHLOROPHYLL-LIPID MEMBRANES

THE ROLE OF ANISOTROPY AND DISPERSION

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(Received June 24th, 1980)

Key words: Chlorophyll-lipid membrane; Linear dichroism; Optical anisotropy; Dispersion

Summary

Polarised absorption and reflection spectra of chlorophyll-containing bimolecular lipid membranes were obtained in the spectral range of 590—710 nm. The spectra were analysed using the formalism of the complex dielectric tensor which characterizes the optical anisotropy of the membrane and the light absorption therein.

The maxima of the absorption spectra recorded at a 45° angle of incidence are located at 665 and 670 nm for light in which the electric vector is oriented parallel and perpendicular, respectively, to the plane of incidence. The analysis of these spectra shows that the spectral shift is wholly due to the dispersion of the real part of the dielectric tensor.

The angle between the dipole transition moment in the red and the normal to the membrane was estimated to be 42.3—45.3°.

On the basis of these results, a model absorption spectrum, simulating the dichroic properties of oriented chloroplasts, was calculated for a system of parallel membranes.

Some of the possible artifacts introduced into the dichroic spectra of chloroplasts due to anisotropy and dispersion are discussed.

Introduction

Almost all studies of light absorption and emission by chlorophyll in chloroplasts or in anisotropic samples are intended to clarify the structure of photosynthetic membranes and the mechanism of excitation energy transfer between chlorophyll molecules.

Macroscopically, the chloroplast may be regarded as a light-absorbing and optically anisotropic 'crystals', the optical axis of which is perpendicular to the plane of thylakoid lamellae. Microscopically, the chloroplast includes a number of parallel, optically anisotropic and light-absorbing membranes separated by isotropic and transparent aqueous layers.

The very specific structural properties of chloroplasts require that some caution be taken in interpreting the linear dichroism spectra. The mean concentration of chlorophyll in chloroplasts is high and approaches a value of the order of 0.1 M [1]. Due to such a high concentration of the strongly absorbing pigment, an exact and valid interpretation of linear dichroism data should take into account not only the light absorption phenomena as expressed by Lambert's law, but also the dispersion of the real parts of the dielectric tensor, which characterizes the dielectric properties of thylakoid membranes at optical frequencies, and the optical anisotropy of membranes (see Theory).

With chloroplasts it would be difficult, if possible at all, to collect sufficient data for a complete determination of the dielectric tensor. In this situation, model experiments with chlorophyll-containing planar bimolecular membranes would be valuable. It has been shown by other authors [2—4] that the concentration of chlorophyll molecules in bimolecular lipid membranes is sufficiently close to that in chloroplast membranes. Moreover, the phenomena investigated here are independent of factors such as specific lipid composition and some protein and additional pigment contents, which distinguish the model from natural membranes. This work presents experiments in which a complete set of optical parameters was determined for a single chlorophyll-containing bimolecular lipid membrane. On this basis, the absorption spectra for a system of parallel membranes were calculated. They are discussed with reference to the polarized absorption spectra of chloroplasts.

Methods

Theory

The large degree of orientation of molecules and high concentration of pigment in the membrane imply that the membrane should be regarded as a thin, strongly absorbing and optically anisotropic layer. The preferential orientation

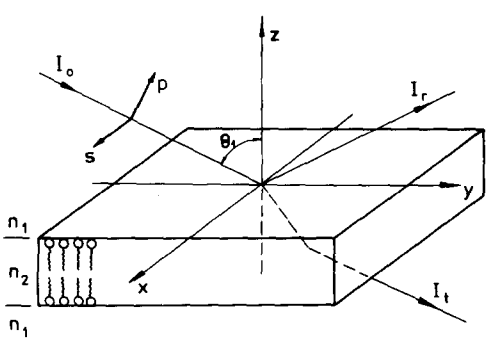

Fig. 1. Schematic representation of the membrane and the coordinate system. The vectors, p and s, indicate the electric vector polarizations for p- and s-polarized waves.

of molecules in the bimolecular lipid membrane as well as in the thylakoid membrane, with their long axes perpendicular to the membrane plane, implies that the membrane is equivalent to a thin slab of a uniaxial crystal in which the optical axis is oriented perpendicular to the surface and immersed in an isotropic liquid (see Fig. 1). In the following we shall consider the membrane to be homogeneous, i.e., the optical properties are assumed to be the same at any point within the membrane.

The optical properties of a bimolecular lipid membrane may be most conveniently described by introducing a complex dielectric tensor, which is diagonal in the coordinate system depicted in Fig. 1; it is represented by only two complex numbers, $\epsilon_{\parallel}$ and $\epsilon_{\perp}$:

$$\epsilon = \begin{bmatrix} \epsilon_{\perp} & & \\ & \epsilon_{\perp} & \\ & & \epsilon_{/\!/} \end{bmatrix} \tag{1}$$

where:

$$\epsilon_{\perp} = \epsilon'_{\perp} + i\epsilon''_{\perp} \tag{2}$$

$$\epsilon_{/\!/} = \epsilon'_{/\!/} + i\epsilon''_{/\!/} \tag{3}$$

The relationship between the dipole moment of a given transition, j and the imaginary parts of $\epsilon_{\perp}$ or $\epsilon_{\parallel}$ may be written in the following form [5]:

$$\epsilon''^{j}_{/\!/} = \frac{4\pi^2}{\hbar} \cdot N_j \langle |\mu^j_{/\!/}|^2 \rangle K^2_{/\!/} \rho(\omega - \omega_j) \tag{4a}$$

$$\epsilon''^{j}_{\perp} = \frac{4\pi^2}{\hbar} \cdot N_j \langle |\mu^j_{\perp}|^2 \rangle K^2_{\perp} \rho(\omega - \omega_j) \tag{4b}$$

where N_j is the number of absorbing molecules in a unit volume; $\mu^j_{\parallel}$ and $\mu^j_{\perp}$ are the components of the dipole transition moment parallel and perpendicular to the optical axis, respectively; and the brackets $\langle\ \rangle$ denote averaging over all possible orientations of transition moments. The function, $\rho(w - w_j)$, is a form factor for the jth transition, normalised to unity. K is the lotal field factor which relates the electric field intensity at the molecule with the macroscopic (average) field intensity which occurs in Maxwell equations.

Fresnel coefficients for transmission and reflection for a single membrane [6] are the following:

$$t = \frac{t_{12} t_{23} e^{i\beta}}{1 + r_{12} r_{23} e^{2i\beta}} \tag{5}$$

$$r = \frac{r_{12} + r_{23} e^{2i\beta}}{1 + r_{12} r_{23} e^{2i\beta}} \tag{6}$$

where:

$$r_{jk} = \frac{m_j - m_k}{m_j + m_k} \tag{7}$$

$$t_{jk} = \frac{2mj}{m_j + m_k} \tag{8}$$

$$\beta = \frac{\omega d}{c} \cdot n_2 \cos\theta_2 \tag{9}$$

$m_i = n_i \cos\theta_i$ for a light wave in which the electric vector is oriented perpendicular to the plane of incidence (s-polarised) and $m_i = n_i \cos\theta_i/\epsilon_{\perp i}$ for a wave in which the electric vector is in the plane of incidence (p-polarised). Owing to the fact that the membrane thickness, d (approx. 6 nm), is small as compared to the wavelength (600—700 nm), it will be convenient to expand the expressions on the right-hand side of Eqns. 5 and 6 in series to the first order of β. This procedure gives the final formulae in the simple and clear form without any significant decrease in the accuracy at the angles of 60° [7]. Additionally, for a membrane with identical solutions on both sides, we have:

$$m_1 = m_3 = m$$

$$r_{12} = -r_{23} = r_0$$

Under these conditions, the expanded expressions for transmission and reflection coefficients are:

$$t = 1 + i\beta \cdot \frac{1 + r_0^2}{1 - r_0^2} = 1 + i\beta \cdot \frac{m^2 + m_2^2}{2mm_2} \tag{10}$$

$$r = -2i\beta \cdot \frac{r_0}{1 - r_0^2} = \frac{i\beta}{2mm_2}(m_2^2 - m^2) \tag{11}$$

These formulae, rewritten in the explicit form, give the following formulae for the transmittance and reflectance of the membrane:

$$T_s = |t_s|^2 = 1 - \frac{2\pi d}{\lambda n_1 \cos\theta_1} \cdot \epsilon_\perp'' \tag{12}$$

$$T_p = |t_p|^2 = 1 - \frac{2\pi d n_1}{\lambda \cos\theta_1}\left(\frac{\cos^2\theta_1}{n_1^2} \cdot \epsilon_\perp'' + n_1^2 \sin^2\theta_1 \cdot \frac{\epsilon_{/\!/}''}{\epsilon_{/\!/}'^2 + \epsilon_{/\!/}''^2}\right) \tag{13}$$

$$R_s = |r_s|^2 = \left(\frac{\pi d}{\lambda n_1 \cos\theta_1}\right)^2 [(\epsilon_\perp' - n_1^2)^2 + \epsilon_\perp''^2] \tag{14}$$

$$R_p = |r_p|^2 = \left(\frac{\pi d n_1}{\lambda \cos\theta_1}\right)^2 \left[\left(\frac{\cos^2\theta_1}{n^1}\epsilon_\perp'' - n_1^2 \sin^2\theta_1 \cdot \frac{\epsilon_{/\!/}''}{\epsilon_{/\!/}'^2 + \epsilon_{/\!/}''^2}\right)^2 + \left(1 - n_1^2 \sin^2\theta_1 \cdot \frac{\epsilon_{/\!/}'}{\epsilon_{/\!/}'^2 + \epsilon_{/\!/}''^2} - \frac{\cos^2\theta_1}{n_1^2} \cdot \epsilon_\perp'\right)^2\right] \tag{15}$$

In the derivation of Eqns. 12 and 13, all terms proportional to $(d/\lambda)^2$ have again been neglected.

The experimentally measurable quantities are the absorbance and the reflectance. Since the transmittance is very close to unity (Eqns. 12 and 13), we may use the approximated expressions for the absorbances. We obtain these from

Eqns. 12 and 13 in the form:

$$A_s = \frac{1}{2.3} \cdot \frac{2\pi d}{\lambda n_1 \cos\theta_1} \cdot \epsilon_\perp'' \tag{16}$$

$$A_p = \frac{1}{2.3} \cdot \frac{2\pi d n_1}{\lambda \cos\theta_1} \left(\frac{\cos^2\theta_1}{n_1^2} \cdot \epsilon_\perp'' + n_1^2 \sin^2\theta_1 \cdot \frac{\epsilon_{/\!/}''}{\epsilon_{/\!/}'^2 + \epsilon_{/\!/}''^2} \right) \tag{17}$$

The quantities on the left-hand sides of Eqns. 14—17 are experimentally obtainable. The solution of these equations gives the real and imaginary parts of the dielectric tensor components as functions of the wavelength.

Having the dielectric tensor for a single membrane completely determined, we may calculate the average components of the dielectric tensor for a system of parallel membranes in which the thickness and spacing of membranes are much smaller than the wavelength. This may be done by making use of the boundary conditions for the $\vec{E}$ and $\vec{D}$ fields [8]. A simple calculation gives:

$$\epsilon_{\perp \mathrm{av}} = \epsilon + f(\epsilon_\perp' - \epsilon) + if\epsilon_\perp'' \tag{18}$$

$$\epsilon_{/\!/\,\mathrm{av}} = \frac{f\epsilon^2\epsilon_{/\!/}' + (1-f)\,\epsilon(\epsilon_{/\!/}'^2 + \epsilon_{/\!/}''^2) + if\epsilon^2\epsilon_{/\!/}''}{[f\epsilon + (1-f)\epsilon_{/\!/}']^2 + [(1-f)\epsilon_{/\!/}'']^2} \tag{19}$$

Here, ϵ denotes the optical dielectric constant of the isotropic (aqueous) layers while f is the volume fraction of membranes in the whole system. The above formulae show that the relationships between the optical parameters of a single membrane and those of a membrane system are not simple. Especially the form of the imaginary part of $\epsilon_{\parallel \mathrm{av}}$ indicates a possibility of distortion of the absorption spectrum by the wavelength dependence of $\epsilon_{\parallel}'$ and $\epsilon_{\parallel}''$ in the denominator of Eqn. 19.

Experimental procedure

Planar bimolecular lipid membranes were formed from solutions of egg lecithin (3 mg/ml ≈ 4 mM) and chlorophyll *a* in chloroform/decane (1 : 4, v/v) on a 2 mm hole in a Teflon wall immersed in a glass cuvette containing 0.1 M KCl (pH 7.2—7.4, refractive index 1.3315). Chlorophyll *a* was freshly extracted from spinach or from dried nettle leaves and purified by thin-layer chromatography. Its spectra in acetone and diethyl ether resembled closely those reported in the literature [9].

The cuvette with the membrane was placed in the light path in a laboratory-constructed double-beam spectrophotometer consisting of a light source (150 W halogen tungsten lamp with stabilised power supply), monochromator, S-20 photomultiplier, lock-in amplifier and *x-y* recorder. Polaroid film was used as polarizer with the dichroic ratio in the red of the order of 800. The two light beams, both passing across the cuvette, were balanced in the absence of the membrane to within 0.3% in the whole spectrum. After the membrane had been formed, the additional unbalanced signal was detected and recorded by scanning the spectrum at least three times. The background and reference signals were recorded immediately after breaking the membrane. Temporal stability and noise filtering were such as to allow an accuracy of somewhat better than 10^{-4} in the absorbance units obtainable in a single experiment. The

spectral resolution was 1.5 nm at 650 nm wavelength. The light beam was collimated to within ±6° around its axis and its cross-section in the membrane plane was 0.2 × 0.7 mm. An appropriate slit was placed in the image plane before the photomultiplier in order to eliminate the effects of scattered light. The same apparatus was also used, after slight modifications, in measurements of light reflection. The experimental data were transferred to paper tape and then computer-processed.

Results

The absorption spectra were obtained at a 45° angle of incidence. Separate membranes were formed for each experiment with a given polarisation of the light beam. It should be mentioned that the individual spectra to be averaged displayed the same qualitative and quantitative features as those presented in Fig. 2. The only consequence of averaging was the reduction of accidental errors due to noise in the detection system and to small lipid droplets crossing the light beam near the membrane.

The bandwidth and relative peak heights here are different from these observed with chlorophyll *a* in diluted solutions. The most interesting feature of the absorption spectra is that the main peak positions are different for light polarized parallel (665 nm) and perpendicular (670 nm) to the plane of incidence. This effect is similar to the phenomena observed with oriented chloroplasts [10,11]. An explanation of this effect may be possible with reference to dielectric tensor dispersion (see below). Here, it should only be noted that the peak shift is not caused by the wavelength-dependent light reflection, which is too small to influence significantly peak positions.

For the determination of the real and imaginary parts of dielectric tensor components, the reflectance for s- and p-polarised waves must be known at each wavelength. Since the reflection for the p-polarised wave is negligible at a 45° angle of incidence, reflection spectra were determined at an angle of incidence equal to 30°. The spectra are shown in Fig. 3. The shapes of these spectra are similar; the essential difference is in the magnitude of the reflection coefficients. The maxima and minima of both these spectra are located at the long- and short-wavelength slopes of absorption spectra (cf. Fig. 2). This suggests that the wavelength dependence of reflection coefficients is an immediate result of dispersion.

On the basis of the experimental results summarized in Figs. 2 and 3, Eqns. 14—17 were solved point-by-point to obtain the real and imaginary parts of the dielectric tensor components. The results are shown in Figs. 4 and 5.

The peak heights of $\epsilon_{\perp}''$ and $\epsilon_{\parallel}''$ reveal proportions different from those of the absorption peaks in Fig. 2. However, the difference is understandable if we take into account the fact that the absorbance spectra were taken at a 45° angle of incidence while $\epsilon_{\perp}''$ and $\epsilon_{\parallel}''$ represent the absorption of s- and p-polarised waves in an imaginary experiment in which the angle of incidence was assumed to be 90°. It is even more important that both $\epsilon_{\perp}''$ and $\epsilon_{\parallel}''$ have their maxima located at the same wavelength (670 nm) as opposed to the absorption spectra, which represent simply the wavelength dependence of membrane absorbance. A closer examination of Eqn. 17 indicates that the shift in the A_p spectrum is intro-

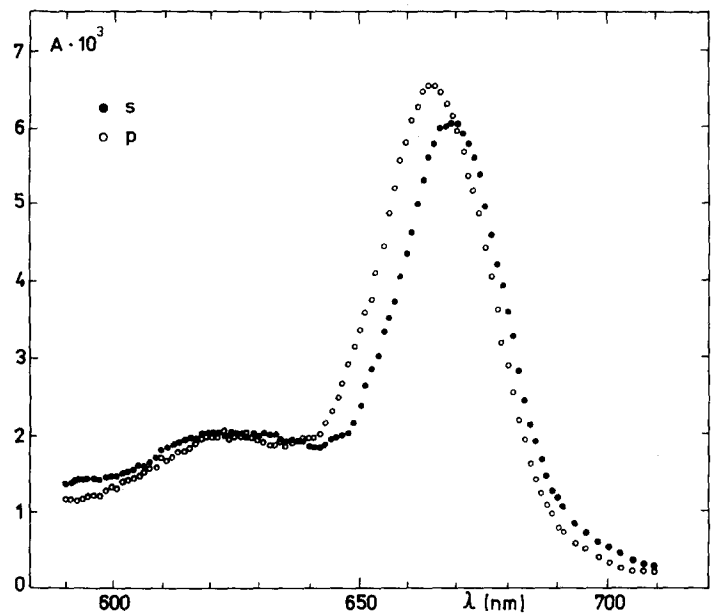

Fig. 2. Polarized absorption spectra of chlorophyll *a* in membranes for s- and p-polarized light. The angle of incidence was 45°. The mole fraction of chlorophyll *a* in the membrane-forming solution was 0.45. Each spectrum is an average from experiments with six membranes.

duced by the second term of this equation:

$$n_1^2 \sin^2\theta_1 \frac{\epsilon_{//}''}{\epsilon_{//}'^2 + \epsilon_{//}''^2} \tag{20}$$

This is the wavelength dependence of $\epsilon_{\parallel}'$ (dispersion) which causes the A_p peak to be shifted to a shorter wavelength with respect to the A_s peak position. This

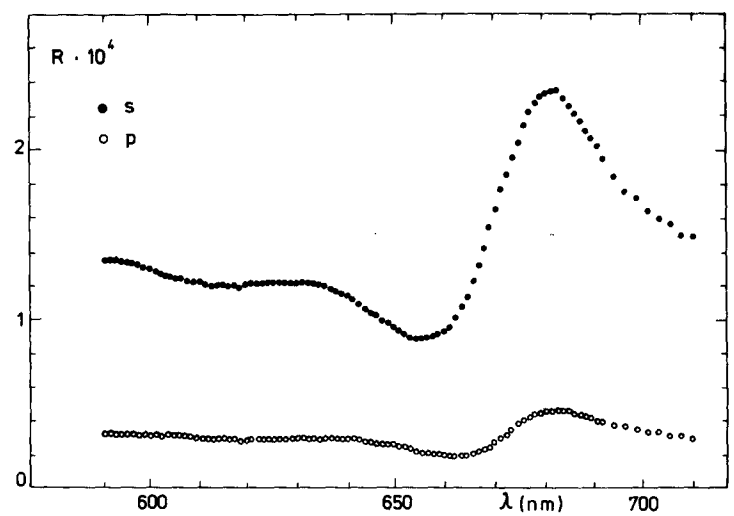

Fig. 3. Reflection spectra of membranes at a 30° angle of incidence. The chlorophyll mole fraction in the membrane-forming solution was 0.45. Each spectrum is an average from four experiments.

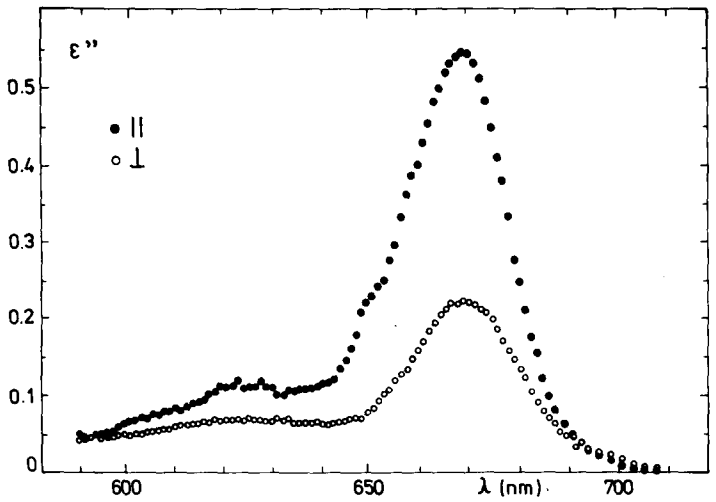

Fig. 4. Wavelength dependence of the imaginary parts of the dielectric tensor components.

effect results from the boundary conditions for the electric field of a light wave passing through the water/membrane interface.

The wavelength dependence of the real parts of the dielectric tensor components is presented in Fig. 5. The $\epsilon'_{\parallel}$ and $\epsilon'_{\perp}$ curves are similar to R_p and R_s in Fig. 3. This picture is quite understandable if we take into account the fact that the reflection intensity depends on the difference between the dielectric properties of the membrane and those of the aqueous solution.

The dispersion and anisotropy of a pigment-containing membrane make the conditions of light propagation within the membrane differ significantly, depending on the orientation of the plane of polarisation. It is interesting to see what changes may be due to these effects in polarised absorption spectra of a

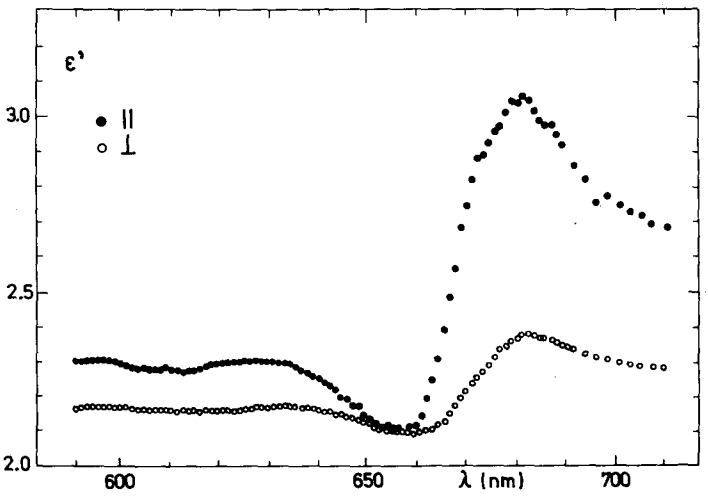

Fig. 5. Real parts of the dielectric tensor components as functions of wavelength.

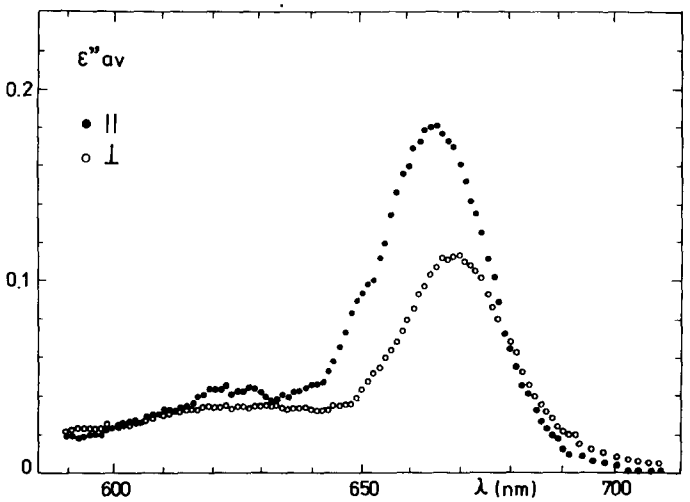

Fig. 6. Wavelength dependence of calculated imaginary parts of the dielectric tensor for a system of membranes. The volume fraction of membranes, $f = 0.5$, and the refractive index of isotropic layers, $n = 1.3315$, were used in the calculations.

lamellar system of such membranes. The imaginary parts of the averaged dielectric tensor for such a system were calculated using Eqns. 18 and 19 and the tensor components for a single membrane shown in Figs. 4 and 5. The membrane fraction in the system (f) was chosen to be 0.5. The resulting ϵ''_{av} spectra for light propagating along the membrane planes with its electric vector perpendicular ($\epsilon''_{\perp av}$) and parallel ($\epsilon''_{\parallel av}$) to the optical axis of such a system are shown in Fig. 6. Again, the peak position for light polarised parallel to the optical axis is shifted to shorter wavelengths, due to dispersion. The dichroic ratio equal to 2.42 for a single membrane (cf. Fig. 4), changed to 1.60 for the system of parallel membranes. This effect is due to differences in the anisotropic properties of a single membrane and those of the membrane system.

Discussion

The relationship between the transition dipole moment orientation and the intensity of light absorption is given by Eqns. 4a and 4b. Let us assume that the dipole moments for the jth transition of all chlorophyll molecules form the same angle with the normal to the plane of bimolecular lipid membranes. Using this assumption, we may write:

$$\langle |\mu_\perp^j|^2 \rangle = \tfrac{1}{2} |\mu^j|^2 \sin^2 \varphi_j \tag{21}$$

$$\langle |\mu_\parallel^j|^2 \rangle = |\mu^j|^2 \cos^2 \varphi_j \tag{22}$$

Insertion of these expressions into Eqns. 4a and 4b gives:

$$\tan^2 \varphi_j = 2 \left(\frac{K_\parallel}{K_\perp} \right)^2 \frac{\epsilon_\perp''^j}{\epsilon_\parallel''^j} \tag{23}$$

The exact determination of φ_j requires the local field factors, $K_{\parallel}$ and $K_{\perp}$, to be known and this is a very difficult problem in the case of a chlorophyll chromophore located at the boundary of isotropic (water) and anisotropic (membrane) media. However, the angle φ_j may be estimated for two extreme cases. Both main peaks at 670 nm in Fig. 4 are most probably coupled to a single transition. The ϵ'' values at this wavelength are $\epsilon_{\perp}'' = 0.225$ and $\epsilon_{\parallel}'' = 0.545$. Thus, if chlorophyll molecules were in an infinite isotropic medium (water), the local field factors would be equal to each other. In this case, Eqn. 23 gives $\varphi = 42.3°$. In the other extreme case, let the chlorophyll molecules be in an infinite anisotropic medium like the membrane. For an approximate estimation of φ in this case, the Lorentz formula may be applied in the form:

$$K_{\parallel} = \frac{\epsilon_{\parallel}' + 2}{3} \tag{24}$$

$$K_{\perp} = \frac{\epsilon_{\perp}' + 2}{3} \tag{25}$$

Taking $\epsilon_{\perp}' = 2.206$ and $\epsilon_{\parallel}' = 2.694$ at 670 nm and introducing appropriate expressions for $K_{\perp}$ and $K_{\parallel}$ into Eqns. 23, we obtain $\varphi = 45.3°$. Thus, the angle between the transition moment and the optical axis is between 42.3 and 45.3°.

As determined in the simplified calculations, based on projections of the transition moment onto the electric vector of the light wave, the angle is about 53.5° [2] or 54—56° [3]. A similar value, $\varphi = 53°$, may be calculated in this manner from the data in Fig. 2, while in fact the angle is 42.3—45.3°.

The peak shift effect related to dispersion, treated in the previous section, was also observed and interpreted in the same way by Breton et al. [15] and Chollet [7]. However, these authors were unable to correct their spectra for dispersion as the experimental data were insufficient.

In many cases, such corrections, consisting of the conversion of absorbance into ϵ'' spectra, seem to be necessary. This is not due to the absorbance, but rather, because the imaginary part of the dielectric permitivity (ϵ'') is directly linked to the projection of the transition moment on the electric field vector and to the form factor of a given absorption band (Eqns. 4a and 4b).

The dependence of peak positions on the polarization plane in linear dichroism or polarized absorption spectra was also observed and interpreted as indicating different spectral forms of chlorophyll in chloroplasts or in isolated lamellae [10—12,14]. The main peak position in an absorption spectrum for p-polarized light is always positioned at a shorter wavelength than for s-polarized light. This leads to the conclusion that the long-wavelength transition moments are closer to the membrane plane than the short-wavelength ones. A similar dependence of the peak position on the polarization plane is found in the present experiments in the absorption spectra of a single membrane (Fig. 2). However, the picture changes radically in the spectra in Fig. 4; here, the peaks are located at the same wavelength and display similar wavelength dependence. Thus, in some cases, it may not be necessary to consider different spectral forms in order to explain the relative peak shift in the polarized absorption spectra. This conclusion refers especially to experiments with chloroplast lamellae oriented, for example, by deposition onto a glass plate.

With chloroplasts the situation is somewhat different. First, the absorbance spectra of chloroplasts should not necessarily follow the ϵ'' spectra due to the usual effect of dispersion which may be illustrated by the relationship between the absorbance and the imaginary part of the dielectric permittivity in a continuous medium [16]:

$$A = \frac{1}{2.3} \cdot \frac{\omega \epsilon'' x}{cn'} \tag{26}$$

where x is the thickness of the sample; n' is the frequency-dependent real part of the refractive index. Second, $\epsilon_{\parallel}''$ and $\epsilon_{\perp}''$ which characterize the individual membrane may differ from $\epsilon_{\parallel av}''$ and $\epsilon_{\perp av}''$ as predicted from Eqns. 18 and 19.

It should be mentioned that the effect considered here is quite different from selective scattering and selective reflection [11]. These artifacts, in principle, may be reduced or substracted from experimental data by an appropriate experimental procedure. However, the linear dichroism spectra obtained in this way still contain some contribution arising from dispersion.

The lack of effect of membrane stacking [11] may be analysed as follows. When the lamellae are superimposed, thus forming grana in chloroplasts, then Eqn. 26 describes the effect together with Eqns. 18 and 19. In the other case, when the lamellae are spread over the sample volume, then the absorbance is the sum of absorbances of individual lamellae, described by Eqns. 16 and 17. The same formulae describe the absorbance of the lamellae deposited on a glass plate. In all these cases, the dispersion effect shifts the A_p spectrum in relation to the A_s spectrum, although the textural dichroism really does not appear.

Thus, even if all artifacts introduced by experimental conditions were eliminated, one should convert the absorbance spectra into ϵ'' spectra in order to obtain adequate and accurate information about the shape, height and position of absorption peaks. This can probably be done with the use of the Kramers-Kronig relationship; appropriate procedures applicable to the problems mentioned above are now being worked out in our laboratory.

The effect of dispersion on polarized absorption spectra is always more remarkable in more strongly absorbing samples. The surface concentration of chlorophyll molecules in membranes used in this work was about $4.5 \cdot 10^{13}$ cm^{-2}. Assuming the mean concentration of the chlorophyll in chloroplasts to be of the order of 0.1 M [1] and the thickness and spacing of membranes to be equal to 7.5 nm, we obtain the surface concentration of chlorophyll molecules of about twice that in model membranes. Thus, it may be inferred that the deviations introduced by dispersion and anisotropy into polarized absorption spectra of photosynthetic membranes may be of the same order as those in model membrane spectra.

Acknowledgement

This work was performed under contract R.III.13.2.7.

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