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DIFFERENTLY ORIENTED CHLOROPHYLLS IN *MESOTAENIUM CALDARIORUM* DETECTED BY MICROPHOTOMETRICAL DICHROISM MEASUREMENTS IN VIVO

BARBARA HEINZE and ARNOLD WARTENBERG

Fachrichtung Mikrobiologie, Universität des Saarlandes, Bau 2, D-6600 Saarbrücken (F.R.G.)

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The chloroplasts of individual cells of *Mesotaenium caldariorum* were examined microphotometrically under non-polarized and polarized measuring light. The measurement with non-polarized light showed different absorption bands of the thylakoids depending on the position of their surface with respect to the incident light beam: in the edge position, the absorption bands lie at 672 nm, in the face position at 678 nm. From this difference in absorption maxima, we conclude that the molecules related to the sub-bands at the two wavelengths are oriented differently. The Q_y transition of the molecules which absorb light at 678 nm must be oriented parallel to the face of the thylakoids (fraction I), while that of the molecules absorbing at 672 nm is oriented perpendicular to the face (fraction II). Measurement with polarized light leads to the same conclusion that two fractions of differently oriented chlorophylls exist: In the edge position, a very large difference between $E_{\parallel}$ and $E_{\perp}$ (dichroism) was found in red light, with a maximum of $E_{\parallel}$ lying at 675 nm and a maximum of $E_{\perp}$ at 670 nm, with a shoulder at 650 nm. In the blue region, especially in the Soret band zone, the chloroplast showed a negative dichroism in the edge position, which changes over to positive values when the wavelength exceeds 450 nm. In the face position no dichroism in red or blue light could be detected. Comparison of the 'edge position dichroism' in red light with that in blue light justifies the supposition that the chlorin planes of the chlorophyll molecules may be oriented perpendicular or parallel to the thylakoid face, in the case of perpendicular orientation with the Q_y transitions of fraction II and the x-transitions (B_x, Q_x) of fraction I projecting out of the plane, and for parallel orientation with all transition moments lying parallel to the plane (fraction I). The relative dichroism, $(E_{\parallel} - E_{\perp})/(E_{\parallel} + E_{\perp})$, measured at the edge position amounts to 0.34 (i.e., 34% of the total absorption) at 680 nm. These data probably do not reflect the total quantity of oriented chlorophyll because from the opposite orientations of the Q_y transition moments of fraction I and II pigment a partial quenching of the measurable dichroism results. The red light absorption bands of the two chlorophylls oriented in an opposite manner (fractions I and II) correspond to the known bands of Photosystem I and II.

Introduction

Observations concerning chlorophyll orientation are related to the plane of the potential box of conjugated double bonds of the chlorin ring of the chlorophyll molecule. Two of the four possible electron transitions (B_y and Q_y) lie along the longitudinal axis of the potential box, while the other two (B_x and Q_x) are directed parallel to the lateral axis [31]. The main red peak of the Chl *a* spectrum and the neighbouring band at 615 nm have been assigned to the Q_y transition, while the Soret band and its satellites are caused by the B_x

Abbreviations; Chl, chlorophyll; PS, photosystem.

and B_y transitions. The Q_x transition has been attributed to the Chl *a* band at 575 nm.

Though our knowledge of the orientation of chlorophyll has increased in recent years, observations reported in the literature are concerned mainly with the orientation of one of the transitions alone and not with the whole chlorin ring of the chlorophyll molecule. For wavelengths around 680 nm, most publications suppose conformably a parallel orientation of Q_y with respect to the thylakoid surface based on measurements of fluorescence polarization, absorption dichroism, circular dichroism, rotation dichroism or photoselection [1,5,6,8,9,15,17]. Only in one case were contradictory results obtained for the same region of wavelengths, indicating an orientation perpendicular to the thylakoid face [14]. Chlorophyll orientation in the region of shorter red wavelengths has remained unclear [1,6,7,9], while a concept regarding the orientation of Chl a_I (P-700) [21] and the chlorophyll Chl a_I dimer (special pair) [20] based on detailed investigation has been reported [16–19]

In the special case of a physiologically active chlorophyll, one of us (Ref. 29, see also Ref. 10) was able to detect the orientation of the whole chlorin ring of the chlorophyll molecule in vivo. The Soret band of Chl *a*, involved in low-light movement of the chloroplast of *Mesotaenium*, shows an action dichroism combined with a stronger absorption parallel to the thylakoid surface [10]. The corresponding transition moment, however, Q_y of the main red peak, turned out to be oriented perpendicular to the face.

Beyond this, the presence of two differently oriented forms of chlorophyll could be detected on the basis of absorption measurements [30], one form being parallel and the other perpendicular to the thylakoid surface. In addition to results on absorption dichroism obtained with polarized light [10,25,30], measurements under non-polarized light have also contributed to the discovery of the two differently oriented forms of chlorophyll [30], considering two directions of light incidence relative to the thylakoid face, i.e., the chloroplast position: the chlorophyll oriented perpendicular to the face absorbs red light of shorter wavelengths (absorption maximum at 670 nm), while the pigment oriented parallel to the face absorbs red light of longer wavelengths (680 nm).

Our new microphotometrical equipment should enable us to confirm the results reported earlier [30] with a higher degree of measuring accuracy, closer positioning of the measuring points on the wavelength scale and a much shorter measuring time. It is intended to present details of the orientation and dichroism of chlorophyll apparatus, with special interest in the question as to whether or not any relationship exists between the absorption of the two differently oriented forms of chlorophyll mentioned above and the absorption of the two photosystems (PS I and PS II) of photosynthesis.

Materials and Methods

Mesotaenium caldariorum var. *caldariorum* (No. 648-1, Göttinger Algensammlung) is a green unicellular alga with a cylindrical round-ended cell shape and a large plate-shaped lamellate chloroplast. The chloroplast has a size of $3.4 \times 8.6 \times 30$–$50\ \mu$m, occupies nearly the whole cell volume and is able to revolve around its long axis. The lamellae of the chloroplast are disposed strongly parallel to the face of the chloroplast and therefore allow definition of the direction of incidence of the measuring light with respect to the thylakoid face, if the chloroplast is positioned in the edge or face position during measurements.

Mesotaenium was grown according to the method described previously [30], using culture solution of the same composition but without agar. Under these conditions, the cells swim as a single cell layer on the liquid surface. During the microphotometrical measurement, the cells, prepared on slides, were selected for edge or face view of their chloroplast and irradiated by monochromatic light with a computer-triggered wavelength change. The transmitted light was split by polarizing filters and then in part amplified and registered by a photomultiplier.

Instruments used: Zeiss Axiomat (microscope photometer UV-NDPC-Scan); grating monochromator, controlled by a minicomputer in steps of 0.125 nm; photomultiplier HTV R 446, spectral type S-20; ultraviolet condenser, $A = 0.8$; Ultrafluar objective $50\times$, $A = 0.5$, glycerol immersion. On-line minicomputer Nova 2/10, Data General Corp.; 24K memory; real-time disk-operating sys-

tem, two Diablo magnetic disks; 12-bit A/D converter, 36 μs converting time. The computer controls the monochromator by means of a step motor (no greater than 200 steps/s), receives the digitized values from the A/D converter and records the values via the real-time disk-operating system on disk.

Statistical analysis: The obtained values and mean values were explored by the Kolmogoroff-Smirnoff test (normal distribution), Bartlett test (homogeneity of variances), F-test (analysis of variances) or Students *t*-test (significance of the difference between two mean values). The results of the tests are given in the texts to the figures.

Systematic errors: The error due to the $\cos^4$-law [26] was eliminated by fixation of the specimen field as well as the reference field in the centre of the luminous-field diaphragm using the same adjustment of the condenser-aperture diaphragm. The luminous field was confined by a hole diaphragm with a 3-fold larger diameter compared to the diameter of the photometric field which was 2.13 μm. The photometric field was exactly positioned in the centre of the hole diaphragm. Glare was determined [26] and amounted to up to 1% of the optical flux which reached the photocathode. False light from the monochromatic device had a level of 0.066% or less. Extraneous light and dark currents were eliminated using a light-chopper (50 Hz), so that only modulated light was amplified in the electronic set. Nevertheless, the photocathode dark current was measured and subtracted from each value recorded. The lamellae of the chloroplast oriented in the edge position caused a distributional error, but because the lamellae are situated with their diameter near the limit of resolution and since this error seems to be much smaller than the standard deviation of the measured values due to overall statistical error it was disregarded. A similar error occurs through the sieve effect [18], i.e., an apparent linear dichroism caused by the fact that membranes viewed face on by the measuring light absorb more light than those viewed edge on, provided the concentration of light-absorbing pigment is equal in both directions. Because of the sieve effect, the differences between face and edge position measurements were estimated qualitatively, whilst for quantitative conclusions only differences between 0 and 90° measurements were considered.

Results

Fig. 1 depicts the spectra of *Mesotaenium* chloroplasts obtained microphotometrically, the measurements being made in the face and edge positions of the chloroplast in living cells. The absorption spectra measured in red light show, compared to the results reported earlier [30], more accurately the difference between the peaks related to the face (678 nm) and edge positions (672 nm). In addition, each of the peaks has a shoulder at the same wavelength, at which the other peak is situated. The difference between the peaks of the two spectra in the red wavelength region can be explained by the different orientation of at least two sub-bands: that of the 672 nm with the transition moment (Q_y) lying perpendicular, and that of the other (678 nm) parallel to the thylakoid surface (see Fig. 4). At the face position of the chloroplast, only Q_y of the 678 nm sub-band absorbs, because Q_y of the 672 nm sub-band is situated parallel with respect to the direction of incidence of light. In the edge position of the chloroplast, Q_y' of both sub-bands absorb, with the value of the 672 nm

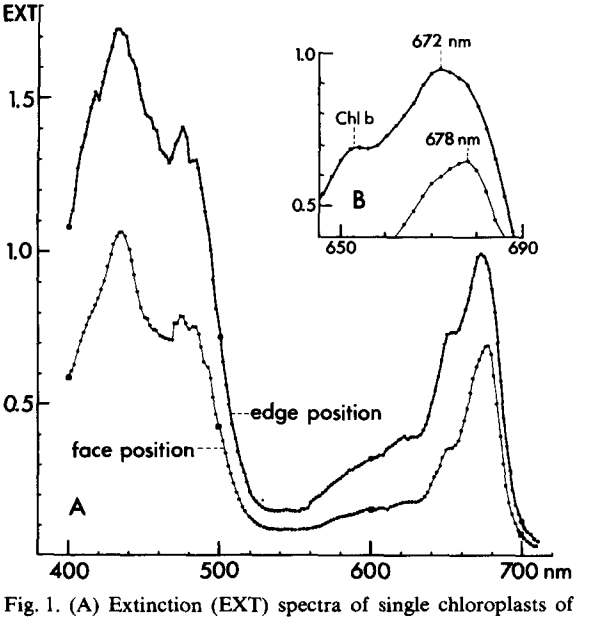

Fig. 1. (A) Extinction (EXT) spectra of single chloroplasts of *Mesotaenium caldariorum* taken at face and edge positions. The direction of incidence of the measuring light is perpendicular to the thylakoid plane at the face position and parallel at the edge position. $\Sigma n = 7800$. (B) Enlarged part of A.

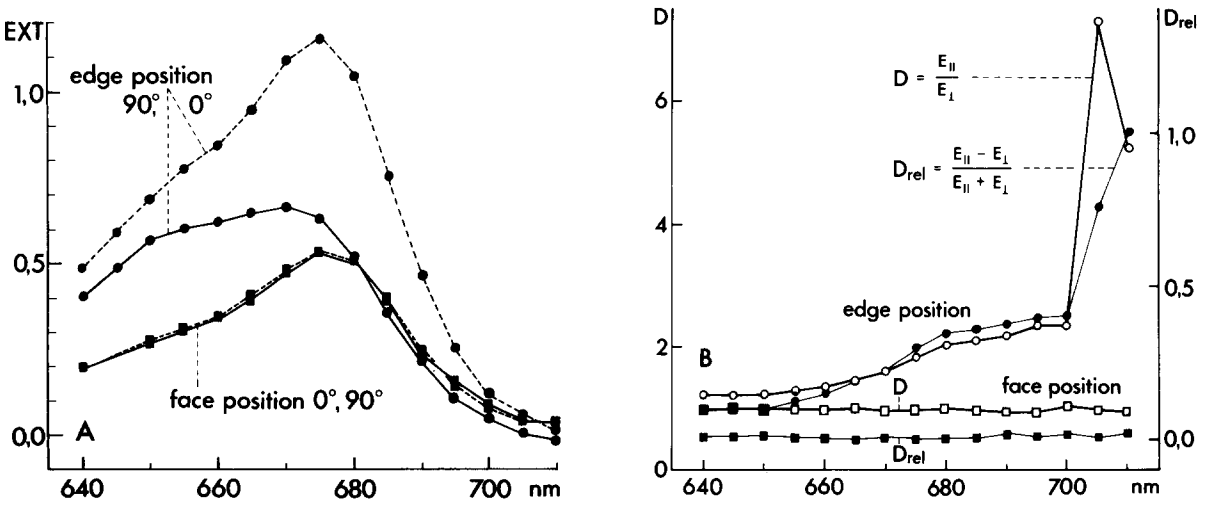

Fig. 2. (A) Extinction spectra of single chloroplasts of *Mesotaenium caldariorum* in relation to chloroplast position (face and edge position) and polarization of the measuring light (0 and 90° with respect to the axis of rotation of the plastid). (B) Dichroism spectra of the values showed in A. Statistical significances ($P = 0.01$): (1) normal distribution is accepted in accord with the central limit theorem or law of large numbers ($\Sigma n = 9000$ for (A); (2) Bartlett test: H_0 accepted for face and edge positions; (3) analysis of variances (*A* wavelength; *B* polarization): H_0 (*A*) refused for face and edge positions, H_0 (*B*) and H_0 (*AB*) refused for edge position; (4) Student's *t*-test for the differences between the 0 and 90° mean values at 640, 645 and 650 nm: H_0 values accepted for face position and refused for edge position.

sub-band exceeding that of the 678 nm sub-band, because the latter is relatively weak.

The corresponding spectra measured with differently polarized light (0 and 90° relative to the longitudinal axis of the chloroplast) are shown in Fig. 2. In the face position of the chloroplast, no dichroism could be detected; the 0 and the 90° spectra in Fig. 2A are exactly the same. The maximum of each spectrum is situated at 675 nm, the difference between the measuring points being 5 nm. The 0° spectrum measured in the edge position corresponds in general to both spectra in the face position, apart from the increased total absorption, due to the greater thickness of the chloroplast at the edge position. This similarity is to be expected, since all Q_y' transition moments which are oriented parallel to the axis of rotation, i.e., the longitudinal axis of the chloroplast, are lying in the absorption direction both at the face and edge position of the chloroplast (see Fig. 4). On the other hand, the edge spectrum measured with 90°-polarized light shows a peak at a lower wavelength (670 nm), which is assigned to Q_y, and a significant absorption at still lower wavelength (655 nm), both oriented perpendicular to the thylakoid face.

The measurements shown in Fig. 2 on the whole support the results given in Fig. 1, showing that there are two forms of Chl *a* oriented differently: first, fraction II absorbing red light of shorter wavelength (672 nm), Q_y being situated perpendicular to the face of the thylakoids, and second, fraction I absorbing red light of longer wavelengths (678 nm and beyond), Q_y being polarized parallel to the face.

With decreasing wavelengths in the range of the Chl *b* absorption, the two edge spectra appear to approach each other. Since both the face position spectra are otherwise alike, an almost random distribution or orientation of Chl *b* molecules is indicated.

A very strong dichroism appears in the region 700–705 nm (Fig. 2B) which is indicative of a high degree of orientation of P-700 or other far-red light absorbing chlorophylls with their Q_y parallel to the face. However, the high values of dichroism are based on very low values of extinction. Therefore, these values of dichroism are not very significant and need confirmation.

The absorption spectra measured in blue light are shown in Fig. 3A and the relevant dichroism spectra in Fig. 3B. In agreement with the measure-

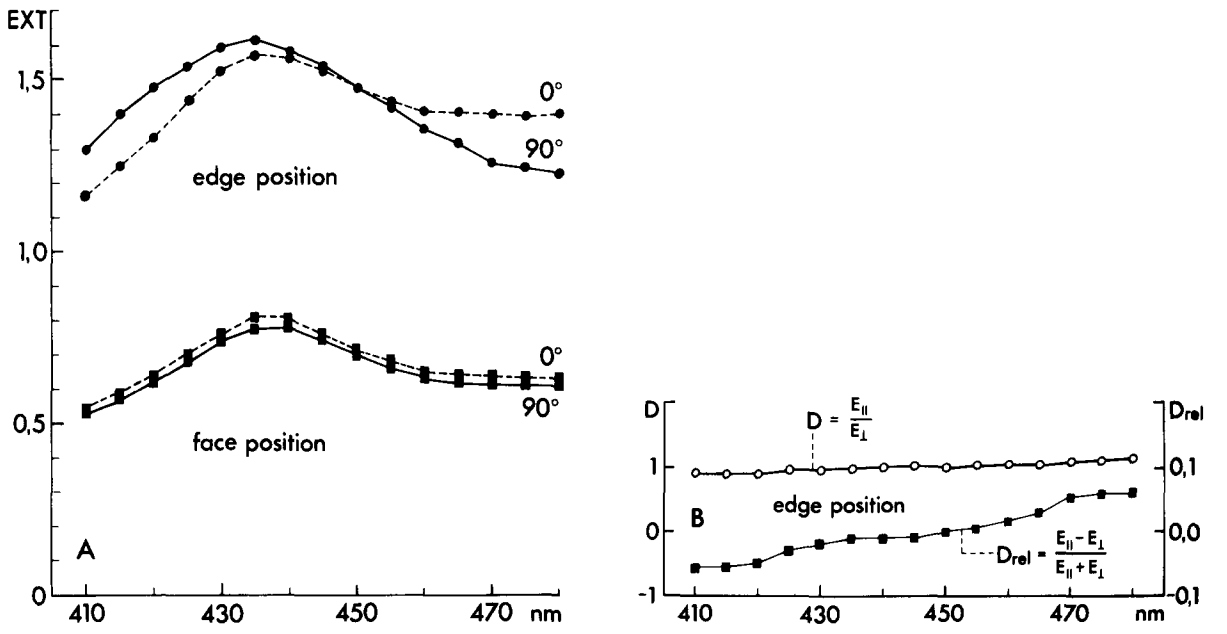

Fig. 3. (A) Extinction spectra of *Mesotaenium* chloroplasts (see legend to Fig. 2). (B) Dichroism spectra of the values shown in A. Statistical significances ($P = 0.01$): normal distribution accepted in accord with the central limit theorem ($n = 25$, $\Sigma n = 1500$); the Bartlett test was used to examine the homogeneity of variances of the 0 and 90° values, differently for the values measured at face and edge positions: H_0 could not be accepted for the edge position series, therefore, the analysis of variances could not be applied; Student's t-test: H_0 is refused for the differences between 0 and 90° mean values of the 410 –420 and 470–480 nm regions of the edge position series alone.

ments which were done under red light at the face position, no significant differences between 0 and 90° absorption were observed, indicating the absence of dichroism. However, at the edge position, a dichroism was observed, which was negative in the Soret region (and below) and which changed over to positive values on reaching the region of Chl *b* absorption (455–480 nm). The spectra lead to the conclusion that the two fractions of Chl *a* molecules oriented differently are also detectable in the blue light zone, although their resolution is not as clear as in the red light region. Moreover, measurements in blue light do not show the orientation degree of the chlorophyll to the same extent as measurements under red light.

Discussion

The microphotometrical measurements with non-polarized and polarized light over the total range of the chlorophyll spectrum on the *Mesotaenium* chloroplasts revealed two different fractions of chlorophyll: fraction I oriented with its Q_y parallel to the thylakoid face absorbing light at 678 nm (and longer wavelengths), and fraction II oriented with its Q_y perpendicular to the face and absorbing at 672 nm (and lower wavelengths).

To summarize all chlorophyll orientations possible within three dimensions, we refer to a model (Fig. 4) based upon earlier results [30]. Therein it was shown that all oriented chlorophyll is situated parallel (0°) or perpendicular (90°) to the thylakoid layer. Other orientations could not be detected. Therefore, the six positions presented in Fig. 4 show all configurations of chlorophyll orientation, and simultaneously they may represent a random distribution of chlorophyll, if all positions exist equally because a random distribution within one plane may result from two oriented states of equal magnitude situated perpendicular to each other. Accordingly, one can explain any possible orientation of chlorophyll molecules as a change of the transition moments of any position pair within the six positions as shown in Fig. 4, involving dif-

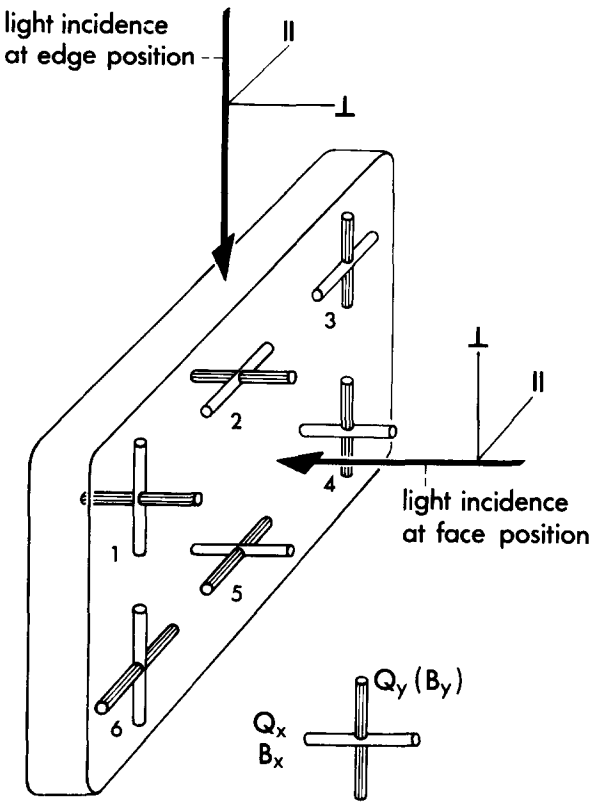

Fig. 4. Model of the chloroplast of *Mesotaenium* showing the six possible orientations of the chlorophyll molecule with respect to the thylakoid plane. The molecule is represented by the two transition moments (x- and y-transition) crossing mutually at an angle of 90°.

ferences in the proportions of the variously oriented forms.

This model may be now applied to our results. Obviously, the molecules of fraction II are represented by positions 1 and 2 of Fig. 4. The transition moments (Q_y) are oriented perpendicular to the face, as concluded from the absorption peak at 672 nm and the strong dichroism in the red region in the edge position. On the other hand, the x-transitions lie in the plane of the thylakoid, as indicated by the absence of chloroplast dichroism during measurements at the face position with blue light. However, it is not possible to distinguish between these two positions (1 and 2) and a random orientation of the x-transitions in the thylakoid plane.

Concerning the situation with fraction I molecules, two possibilities can be assumed: (1) The molecules exist only in two configurations, positions 4 and 5; (2) the molecules can have the four configurations, positions 3–6.

First case: In the long-wavelength region of red light absorption at the edge position of the chloroplast, the relative dichroism (Fig. 2B) has positive values due to the action of position 5, while in position 4 there is no absorption. In the blue light absorption, in the region of the Soret band, at the edge position the relative dichroism must be negative as shown in Fig. 3. A change of wavelength up to 450 nm, and above until 480 nm, causes a change of dichroism to positive values due to the action of fraction II molecules of position 2, while position 1 is not absorbing. At the face position of the chloroplast, only fraction I molecules participate in the absorption of red light.

Second case: In the red light region of absorption, especially near 680 nm, and in the edge position the positive relative dichroism may be stronger than proposed in the first case as in fact shown in Fig. 2, because not one but two transitions share the red absorption (positions 5 and 6). In the blue light region near the Soret band, fraction I due to position 3 participates in the positive dichroism, which in the first case would only be characteristic of fraction II. Moreover, compared to the strong red light dichroism, the weak dichroism in the blue light zone may be explained by overlapping of position 3 with position 4 or 5. Therefore, this case seems to be more likely.

The wavelengths at which the two chlorophyll fractions in different orientations have their absorption peaks (678 and 672 nm) suggest a connection between the two fractions and the two photosystems of photosynthesis as proposed in the Introduction. This conclusion is substantiated by results [3,34] which showed absorption peaks for PS I and PS II at 679 (682) and 671 (673) nm, respectively. Earlier reports on the absorption peaks of PS I and PS II [4] are less precise. These values agree with the absorption peaks of the two fractions of chlorophyll orientation presented here, particularly if the differences between the measuring points of $\Delta\lambda = 2$ nm used in our present microphotometrical determination are taken into

account. Our conclusion is supported additionally by the absorption characteristics of the PS I active P-700-Chl *a*-protein complex, which peaks at 677 nm [28]. The absorption features of the light-harvesting Chl *a*/*b*-protein complex, peaking at 672 nm [28] or 675 nm [3], permit the conclusion that the transitions of this pigment-protein complex are oriented perpendicular to the face of the thylakoid membrane. However, the light-harvesting complex peaking at 675 nm is reported to show maximum orientation at 682 nm [3].

By changing from the face to edge position the chloroplast obviously controls the relationship between PS I and PS II activity. With respect to red light absorption, the face and edge positions correspond to PS I and PS II positions respectively; or the edge position may correspond to a PS I + PS II position or to a light-harvesting complex position. According to the view [35] that differences in the orientation of Chl *a* 670 (PS II) and Chl *a* 685 (PS I) may control the energy transfer from PS II to PS I, we assume a similar control for the energy transfer from fraction II pigment (PS II) to that of fraction I (PS I).

Contrary to the orientation of Chl *a* (fractions I and II) the molecules of Chl *b* seem to be oriented relatively poorly. Similar differences regarding the dichroism of Chl *a* and Chl *b* have been reported in earlier investigations [10,29,30]. However, this assumption of a small degree of orientation of Chl *b* may be in need of correction: differently oriented forms of Chl *b*, as reported recently to be associated with the light-harvesting complex and with the antenna of PS I, respectively [3], may have simulated the weak orientation of Chl *b*.

Because of the apparent weak orientation of Chl *b* in the present study, the dichroism of the chloroplast has small values around 650 nm, however, it increases with wavelengths up to about 680 nm. The overlapping of the dichroism of fraction I pigment and fraction II pigment causes the total dichroism of the chloroplast to remain between 30 and 40% (relative dichroism). These values appear at higher wavelengths where dichroism is induced predominantly by fraction I. A similar increase in dichroism occurring with increasing wavelength between 650 and 680 nm has been observed by other authors [6,8,32].

The dichroism of the *Mesotaenium* chloroplast in the present study is very strong when compared to the values obtained earlier [24,30]. The fast computer-controlled measurements may account for this difference. One cannot exclude the possibility that under the present experimental conditions, nearly 100% of the total chlorophyll was in the state of orientation. The values of relative dichroism between 30 and 40% may be caused by the quenching of dichroism due to overlapping of the two fractions of oriented chlorophyll.

However, the results of photometric measurements of the chloroplasts of living cells must be examined also for effects of light scattering [22] and the so-called form dichroism [12,23]. Two kinds of light scattering can occur: scattering with non-selective and selective dispersion. The effects of non-selective scattering strongly depend on light wavelength, the number of quanta decreasing with the fourth power. This kind of wavelength dependence, characteristic of light scattering, is not seen in the absorption differences shown in Figs. 1 and 2A. Moreover, a regression analysis on *Mesotaenium* chloroplast (unpublished data) estimated a 9-fold reduction of scattering between 550 and 670 nm from 45 to 5% of the total absorption. Furthermore, the chloroplast of *Mesotaenium* is very transparent, provided the alga has been grown under low-light conditions, the same being true for the chloroplast of *Mougeotia* [12], but not for the granal chloroplasts of higher plants. Concluding it can be argued that scattering visible during microscopical observation is very small and does not disturb the measurements.

The selective scattering is shown to peak at 703 nm [2,27] for intact chloroplasts, but the peak shifts to 690 and 682 nm for non-intact granal and non-granal chloroplasts, respectively [2]. Therefore, the major part of the 'in vivo dichroism' of *Mesotaenium*, presented in the shorter red-wavelength range (Fig. 2A), cannot be deduced from light scattering. Only the peaks of dichroism, shown in Fig. 2B at 705 nm, may be caused by selective scattering. Moreover, the two curves (0 and 90°) measured at the face position and the one taken at the edge position (0°) of Fig. 2A are exactly the same or similar, respectively, which is in contrast to those expected if absorption changes were induced by different amounts of light scattering in the two positions of the chloroplast. This

observation confirms the assumption that visible dichroism is not influenced to any appreciable extent by light scattering.

Form dichroism is a well known property of the living chloroplast [12,13,23] as it is a composite body [33]. Like the respective types of birefringence, the form and intrinsic dichroism are not distinguishable from each other in the living chloroplast [12]. Therefore, the dichroic effects reported in this paper may depend on form dichroism, which means that these effects may be due to the arrangement of chloroplast layers with unoriented pigment alternating with non-pigmented layers of a different refractive index. However, one has to assume that the form dichroism of the chloroplast should reach nearly the same extent in blue as well as in red light, provided the absorptions in these two regions are the same. After all, the measured dichroism of the *Mesotaenium* chloroplast, had it been a form dichroism, should have attained greater values in blue than in red light. Our measurements, however, show the opposite, considerably higher dichroism in the red than in blue light. Therefore, there can be no doubt that the pigment in the living chloroplast of *Mesotaenium* is oriented, at least in part, which leads to the conclusion that the chloroplast must be dichroic in the sense of intrinsic dichroism.

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