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DICHROISM OF TRANSIENT ABSORBANCE CHANGES IN THE RED SPECTRAL REGION USING ORIENTED CHLOROPLASTS

I. FIELD INDICATING ABSORBANCE CHANGES

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SUMMARY

The light-induced transient absorbance changes which are affected by valinomycin have been studied using magnetically oriented spinach chloroplasts and a polarized measuring beam. The ΔA spectra for the two polarizations parallel and perpendicular to the plane of the photosynthetic membranes have been recorded in the spectral range 630–750 nm. Large polarization effects are found in all the bands of the ΔA spectrum, shifts in the position of the extrema are observed and the two spectra cross each other at various wavelengths. A comparison of these spectral features with available data on the dichroism of the Stark effect on monomolecular films of chlorophyll *a* and *b* indicates similarities favoring the already well documented hypothesis of the electrochromic nature of these absorbance changes in vivo.

The data on this electrochromic effect can be correlated with the linear dichroism of oriented chloroplasts and the $\Delta A_{//}-\Delta A_{\perp}$ spectrum in the 645–655 nm region gives further evidence of the orientation out of the membrane plane of the red transition moment of chlorophyll *b*.

INTRODUCTION

Transient absorbance changes observed when a suspension of chloroplasts is excited by a flash of light have been attributed to an electrochromism due to a light-induced electric field originating from the charge separation occurring in the reaction centers [1]. After delocalization of these electric charges over the thylakoid, the membrane behaves as a charged capacitor and a Stark effect on the photosynthetic pigments is observed. Several lines of supporting evidence for this interpretation have been obtained mainly through (i) the very rapid rise-time of the transient [2], linking it to a primary photochemical process, (ii) the concentration dependent effect of antibiotics collapsing the field by increasing the ionic permeability of the membrane [3], (iii) the similarities of the in vivo spectrum and of the electrochromic spectrum obtained with monolayers of photosynthetic pigments [4] and (iiii) the photo-EMF

experiments showing movements of charges at the thylakoid surface [5, 6]. The wavelength dependence of the magnitude of this transient presents a broad maximum around 515 nm (explaining why this absorbance change is often named "515 effect"), a minimum at 475 nm and a complex set of bands in the red spectral region [3].

From polarized light spectroscopy on oriented chloroplasts, it has been recently shown [7–10] that the degree of orientation of the bulk of the pigments with respect to the plane of the photosynthetic membrane is considerably higher than was previously thought [7–10]. Furthermore, the possibility to orient chloroplasts in a magnetic field [8–9] under physiological conditions and with convenient geometry for optical measurements in polarized light had led us to study the dichroism of some already known transient absorbance changes [11–14], in order to get information both on the orientation of the pigments undergoing the absorbance changes and on the mechanisms underlying these changes. In a previous report [11], we have described the dichroism of the electrochromism in the spectral range 450–540 nm using magnetically oriented chloroplasts. Although polarization effects were detected, the conclusions that we could derive from this study were somehow limited because the overlap of the absorption bands of different species precluded any precise assignment of the signal observed at a particular wavelength and also because no data on the polarization of the Stark effect on carotenoid molecules were available.

In these two respects the red region seemed more promising: the Q_y transition moments of chlorophyll *b* and of the different chlorophyll *a* forms give rise to successive absorption bands whose orientations have been estimated by linear dichroism [7, 14] and data on the polarization of the Stark effect on chlorophyll monolayers are already published [15]. These facts and also the possibility to study under very similar conditions the *P*-700 absorbance changes (see accompanying paper, [16]) led us to investigate the dichroism of the light induced field indicating transient absorbance changes on magnetically oriented chloroplasts in the 630–750 nm spectral range.

MATERIALS AND METHODS

Freshly harvested leaves from young (3–6 weeks) spinach plants were chopped for 5 s in a sucrose (0.4 M)/Tris(20 mM, pH 8)/KCl (20 mM) buffer using a low speed blendor. The homogenate, filtered through nylon cloth (30 μm mesh) was centrifugated at $1000 \times g$ for 1 min. The upper fraction of the pellet was resuspended in the same buffer to which 5 % Ficoll was added to prevent settling of the chloroplast. Valinomycin (10^{-6} M) was added as necessary. All the measurements were performed at approx. 5 °C with a chlorophyll concentration of $2 \cdot 10^{-5}$ M in a 8×8 mm cuvette.

The experimental arrangement is outlined in Fig. 1. A 10 kG magnetic field was applied to the suspension of chloroplasts which align with the plane of the photosynthetic membranes perpendicular to the direction of the magnetic field. Saturating excitation was provided by flashes from a tunable dye laser (model 23, Electro Photonics Ldt.) operated near the maximum of Rhodamin 6 G (around 600 nm). The flashes, 500 ns in duration and 10^{-2} nm in half-band width, were fired at a frequency of 2 Hz. The measuring beam (half-band width 2 nm), the intensity of which was kept low enough to avoid any noticeable effect on the magnitude of the absorbance changes, was polarized before impinging on the sample by a Rochon polarizer secured in a hand rotating mount. The phototube (Hamamatsu R 712), magnetically

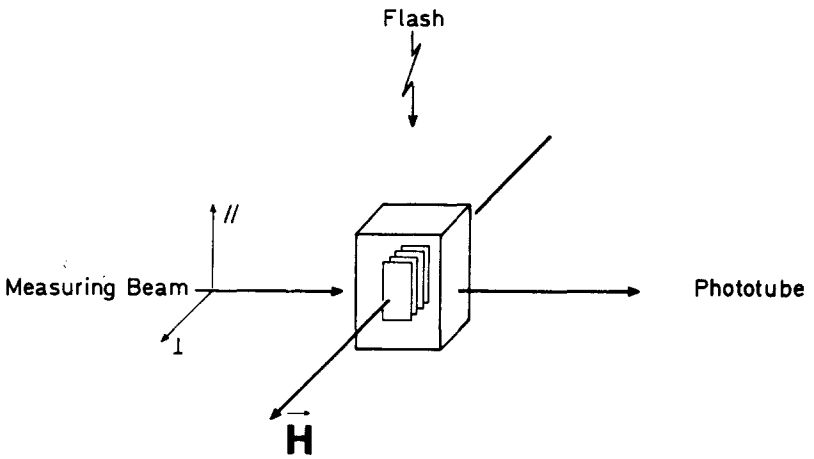

Fig. 1. A schematic representation of the spatial position of the cuvette, of the measuring beam, of the excitation flash and of the magnetic field $\vec{H}$. The small rectangular slabs inside the cuvette represent the stacked photosynthetic membranes.

shielded, was located 40 cm from the cuvette and protected by a narrow (usually 3 nm half-band width) interference filter and a selected Schott blocking filter. An adjustable aperture was positioned between the sample and the detector to minimize the collection of fluorescence light. The output of the phototube, amplified through a differential amplifier (type 127, Tektronix) was fed into a signal averager (Didac 800, Intertechnique) with a resolution set at 2.5 ms. The time constant was 0.2 ms.

RESULTS

400 transients were averaged at each wavelength and for both polarizations of the measuring beam. A measurement at any particular wavelength was always compared to a reference measurement at 650 nm on the same sample. 4–8 measurements were taken on a given sample before a modification in the amplitude and/or in the dichroic ratio would indicate that the sample has to be changed.

Such a series of measurements performed with untreated chloroplasts was compared to an identical series obtained with valinomycin treated chloroplasts. At the rather high concentration (10^{-6} M) of valinomycin used, the transient decayed much more rapidly so that it was no longer resolved on the time-scale of our measurement [17]. For one particular wavelength and a given polarization, the difference between the signals obtained with untreated and valinomycin treated chloroplasts gives an amplitude value which is plotted versus wavelength in Fig. 2.

As a check, the decay half-time of the transient (100–250 ms depending on the chloroplast preparation) obtained at every wavelength was compared to the one obtained at 515 nm; no significant difference was observed when the effect of the transient remaining after valinomycin treatment was taken into account. This residual absorbance change is described in the accompanying paper [16].

Using oriented chloroplasts, large polarization effects are observed in all the bands of the spectrum with either $|\Delta A_{//}| > |\Delta A_{\perp}|$ (around 650, 670, 680, 700 nm) or the

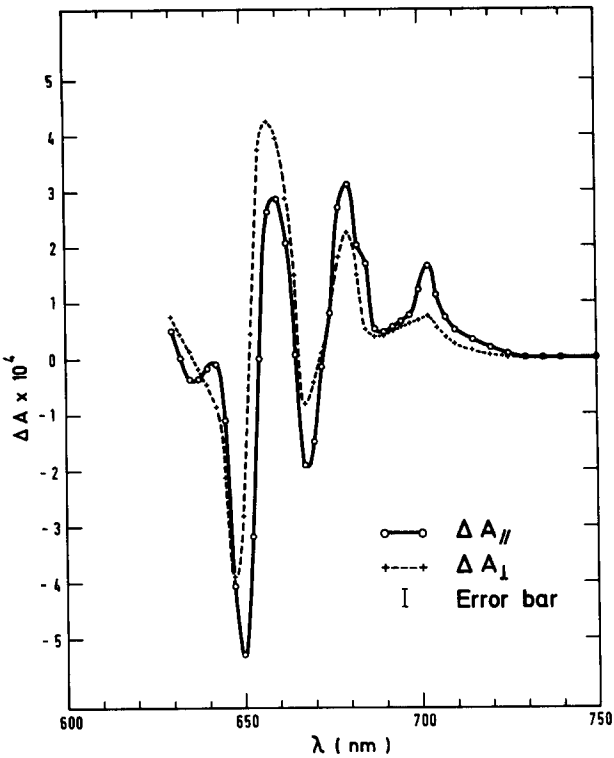

Fig. 2. Wavelength dependence of the field indicating absorbance changes on magnetically oriented spinach chloroplasts for the two polarizations of the measuring beam. Negative values for ΔA correspond to a decrease in absorbance.

reverse (around 640, 660 nm). The maximum dichroic ratio $D = \Delta A_{//}/\Delta A_{\perp}$ observed at 650 nm was 1.9 ± 0.1. Under the same conditions, $D = 1.35$ at 515 nm which corresponds to $p = \Delta A_{//} - \Delta A_{\perp}/\Delta A_{//} + \Delta A_{\perp}$ of 15 %, a value very close to the one obtained in a previous study using a different apparatus [11]. At some wavelengths (e.g. 654 nm) $2\,\Delta A_{//}$ and $\Delta A_{\perp}$ are of equal amplitudes but opposite signs; with unoriented chloroplasts, no field indicating transient is observed at such wavelengths. In the chlorophyll *b* absorbing region near 650 nm $\Delta A_{//}$ extrema are red shifted by approximately 3 nm as compared to the $\Delta A_{\perp}$ extrema. Finally $\Delta A_{//}$ and $\Delta A_{\perp}$ have the same magnitudes and signs at several wavelengths around 638, 647, 675 and 690 nm.

DISCUSSION

The ΔA spectrum for unoriented chloroplasts can be estimated from data presented in Fig. 2 by adding $2\Delta A_{//} + \Delta A_{\perp}$ [9]. Such a calculated spectrum closely resembles the one obtained by Emrich et al. [3], using broken chloroplasts. Furthermore, the accelerating effect of valinomycin and the identical kinetics of the transients at 515 nm and in the red bands when considering the untreated minus valinomycin treated chloroplasts signals indicate that the transients described here correspond to the electrochromic effect [1–3].

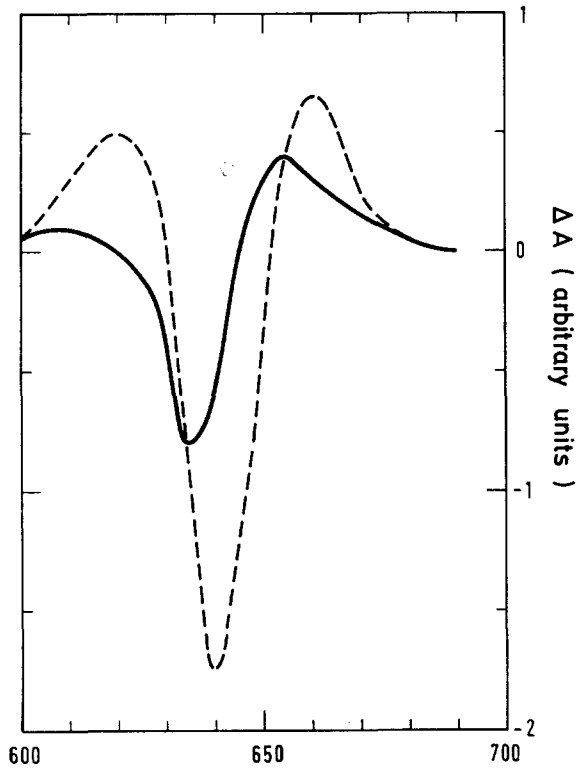

Fig. 3. Quadratic Stark effect spectra on a monomolecular layer of chlorophyll *b* obtained with a polarized measuring beam. The two spectra correspond to different polarizations for which the electric vector of the measuring beam has a component parallel (continuous line) or perpendicular (dashed line) to the plane of the monolayer. Spectra redrawn from Kleuser and Bücher work [15].

Kleuser and Bücher [15] have studied the Stark effect using monolayers of chlorophyll *a* and chlorophyll *b* as a function of the polarization of the analyzing beam. Their study was conducted at liquid nitrogen temperature, but there does not seem to be any large temperature effect on the overall shape of the spectrum (compare Fig. 3 redrawn from ref. 15 with Fig. 1 b of ref. 4). The spectrum in Fig. 3 and those of ref. 15 show (i) very large polarization effects, (ii) shifts in the position of the extrema and (iii) differences in the shapes of the spectrum for the two polarizations, inducing crossings of the two spectra at several wavelengths different from the zero-line crossing positions. From a qualitative point of view, this behaviour is very similar to what is observed in the polarization spectra of in vivo chlorophyll (Fig. 2). We will take this observation as further evidence that the absorbance changes discussed here are linked to a Stark effect.

However, discrepancies exist between in vivo and in vitro spectra when analyzing the relative signs and magnitudes of the polarization effects. We think that these discrepancies can be best explained in terms of different orientations of the transition moments of the absorbing species with respect to the membrane plane and to the applied electric field.

As a first approximation, we can describe a Stark effect on a pigment as a shift

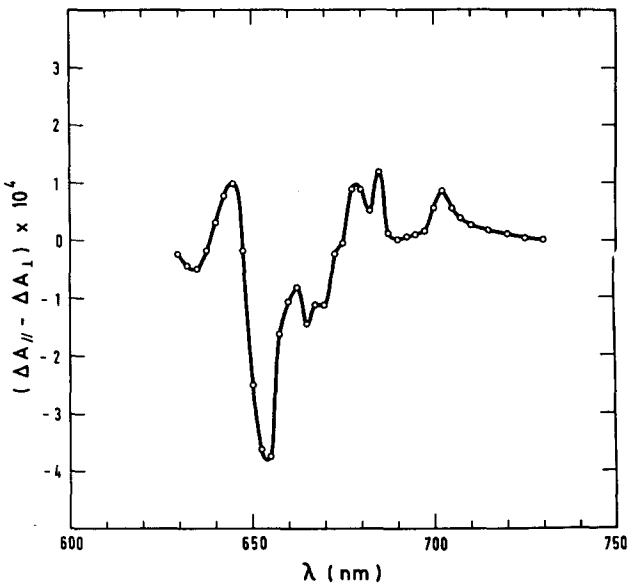

Fig. 4. Difference spectrum $\Delta A_{//} - \Delta A_{\perp}$ obtained from the data plotted in Fig. 2.

in frequencies of the absorption band of this pigment. In vivo the observed shift is directed towards the red [18], so the shape of the ΔA spectrum will be a derivative of the absorption band [4] with ΔA positive towards the long wavelengths. If we now consider a set of absorbing oscillators oriented with respect to a plane, they will present a linear dichroism signal $A_{//} - A_{\perp}$ which has the shape of the absorption band but the sign of $A_{//} - A_{\perp}$ will depend upon the orientation of the oscillators with respect to the plane [9]. If the oscillators are close to the plane (between 0 and 35° of the plane), $A_{//} - A_{\perp}$ will be positive and $\Delta A_{//} - \Delta A_{\perp}$ will be a derivative of the absorption band with the positive signal towards the long wavelengths. However if the oscillators are tilted out of the plane by more than 35°, $A_{//} - A_{\perp}$ will be negative and the derivative $\Delta A_{//} - \Delta A_{\perp}$ will be negative towards the long wavelengths.

In the chlorophyll *b* absorbing region around 650 nm, the field-indicating absorbance changes are well resolved and the overlapping of the absorption bands is smaller than for the region of absorption of chlorophyll *a*. The $\Delta A_{//} - \Delta A_{\perp}$ spectrum drawn in Fig. 4 indicates that the sign of the derivative signal centered around 649 nm is negative towards the long wavelengths. From the arguments described above, it can be concluded that the red transition moments of the chlorophyll *b* molecules are tilted out of the membrane plane by an angle greater than 35°. The same conclusion has been drawn independently from comparison of the linear dichroism spectra of normal and chlorophyll *b*-less mutant of barley (S. Demeter, personal communication) and from polarized absorption spectroscopy on oriented spinach chloroplasts at low temperature [14].

In the 660–710 nm region, there is an overlap of the absorption bands of the different chlorophyll *a* forms and Fig. 4 cannot be used to analyze the orientation of these species. A rigorous treatment of the Stark effect must take into account the

differences in (i) the permanent dipole moment and (ii) the polarizability between the excited and ground states of the molecules submitted to the electric field [14]. However, we can use the magnitude of the ΔA signals for different chlorophyll forms as a qualitative estimation of the interaction between the electric field and the photosynthetic pigments. The magnitude of the ΔA signal (Fig. 2) does not follow the absorption curve. It is very large in the chlorophyll *b* absorbing region, because the delocalized electric field which is directed perpendicularly to the membrane plane can strongly modify the frequencies of the chlorophyll *b* molecules oriented also rather perpendicular to the membrane. It is also appreciable in the 665–675 nm region, where the chlorophyll *a* oscillators are known to be either at random or close to the "magic angle" of 35° [7]. In the 675–720 nm region, the ΔA signals are small compared to the absorption, an observation that indicates a small effect of the electric field on the transition dipoles absorbing in this spectral range. From linear dichroism and polarized fluorescence spectroscopy, it has been established that these oscillators are oriented very close to the membrane plane [7–10].

Although there is a good correlation between the linear dichroism of the pigments and the data on the electrochromic effect obtained with oriented chloroplasts, more quantitative conclusions could be derived from those spectra by mathematical analysis and by the introduction of physical parameters. As an example, it can be calculated that a distribution of orientation close to the magic angle (35°) of the chlorophyll *a* oscillators absorbing around 670 nm fits better the experimental results than a random distribution of these oscillators. Such calculations are beyond the scope of this article and will be described elsewhere (G. Paillotin and J. Breton, in preparation).

Finally it is interesting to note that the magnitude of the ΔA signals in the carotenoid region is much larger for chromatophores of photosynthetic bacteria [18] than for chloroplasts [3, 11] (by an order of magnitude even when taking into account the residual absorption of the chlorophylls around 515 in chloroplasts). This is relevant to the observation that carotenoids in higher plants are rather planar to the membrane plane [7], in contrast to the out of plane orientation of the carotenoids of photosynthetic bacteria [19]. Such a difference in orientation would led to an effect of the electric field on the transition dipole moments of the carotenoids much greater for bacteria than for chloroplasts. In this respect , the measurement of the dichroism of the field-indicating absorbance changes on oriented material from photosynthetic bacteria seems worthwhile.

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