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PROTOCHLOROPHYLL FORMS WITH DIFFERENT MOLECULAR ARRANGEMENTS

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Summary

Spectral properties of protochlorophyll (PChl) forms were investigated in solid-film model systems by absorption. fluorescence and circular dichroism (CD) spectroscopy. The solid films were prepared from diethyl ether solution of PChl on a cover glass surface by evaporation of the solvent. After preparation the films usually showed an absorption maximum at 635 nm or in some cases at 640 nm. The PChl form with 635 nm absorption maximum had no CD signal, whilst the films with absorption maximum at 640 nm gave an intense negative CD band at about 640 nm and a positive one at 668 nm. The treatment of the films with ammonia or acetone vapour resulted in a red shift of the absorption maximum from 635 nm or 640 nm to 650 nm. The study of the CD spectra of the films with different PChl forms showed that, depending on the treatment, forms of PChl with similar absorption and fluorescence spectra, but with opposite CD signals, can exist. It is suggested that the differences of the CD spectra are mainly due to different arrangements of the aggregates.

Introduction

A large body of data derived from experiments utilizing different spectroscopic methods has led to the conclusion that PChl and/or PChlide form different complexes interacting with one another and with the lipoprotein mole-

Abbreviations: PChl, protochlorophyll; PChlide, protochlorophyllide; $PChl_{635}$, $PChl_{640}$, $PChl_{650}$, protochlorophyll form with absorption maximum at 635, 640 or 650 nm, respectively; CD, circular dichroism.

cules of the etioplast membranes [1,2].These complexes are called 'forms' and are characterized by their spectroscopic properties: red absorption and/or fluorescence maxima. The forms observed in etiolated leaves have absorption maxima at 628—630, 635—637 and 650 nm [3,4]. Treatment of the etiolated leaves with δ-amino levulinic acid results in the appearance of a form absorbing at about 640 nm [5]. These forms show maxima in the low-temperature fluorescence spectrum of etiolated leaves at 633—635, 640—645 and 655 nm, and there are also bands at 675 and 705 nm [6,7]. The isolated PChl or PChlide lipoprotein complexes have an absorption maximum at 639—644 nm [8,9] and a fluorescence band at 640—652 nm [10,11]. These well-known forms were also found in the pumpkin seed coat, in the absorption spectrum of which peaks at 635 and 650 nm can be found [12,13].

To study the nature and structural properties of the PChl and PChlide forms, several authors have experimented with different model systems of PChl pigments. Using these systems, it is possible to investigate the nature of the pigment forms with sensitive spectroscopic methods, such as infra-red, EPR, NMR, CD and so forth. In non-polar solvents the red absorption maximum (the $Q_{y(O-O)}$ electronic transition band) of the PChl pigments was found to be red-shifted and the concentration of the pigment and the properties of the solvent are involved in this shift. In diethyl ether solution of PChl the $Q_{y(O-O)}$ band appeared at 622 nm [14,15]; in dry CCl_4 solution, however, it was shifted to 624 nm [15] or to 630 nm [16]. Similarly, a red-shifted absorption maximum of PChl was found in the case of other non-polar solvents such as benzene, cyclohexane or chloroform [16]. In mixed water/dioxan solutions this maximum appeared at 626, 629, 633, 638 or 645 nm, depending on the ratio of water to dioxan. The low-temperature fluorescence spectra of these same water/dioxan solutions showed maxima at 632, 639, 640, 642 and 646 nm, respectively [17]. A pyridine solution of PChl was found to have an absorption maximum at 634 nm [15] and fluorescence maxima at 640 and 700 nm at 20°C and 655, 690 and 700 nm at −120°C [18]. PChl forms, having similar to in vivo spectroscopic properties, could be prepared in solid films by treatment of the films with vapour of different solvents: the forms prepared in this way exhibited absorption maxima at 635, 645, 650 and 675—680 nm and fluorescence maxima at 640, 655, 685 nm and several long-wavelength bands at about 700 nm [19,20].

The main pigment of etiolated leaves, PChlide, showed strong aggregation properties. Forms of PChlide with absorption maxima at 628 nm in methanol [14,15], at 650—655 nm in dry chloroform, benzene and liquid paraffin [21, 22] were prepared. The fluorescence spectrum of the liquid paraffin solution of PChlide showed maxima at 633 and 690 nm [21].

In infra-red spectroscopy, the existence of a band at 1668 cm^{-1} provided clear evidence that the forms of PChl and PChlide with red-shifted absorption and fluorescence maxima are aggregates of pigment molecules interacting via the C-9 ketone group of one molecule with the central Mg atom of the another [16,23,24]. The long-wavelength forms were found to be 'hydrated' aggregates in which water molecules connect the porphyrin rings [24].

These data are compatible with the results of the CD spectroscopy measurements. While the PChl in the monomeric state in diethyl ether solution had a

positive CD signal at 620 nm [15], the $PChl_{624}$ had a large negative Cotton effect in CCl_4 solution [15,16], the $PChl_{637}$ showed a positive Cotton effect in water/dioxane solution [17], and, in the CD spectrum of the $PChlide_{650}$ in liquid paraffin solution, a large negative Cotton effect (with a negative signal at 655 nm) was found [25]. The CD spectrum of the PChl or PChlide holochrome showed negative bands in the vicinity of 613, 635 and 647 nm [9,11]. $PChl_{650}$ exhibited a positive CD signal of high intensity at about 650 nm in the CD spectrum of the pumpkin seed coat [13]. The strong Cotton effects and CD signals observed in the CD spectra of the above mentioned systems suggest a molecular-exciton type interaction between the pigment molecules [26]. On the basis of the shape of the CD signals, the arrangement of the aggregates can be elucidated [27]. Using these data, together with the data of NMR spectroscopy, models of PChl dimers (absorbing at 624—628 nm) have been constructed [15,16].

Despite abundant experimental data, many questions remain open concerning the structure and aggregation properties of the in vivo and in vitro PChl and PChlide forms.

The present work was undertaken to investigate the following problems: (1) to prepare PChl forms with similar spectroscopic properties to in vivo PChlide forms; (2) to study the fluorescence properties of the different PChl forms, and, (3) to investigate the arrangement of PChl aggegrates by CD spectroscopy.

Materials and Methods

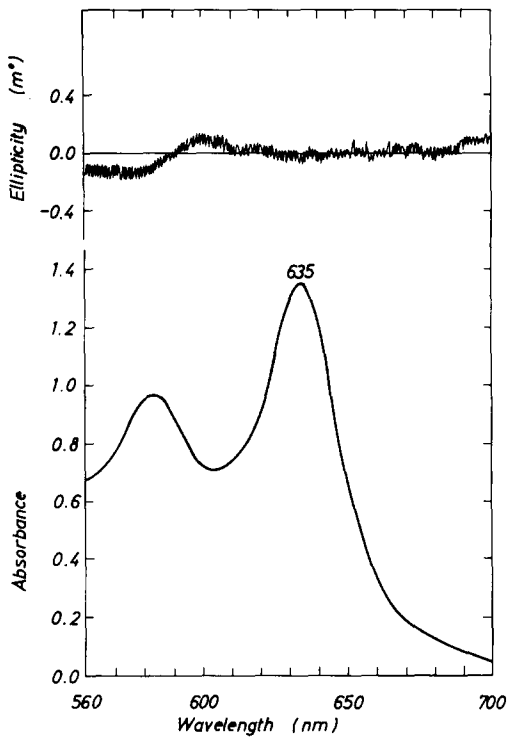
For preparation of solid films PChl was isolated from pumpkin seed coats and was purified by sugar column chromatography [23]. Solid films were prepared on cover glass by evaporation of a diethyl ether solution of PChl; traces of the solvent were removed in vacuum. In several cases the solid films were then treated with acetone vapour or ammonia in a Thunberg tube.

Absorption spectra were measured with an Unicam SP 1800 spectrophotometer. Fluorescence spectra were recorded with a spectrofluorometer consisting of a Hilger and Watts grating monochromator with motor drive, a FEU 38 photomultiplier and a Kipp and Zonen BD 5 recorder. Excitation was carried out with a high-pressure mercury lamp with cut-off filters. The excitation wavelengths were 405 and 436 nm. The CD spectra were recorded with a Jasco J 40 spectropolarimeter. CD was measured in terms of ellipticity (Θ), in units of millidegrees (mdeg). In all cases, the CD spectra were registered directly at the photomultiplier. The anisotropic properties of the samples were measured with a Jasco 5 spectropolarimeter. The samples were fixed on a rotatable mount, and the ORD spectra were recorded at three different angles. These measurements showed that the spectra were rotation invariant.

Results

The region of the $Q_{y(0-0)}$ and $Q_{x(0-0)}$ electronic transitions were studied in the absorption and CD spectra of the PChl solid films.

The solid films usually had the absorption maximum at 635 nm after the evaporation of the diethyl ether. This form of PChl showed no CD signal, even at the highest sensitivity of the spectropolarimeter (Fig. 1). In some cases, the



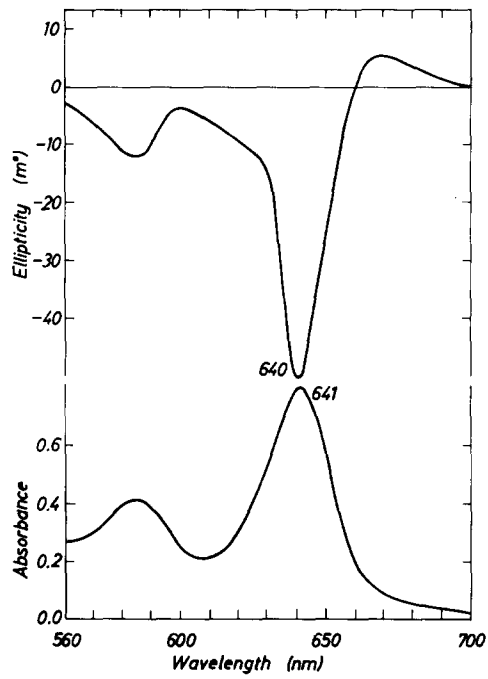
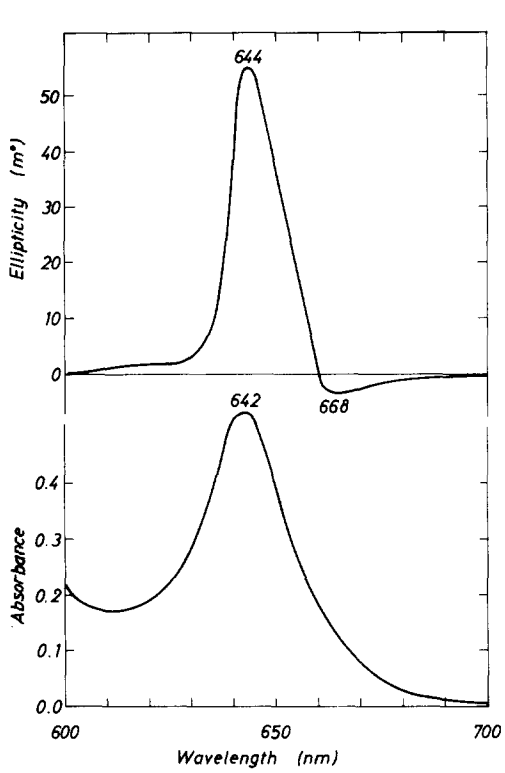


Fig. 1. Absorption and circular dichroism spectra of PChl solid films immediately after preparation.

Fig. 2. Absorption and circular dichroism spectra of PChl solid films with spontaneously red-shifted absorption maximum.

solid films exhibited the main absorption maximum at about 640 nm, instead of 635 nm after the preparation. These films had a characteristic and very intense CD signal: there was a large negative asymmetric band at 640 nm and a positive one of lower intensity around 668 nm (Fig. 2). Often, the films with absorption maximum at 635 nm had a more-or-less asymmetric absorption band, and CD signals with absorption maximum at 640 nm. This refers to the simultaneous existence of the two forms in these films.

The treatment of films containing only $PChl_{635}$ with ammonia vapour resulted in a rapid shift of the 635 nm absorption maximum to 640—642 nm. The CD spectra of the treated films showed an intense positive band at 644 nm and a negative one, of lower intensity, at 668 nm (Fig. 3). Treatment of the films of $PChl_{640}$ for a longer time (about 60 min) with ammonia vapour resulted in transformation of $PChl_{640}$ into $PChl_{650}$. A similar transformation was observed in the case of treatment of $PChl_{635}$ or $PChl_{640}$ with acetone vapour. After treatment, the main absorption maximum appeared at about 650 nm and there was a shoulder around 635 nm. This absorption spectrum is very similar to the absorption spectra of etiolated leaves and pumpkin seed coats. In the CD spectra of the films with absorption maximum at about 650 nm, a very intense positive band at 654 nm and two negative signals of lower intensity at 632 and 644 nm were found (Fig. 4). The latter films were stable; their spectroscopic



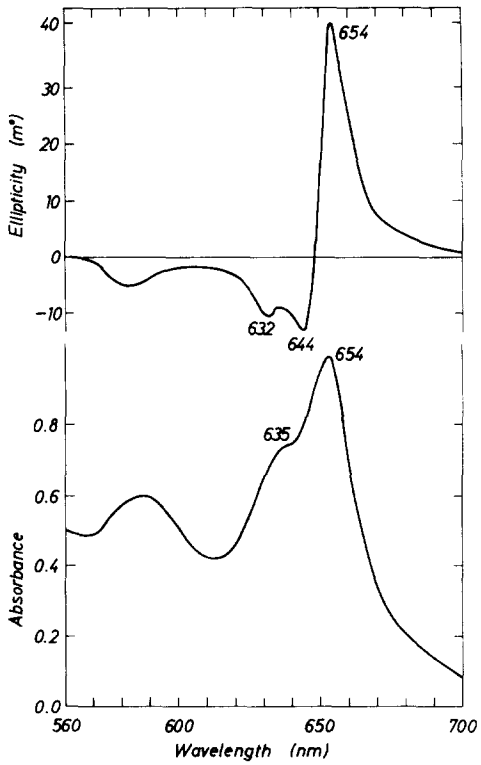


Fig. 3. Absorption and circular dichroism spectra of PChl solid films treated with ammonia for 5 min.

Fig. 4. Absorption and circular dichroism spectra of PChl solid films treated with vapour of acetone for 45 min.

properties were changed only by strong disaggregation effects.

The spectra presented in Fig. 4 demonstrate the final state of the film after acetone treatment. During the treatment, however, a $PChl_{650}$ form with a negative CD signal at about 650 nm was found as an intermediary product of the transformation (Fig. 5). This negative CD band disappeared during further treatment and a positive maximum at 654 nm developed (Fig. 4).

For more exact identification of the PChl forms in the solid films, we recorded the low-temperature fluorescence spectra of the films. All types of film showed strongly red-shifted bands in their fluorescence spectra which are due to the so-called crystallic forms of PChl, and can probably arise as in chlorophyll microcrystals [20]. The film with an absorption maximum at 635 nm had the main fluorescence peak at 675 nm, and there were two smaller bands at 625 and 720 nm (Fig. 6A). The film with $PChl_{640}$ showed the main fluorescence maximum at 710 nm, and it had several fluorescence bands of lower intensity at 625, 635 and 675 nm (Fig. 6B). The fluorescence spectra of the spontaneously red-shifted and ammonia-treated films having absorption maximum at 640 nm were practically identical. The films with $PChl_{650}$ exhibited the main fluorescence band at 705 nm, and there were smaller peaks in the spectrum at 625 and at about 680 nm (Fig. 6C).

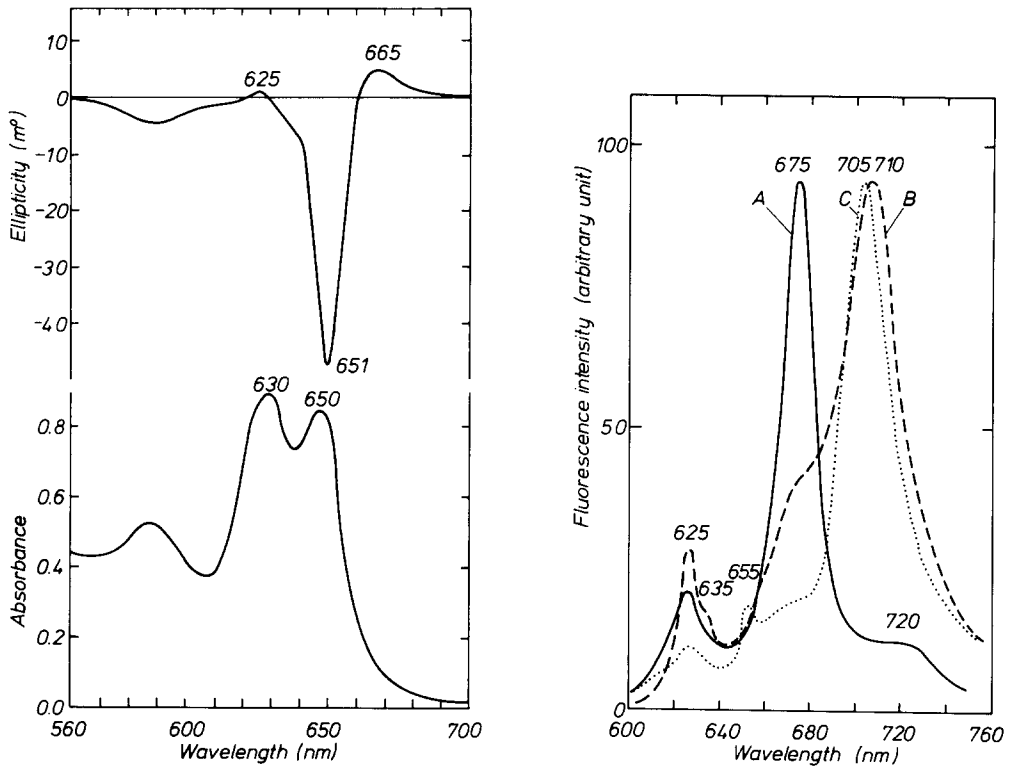


Fig. 5. Absorption and circular dichroism spectra of PChl solid films with intermediary $PChl_{650}$ appeared by acetone vapour treatment of the films for 2 min.

Fig. 6. Low-temperature fluorescence emission spectra of solid films containing different forms of PChl: A, (———) with absorption maximum at 635 nm; B, (- - - - - -) film with absorption maximum at about 640 nm; C, (· · · · · ·) film with absorption maximum at about 650 nm.

Discussion

There is good evidence for the existence of PChl and PChlide in different forms in vivo. In solid-film model systems we could prepare forms of PChl with spectroscopic properties similar to that in vivo. On the basis of these similarities it is tempting to assume that the forms of PChl prepared in solid films are good models for the in vivo PChl and PChlide forms.

Whereas the solid films showed absorption spectra similar to that of the etiolated leaves, there are significant differences in the fluorescence spectra: in the fluorescence spectra of the solid films the long-wavelength bands at 675, 705 and 710 nm are dominating. It can be concluded that the solid films contain a small amount of long-wavelength forms of PChl, and there is an intense energy migration to them. It may be possible that etiolated leaves also contain a very small amount of the long-wavelength form of PChl or PChlide, but the efficiency of the energy migration is very low. Therefore, these bands at 675 and 705 nm show low intensity in the fluorescence spectra of etiolated leaves.

The results presented in this paper and earlier data indicate that the different

CD spectra of the solid films are due to different forms of PChl, and the different PChl forms do have different CD spectra. We suggest that the differences in the CD spectra of the solid films can be caused by the superimposition of several factors. The position and the large intensity of the CD signals are obvious evidence for degenerate pigment-pigment interactions [26,27]. The large asymmetry of the Cotton effects of the CD spectra can be caused by overlapping of CD signals of several forms of PChl existing together in the same film. The shape of the Cotton effect is strongly determined also by the intrinsic geometry of the pigment aggregates, because the angle and the distance between the transition electric and transition magnetic moment vectors of interacting molecules play an important role in the optical properties of the molecular complexes. A very important factor is also the direction of these vectors in relation to each other [26—29]. Thus, the arrangement of the aggregates is important in determining the shape of the CD signals in our systems.

Comparing our data with earlier results, we think that the data of the present work permit the following conclusions. After the evaporation of diethyl ether, a red shift of the absorption maximum to 635 nm was obtained, which is probably due to the interactions between the PChl molecules. The interactions existing in films were explained as 'non-specific π-π interactions' [30]. This type of interaction may play an important role in producing the 635 nm PChl form. The transition (or transitions) giving the absorption band at 635 nm were found to be optically inactive. Similarly, optically-inactive transitions of chlorophyll *a* complexes were observed in Nujol films [31]. No explanation of these phenomena is yet known.

It seems likely that, often specific, (C-9 ketone-Mg) interactions also can be formed immediately after evaporation of the diethyl ether. These 'accidental' specific interactions are thought to cause the appearance of the $PChl_{640}$ with a negative CD signal. By ammonia or acetone vapour treatment of the films with $PChl_{635}$, the π-π interactions disappear and the molecules of PChl can link to each other via (C-9 ketone-Mg) interactions, which form aggregates with definite geometry [15]. Treatment of the $PChl_{635}$ with ammonia probably resulted in an aggregate with a similar degree of aggregation to the spontaneously formed $PChl_{640}$. We assume that the degree of aggregation of the $PChl_{650}$ with negative CD signal is also similar to that of the $PChl_{650}$ with positive CD signal.

The differences in the spectroscopic properties of the $PChl_{640}$ and $PChl_{650}$ forms can be interpreted in two ways: (1) $PChl_{650}$ is a higher aggregate than the $PChl_{640}$, (2) these aggregates have a similar degree of aggregation but other molecules (acetone, ammonia or water) play an important role in forming the aggregate; and the differences in spectroscopic properties are due to the differences in the type of interaction of these molecules with the aggregated PChl molecules.

Our data demonstrate that two different PChl forms can exist with similar absorption and fluorescence properties; they exhibit, however, opposite CD signals. For example, we found $PChl_{640}$ or $PChl_{650}$ forms with opposite (with positive or negative) main CD signals in a similar position. The opposite CD signs refer to the different arrangement of the molecules in the aggregates. As the absorption and fluorescence properties depend mainly on the size of an aggre-

gate, it is suggested that a form of PChl of similar degree of aggregation can be formed with two different geometries. In our opinion, these aggregates cannot be taken as one and the same form. In all probability, other pigment forms exist with two different geometries.

Our results prove that for the exact characterization of different forms of pigments, other spectroscopic properties also have to be considered, besides the absorption and fluorescence characteristics.

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