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Spectroscopic forms of divinylprotochlorophyllide in solid films

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Solid films were prepared from diethyl ether solutions of divinylprotochlorophyllide on glass surfaces and then treated with vapours of acetone. The transformation of the films and the appearance of different spectroscopic forms of divinylprotochlorophyllide were observed by absorption spectroscopy. Computer analysis allowed resolution of the absorption spectra into forms with maxima in the red region at 628–630, 635, 644, 650, 655, 665 and 676 nm. These Gaussian peak positions are very similar to those of protochlorophyllide species described in etiolated leaves (Litvin, F.F. and Stadnitshuk, I.N. (1980) Fiz. Rast. (Moscow) 27, 1024–1030, in Russian) or of protochlorophyll forms found in solid films (Böddi, B., Rákász, É. and Láng, F. (1983) Photobiochem. Photobiophys. 5, 27–33). This indicates a similarity in the electronic structures of these pigment aggregates and shows that the additional vinyl group in the divinylprotochlorophyllide does not modify this structure remarkably.

Introduction

The chlorophyll-type pigments usually form spectroscopically heterogeneous species in plant materials, a large number of chlorophyll, PChl and PChlide species called 'forms' have been also described in vivo and in vitro [1,2]. Possible reasons for the spectral multiplicity of these pigments are their molecular interactions among themselves or with other molecules [3]. The knowledge of these interactions and of the molecular structure of these species is necessary for understanding their role in the formation and the function of the photosynthetic apparatus.

The interpretations of the structure of the spectral forms of chlorophyll, PChl and PChlide can be divided into three different groups. In the first group the spectral alterations were explained by the binding of the pigment molecules to carrier proteins [4]; these proteins may have either a structural role of an enzymatic activity as in the case of protochlorophyllide oxidoreductase. The active form of this enzyme has been proposed to be a ternary complex between PChlide, an apoprotein and NADPH; the redox state of the coenzyme may also influence the spectral characteristics of this complex [5,6]. The second interpretation is based mainly on in vitro measurements and takes into consideration either the possibility of the direct self-aggregation of the pigment molecules [7] or their indirect aggregation through bifunctional ligands which form bridges between them, for example, as water molecules [8]. As water can effectively coordinate with its electron donor part to the central magnesium ion and with its proton to the N or O proton acceptor atoms of the porphyrine ring, the number of the coordinated water molecules and the way they are bound may also cause spectral differences in the absorption and fluorescence spectra of chlorophylls [9]. The role of water for determining the spectral properties of PChl forms has been previously shown [10]. The third interpretation combines the two former ones and proposes that the pigment forms are aggregates of pigment molecules connected to protein molecules [11]. The finding of several chlorophyll-*a* and chlorophyll-*b* derivatives in plants, such as monovinyl and divinyl chlorophyll *a*, still increased the complexity of the problem, since the alteration of the primary chemical structure of chlorophyll pigments can also cause spectral multiplicity of the photosynthetic pigments [12]. A similar chemical heterogeneity of pigments was found also in etiolated leaves, which implies the presence of different PChlide esters [13] and of the accumulation of PChlide and DV PChlide [14,15]. DV PChlide is a magnesium phaeoporphyrin a_5 derivative

Abbreviations: DV PChlide, divinylprotochlorophyllide; DV PChl,divinylprotochlorophyll; PChl, protochlorophyll; PChlide, protochlorophyllide; CD, circular dichroism.

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which contains two vinyl groups at positions 2 and 7, while PChlide contains only the vinyl group at position 2. Results were reported also on the simultaneous phototransformation of PChlide and DV PChlide [16].

DV PChlide ester (DV PChl) together with PChl was found in a bigger amount in pumpkin seed coats [17]. The absorption spectra of these seed coats in the red region were very similar to those of etiolated leaves [18,19]. These results on the common occurrence of monovinyl PChlide and DV PChlide or monovinyl PChl and DV PChl in biological materials show the importance of the analysis of the evolution and the spectral properties of DV PChlide aggregates. Many works have described these data of PChl [20,21], PChlide [22–26], and there were also results on DV PChl [27,28], though the DV PChlide has not been analysed in this respect. A possible reason for this can be the small amount of DV PChlide in etiolated leaves. In previous studies concerning the pigment forms in model systems, PChl and DV PChl extracted from seed coats [10,20,21] and PChlide from etiolated tissues [22–26] have been used. Recently, a simple method was developed for preparation of DV PChlide [29].

The question arises whether the additional vinyl group at position 7 on the porphyrin ring of DV PChlide modifies the spectral properties of aggregates of this pigment. In this work the spectral forms of self-aggregated DV PChlide molecules were studied in solid film model systems.

Materials and Methods

DV PChlide was purified from cell-free medium of nicotinamide-enriched culture of *Rhodobacter sphaeroides* according to the method described earlier [29], except for the DEAE-Toyopearl chromatography. DV PChlide in cell-free medium was precipitated by ammonium sulfate at 65% saturation. The collected pigments were dissolved with a small volume of aqeous acetone. The pigments were then transferred into ether, the ether was then evaporated and the pigments were dissolved with a small volume of absolute acetone. This solution was applied onto a column (1×10 cm) of polyethylene (Polyscience, chromatography grade) and eluted with 90% (v/v) aqueous acetone as eluent. The separated DV PChlide was finally transferred into ether. The purity of the isolated DV PChlide was checked spectrophotometrically on the basis of the absorption peak ratios [30]. The purified pigment was also analysed by high-performance liquid chromatography with a polyethylene column and 65% (v/v) aqueous acetone as the moving phase [15].

The solid films were prepared from ether solution of DV PChlide on the inner surface of spectrophotometric cuvettes by evaporating the solvent [10]. The films were treated with acetone vapour [20,27]. The absorption spectra were measured with a Shimadzu UV-240 spectrophotometer and registered into the computer memory. Baseline correction, calculation of the 2nd and 4th derivative and a resolution into Gaussian components of spectra were carried out with help of a computer. The parameters for the spectrum resolutions at the beginning of the experiment were calculated from the derivative spectra; the halfbandwidths were fixed to be 20 nm. A resolution was accepted that fitted the experimental curve with a minimal number of components and that had an error of the magnitude of the experimental error, i.e., the signal-to-noise ratio of the spectra.

Results

To obtain the spectral forms of DV PChlide in solid films their absorption spectra were studied and analysed in the red region. Two main types of DV PChlide solid films were observed after the preparation: the first group of films exhibited red absorption maximum at 632 nm; and the second group of films had a red-shifted absorption peak with maxima at 640 and 650 nm, including intermediary positions, e.g., at 641, 642, 645 nm (Fig. 1). The spectra of films with the 632-nm maximum had Gaussian components at 628 (main component), 644 and 665 nm with 12-nm halfbandwidths. Other components were found in the spectra with 640–650-nm maxima at 635, 655 and 676 nm. The

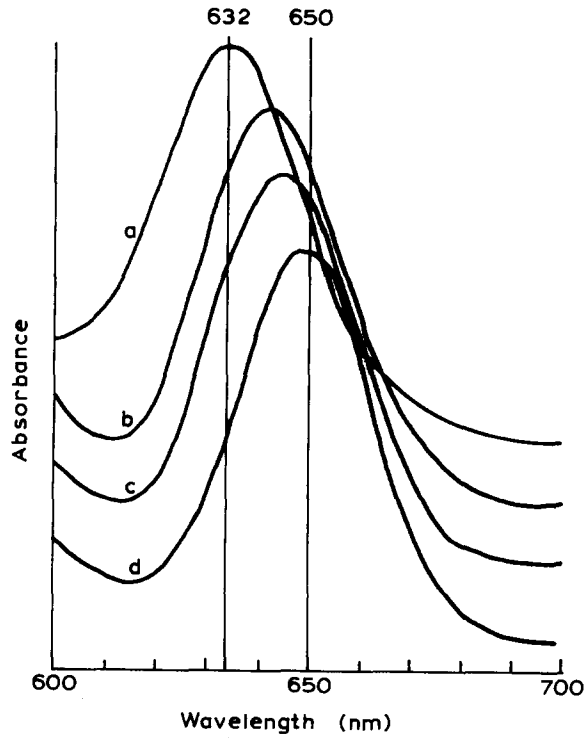

Fig. 1. Absorption spectra of four different DV PChlide solid films after preparation. Films have absorption maxima at: 632 nm (a); 641 nm (b); 645 nm (c); and 650 nm (d).

TABLE I

Ratios of Gaussian components in absorption spectra of divinylprotochlorophyllide solid films after preparation

Ratios of Gaussians	Position of the experimental maximum			
	641 nm	642 nm	645 nm	650 nm
655/635	0.63	0.77	0.94	1.40
676/635	0.10	0.12	0.17	0.30

different ratios of these components in the films may explain the position of the experimental maxima (Table I). In parallel with the appearance of the red-shifted absorption bands remarkable spectral shifts were observed also in the Soret region of the spectra: films with 632-nm red maximum exhibited a broad peak at 440 nm, films with 640–645-nm red bands had a 452-nm blue peak and the 650-nm absorbing films showed blue maximum at 480 nm. These Soret bands were complex due to the overlap of several absorption peaks. This region has not been analysed in detail in this work.

In order to study the evolution of the different spectral forms (to which the Gaussians can be attributed) and to compare the spectral properties of DV PChlide films with earlier studies on PChl [20,21], PChlide [22–26] and DV PChl [27,28] containing models, the above-described DV PChlide films were treated with vapours of acetone. This treatment resulted in a labile structure of the films giving this way a mobility for the DV PChlide molecules. When the treatment was carried out at a temperature higher than 25 °C and the acetone vapour tension was too high, the acetone condensed on the surface of the films and dissolved the DV PChlide. In case of lower temperatures and at low acetone vapour tension a blue-shift was observed in the first minutes of the treatment: the main absorption peak shifted to 630 nm. As a second step of this process a shoulder appeared around 650 nm, the intensity of which increased gradually and the new absorption maximum appeared at 650 nm at the end of the treatment. The whole process is demonstrated in a three-dimensional graph which shows spectra registered continuously during the treatment (Fig. 2). The grids in this figure clearly show the blue-shift at the beginning of this process which is followed by the red-shift. The topological representation (Fig. 3) shows a 'saddle' between the two sets of intensity lines. As this figure demonstrates the changes of the contour lines in relation to time, the presence of this 'saddle', i.e., the absence of contour lines in this region, means that there was no absorbance change here in relation to time, which is an obvious proof for the existence of the isosbestic point in this process. Calculating the intensity divide (the lines of relatively highest intensity values) of the two sets of peaks, an intersection point can be found at 628 nm; this demonstrates that the position of

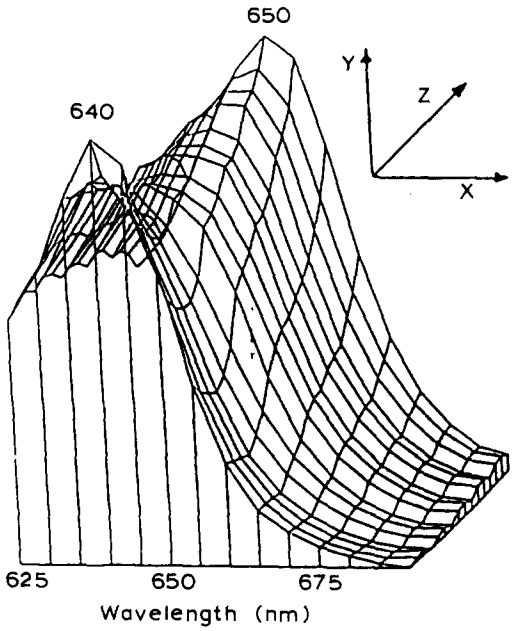

Fig. 2. Changes of the absorption spectrum of a DV PChlide solid film during acetone vapour treatment. Before the treatment started the main maximum was at 640 nm, then it shifted to 632 nm during the treatment and at the end of the treatment it was at 650 nm. The spectra were recorded every 2 minutes. The coordinates are: *X* for wavelength (nm), *Y* for time (min), and *Z* for extinction.

the shortest wavelength form appeared after the 16.5th min of the blue-shift. The conventional demonstration of this process, which shows the spectra registered between the 15th and the 45th minute of the treatment (Fig. 4), is in good agreement with the data of Figs. 2 and 3; it shows maxima at 628 and 650 nm and an isosbestic point around 632 nm (Fig. 4).

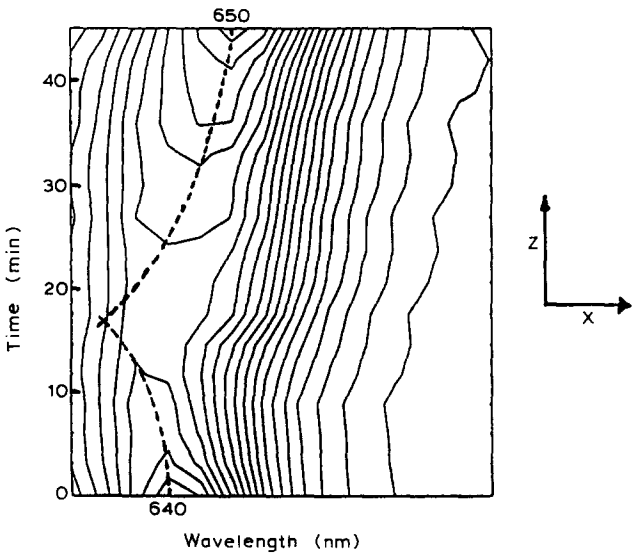

Fig. 3. Topological representation of the transformation process shown in Fig. 2. The grids represent isoextinction lines. The spectra were recorded every 2 minutes. The coordinates are: *X* for wavelength (nm), and *Z* for time (min). The dotted line is the 'intensity divide'.

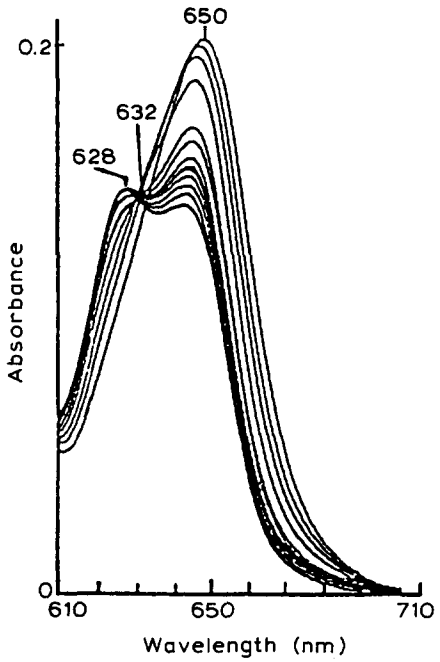

Fig. 4. The second part of the transformation process of the DV PChlide solid film is shown: the transformation of the 632-nm form into the 650-nm form.

For an analysis of this transformation, the extinction values of the Gaussian components were determined. To demonstrate the good resolution of our method two spectra are presented in Figs. 5 and 6. The spectrum in Fig. 5 was registered after a treatment of 10 min and shows an intermediary state of the film. Fig. 6 represents the final state of the film after a treatment of 45 min. In both spectra components at 632, 650 and 666 nm were found with different intensities. The similarity of the spectrum in Fig. 6 to those of the etiolated leaves is remarkable.

Discussion

Direct information on the in vivo molecular structure of chlorophyll-like pigment forms is not easily available as different types of interaction are present simultaneously. Therefore, model systems of different nature are often used to study this question. Solid films were found to be advantageous for preparing different PChl [20,21] and DV PChl [27] forms. After evaporation of the ether solvent, i.e., the preparation of the films, 'accidental' interactions take place producing aggregates with random geometry [31]. However, in some cases, aggregates with definite geometry and with strongly red-shifted absorption bands and intense CD signals can occur [20]. The treatment of the films with vapours of acetone results in the desaggregation of the initial species which appeared after the preparation of the films; then a secondary aggregation takes place in which the pigment molecules are connected to each

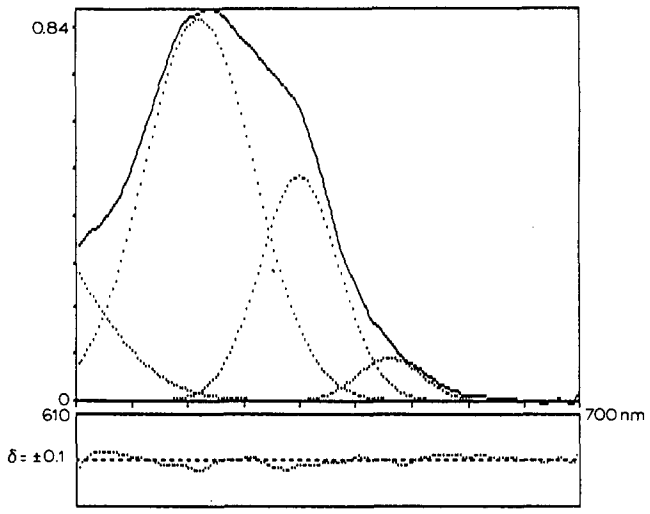

Fig. 5. Computer resolution of the absorption spectrum of a DV PChlide solid film after 10 min acetone vapour treatment.

other with specific molecular sidegroups forming coordination interactions and giving this way definite geometry for the aggregates [20]. Infrared spectroscopy of PChl films treated with acetone vapour gave evidence for the interactions of keto-groups and magnesium ions of the PChl molecules in species having an absorption maximum of 650 nm [21]. By varying the tension of the acetone vapours during the treatment of the films the transformation process could be slowed down and in this way the transformation process could be followed by registering continuously the absorption spectra. These spectra were presented in Figs. 2–4. The resolution of the absorption spectra in different transformation stages of the films made it possible to observe the evolution of the DV PChlide forms. The results shown in Figs. 5 and 6, together with the 3-dimensional and topological visualization of the transformation process, proved that in

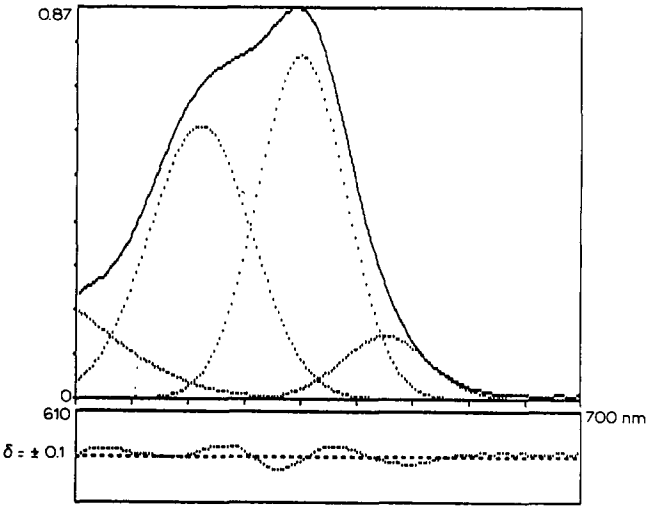

Fig. 6. Computer resolution of the absorption spectrum of a DV PChlide solid film after 45 min acetone vapour treatment. Further treatment did not change the ratio of the Gaussians.

the first phase a 632-nm absorbing form was dominating in the films. This form arose from a 628-nm absorbing form, the existence of which could be calculated from the topological representation of the process (Fig. 3). In the second phase a 650-nm absorbing form had the highest absorption band and, in parallel, a long-wavelength absorbing form with peak at 666 nm appeared. The intensity of this band in the spectrum registered in the final stage (after a treatment of 45 min) was high enough to overlap the shorter-wavelength bands; therefore it hindered the observation of the true isosbestic point (Fig. 4). The 650-nm absorbing DV PChlide form was found to be most stable similarly to PChl and PChlide model systems [10,20,23]. The positions of the red absorption bands (i.e., of the Gaussian components) of other DV PChlide forms were also very similar to those of PChl or PChlide forms in other systems [10,20,32]: despite the difference of their absorption spectra in monomeric forms (in their polar solvents) [30]. A larger difference was found between the Soret band positions of diethyl ether solutions of DV PChlide and PChlide: 438 and 432 nm, respectively [14,30]; therefore a comparison of the absorption spectra of their aggregates in this region seemed to be promising. The aggregation of the DV PChlide resulted in red-shifts and also in band splittings in the Soret region. Unfortunately, there are no data to be found in the literature about most of these pigment aggregates. A 632–634-nm-absorbing PChlide species prepared in dry benzene had a Soret maximum at 440–443 nm, and a 648–651 nm absorbing PChlide species in the same system at 469–472 nm [22,24]. PChl solid films with red maxima at 630, 647 and 651 nm had Soret bands at 440, 475 and 480 nm, respectively [21]. A 650-nm absorbing DV PChl solid film exhibited Soret band at 485 nm [27]. The red-Soret band pairs of DV PChlide were found in this work 632–440, 642–464 and 650–480. Obviously, the exact description of these bands needs a resolution of the Soret region because of the complexity of the films. Such a resolution, however, is a complicated procedure as in this region the B_x and the B_y electronic transitions strongly overlap [33] and there are no data available about the exciton splitting of these transitions in aggregated systems. On the other hand, the comparison of this spectral region to those of in vivo systems is rather complicated due to the carotenoid absorption.

The results of this work demonstrated that the DV PChlide aggregates have similar absorption bands to those of PChlide or PChl aggregates; the additional vinyl group does not have an important effect on the electronic structure of these pigment aggregates, neither on the process of their aggregation. Therefore, it cannot be excluded that the absorption bands observed in spectra of etiolated leaves do not only belong to PChlide but also to DV PChlide forms.

Acknowledgement

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