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CHLOROPLAST BIOGENESIS XXVII

DETECTION OF NOVEL CHLOROPHYLL AND CHLOROPHYLL PRECURSORS IN HIGHER PLANTS*

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SUMMARY: In etiolated higher plants, chlorophylls a and b are formed from protochlorophyllide and its divinyl analog via; a) light induced conversion to distinct chlorophyllide a species, b) dark esterification to distinct chlorophyll a species. Each pair is distinguishable by the Soret excitation maxima in ether at 77K.

INTRODUCTION

In higher plants, only two chemically distinct chlorophyll species (termed Chl a and Chl b) are recognized as participants in the collection and conversion of solar to chemical energy (1). They differ in the presence of a methyl group at position 3 of the macrocycle in Chl a and a formyl group at that same position in Chl b, (2); Chl b is believed to be synthesized from Chl a (3). The synthetic route to the chlorophylls has been documented (2) as follows: Mg-2-vinylpheoporphyrin a_5, i.e. Pchlide, is the immediate precursor of Chlide a. The latter is esterified in the dark with a long chain alcohol to yield Chl a. Protochlorophyllide and Chlide a differ by a double bond in the 7-8 position of the macrocycle and the conversion occurs by a photochemical reaction (2). The Immediate precursor of Pchlide is believed to be 2,4-divinyl Pchlide, i.e. a Pchlide with a vinyl group instead of an ethyl group at the fourth position of the

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Abbreviations: DVPchlide - divinyl protochlorophyllide; Pchlide - protochlorophyllide; Pchl - protochlorophyll; Chlide - chlorophyllide; Chl - chlorophyll

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macrocycle (4). This compound was detected in inhibited R. spheroides cultures (5) but was never before detected in higher plants. In this work we present evidence for the presence of Pchlide and DVPchlide in etiolated tissues which appear to give rise to at least two different Chlide a, two different Chl a and two different Chl b species in higher plants.

MATERIALS AND METHODS

R. spheroides mutant MC-7 , that accumulates DVPchlide, kindly supplied by Dr. June Lascelles, was cultured according to Lascelles and Hatch (6). The pigments were isolated from the culture medium by precipitation with ammonium sulfate (6) and the precipitated pigments were dissolved in acetone. The pigments were transferred to ether as described by Koski and Smith (7). Barley (Hordeum vulgare L. var. Beacon Spring) and cucumber (Cucumis sativus L. cv. Beit Alpha MR) seeds were germinated either in the dark or under a photoperiodic (14 hr light, 10 hr dark) regime as described before (8). In order to extract the pigments from the etiolated or green tissues, three g of tissue were homogenized in 20 ml acetone-0.1 N NH_4OH (9:1 v/v) at 0-4°C for two min in a Sorvall Omni-mixer (9). After centrifugation at 39,000g for ten min, the 80% acetone extract was adjusted to 75% acetone with H_2O. The pigments were partitioned into a hexane fraction containing the phytylated tetrapyrroles and a hexane-extracted acetone fraction containing the unphytylated pigments (9). The unphytylated tetrapyrroles were transferred to ether (9) before chromatography and spectroscopic monitoring. The DVPchlide extracted from R. spheroides in ether was purified by chromatography on thin layers of silica gel H developed in toluene:ethyl acetate:ethanol (8:2:2 v/v/v) at 4°C. It was eluted in methanol:acetone (4:1 v/v) (9), dried under N_2 gas and redissolved in ether for spectroscopic determinations. Chlorophyll a and b in the hexane fraction were chromatographed on thin layers of cellulose MN 300 developed in ligroin (60-80):n-propanol (9.9:0.1 v/v) at room temperature (10) or on thin layers of silica gel H as for DVPchlide. The segregated pigments were eluted in ether, dried under N_2 gas then redissolved in ether for spectroscopic measurements. Photoreduction of the unphytylated Pchlide was achieved by illuminating the etiolated tissues either for two sec with three mw cm^{-2} of incandescent light or for one min with 320 $\mu w\ cm^{-2}$ of white fluoresecent light (8). Spectrophotometric and spectrofluorometric determinations were as reported elsewhere by Cohen and Rebeiz (8).

RESULTS

The unesterified protochlorophyll fraction of etiolated higher plants has always been considered to consist of one chemical species, namely Pchlide (2), with a red absorption maximum at room temperature, in ether, at 621-623 nm, an emission maximum at about 629-630 nm and a Soret absorption/excitation maximum at about 432-433 nm (7, 11, 12). The DVPchlide

of R. spheroides was distinguishable from Pchlide only by its red-shifted Soret absorption/excitation maximum which was found at about 437-438 nm in ether solutions at room temperature (11). The DVPchlide purified from the R. spheroides (MC-7) mutant exhibited an absorption spectrum that corresponded to those reported by others (5, 11). The similarities and differences of the R. spheroides (MC-7) DVPchlide and the Pchlide of etiolated barley leaves, in ether at room temperature are depicted in Fig. 1. Both exhibited an emission maximum at about 627 nm and Soret excitation maxima at about 438 and 432 nm, respectively. A Soret excitation shoulder at 438 nm that had been previously overlooked in extracts of etiolated tissues by other workers (12) was also obvious in the excitation spectrum of the barley Pchlide fraction (Fig. 1).

Since the Soret excitation shoulder at 438 nm may correspond to significant amounts of DVPchlide in the barley extract which is masked at room temperature by the higher concentrations of Pchlide, the spectroscopic properties of the extracted, unesterified Pchl fraction of several etiolated tissues were reinvestigated at 77°K. At this temperature the fluorescence emission and excitation bands narrow significantly and peak resolution improves considerably (Fig. 1, 2). In Figure 2, the fluorescence emission and excitation spectra, in ether, of the unesterified Pchl of etiolated barley leaves and cucumber cotyledons are compared to those of standard DVPchlide. In all three cases the emission maximum was found at 627 nm (Fig. 2A). Surprisingly, however, a distinct divinyl Soret excitation maximum which corresponded to that of the standard DVPchlide was detected at 444 nm in both barley and cucumber (Fig. 2B). In addition, both the barley and cucumber ether extracts exhibited also a blue-shifted Pchlide Soret excitation maximum at 437 nm which was not present in the DVPchlide profile. The Soret excitation shoulders clearly shown at 451 nm in the DVPchlide standard and the cucumber extract are ascribed to an intermediate of Chl biosynthesis and will be discussed elsewhere. The barley unestri-

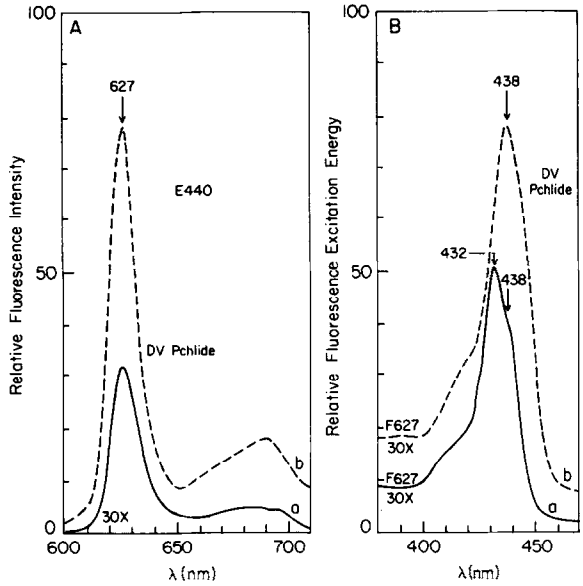

Figure 1. Fluorescence emission (A) and excitation (B) spectra of the unesterified Pchl of etiolated barley (a) and of standard DVPchlide (b), in ether at room temperature. Ordinate scale attenuation is indicated on the spectra, 350X being the least attenuation possible. The emission spectra were elicited by the E-wavelength indicated. The excitation spectra were recorded at the F-wavelength indicated. Arrows point to wavelengths of interest.

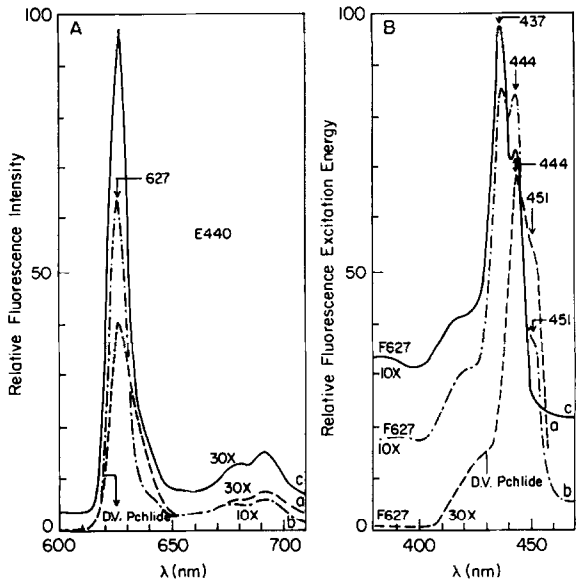

Figure 2. Fluorescence emission (A) and excitation (B) spectra of standard DVPchlide (a), and of the unesterified Pchl of cucumber (b) and barley (c) in ether at 77°K. All symbols are as in Figure 1.

fied Pchlide profile was similar to that of cucumber while that of bean was similar to that of corn. Altogether, the above data indicated that contrary to previous assumptions, the unesterified Pchl fraction of etiolated higher plants is heterogeneous and appears to consist of both DVPchlide and Pchlide.

In order to find out whether the DVPchlide and the Pchlide of etiolated tissues were phototransformable into two distinct Chlide a species, etiolated cucumber cotyledons were illuminated for one min with 320 μw cm^{-2} of white fluorescent light at 28°C. The pigments were then extracted in acetone and transferred to ether. It was shown earlier that this illumination photoconverted most of the unesterified Pchl into a Chlide a emitting pigment (8). As depicted in Fig. 3A, most of this unesterified Pchl was converted into a Chlide a emitting species with an emission maximum at 673 nm. The unreacted unesterified Pchl is observable at 627 nm (Fig. 3A); it was made up of about the same proportions of DVPchlide and Pchlide. The Soret excitation spectrum of the newly formed Chlide a fraction recorded at the emission maximum of Chlide a at 673 nm exhibited two Soret excitation maxima: one at 438 nm, and a red-shifted maximum at 445 nm (Fig. 3B). Altogether, the above results indicated that the Chlide a fraction that was produced by photoreduction of the unesterified heterogeneous Pchl was also heterogeneous and appeared to consist of two distinct components.

The conversion of the two Chlide a components into Chl a was next investigated. The unesterified Pchl of etiolated cucumber cotyledons was converted to heterogeneous Chlide a by illumination for two sec with about 3.0 mw cm^{-2} of incandescent light (8) and the Chlide a fraction was converted to Chl a *in vivo* by incubating the irradiated tissue for 45 min in the dark at 28°C (13). The Chl a was extracted in acetone, transferred to ether and purified on thin layers of silica gel H as described in Methods. The Chl a, eluted in ether, exhibited an emission maximum at 673 nm, simi-

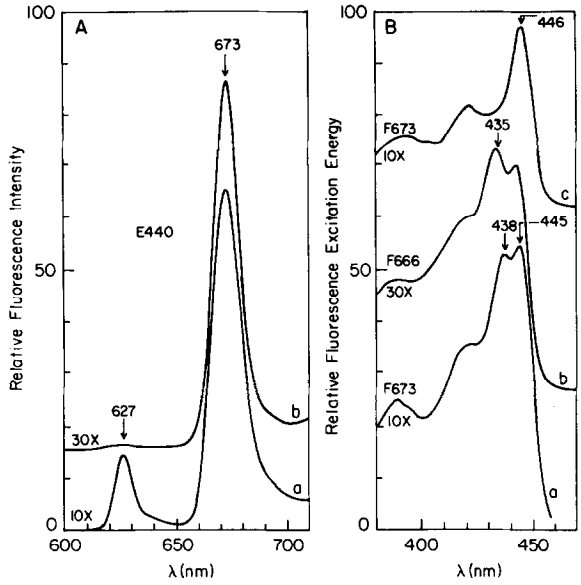

Figure 3. Fluorescence emission (A) and excitation (B) spectra of the Chlide a (a) and newly formed Chl a (b,c) fractions of cucumber, in ether at 77°K. All symbols are as in Fig. 1.

lar to that of Chlide a (Fig. 3A). However, the Soret excitation spectrum recorded at the Chl a emission maximum, i.e. at 673 nm, exhibited a single Soret excitation maximum at 446 nm (Fig. 3B). In order to uncover any putative short wavelength Chl a that may be masked by higher concentrations of the red-shifted component, a Soret excitation spectrum was also recorded on the short wavelength emission tail of the Chl a band, at 666 nm. As depicted in Fig. 3B, the Soret excitation spectrum thus recorded exhibited a blue-shifted maximum at 435 nm in addition to the red-shifted component. These results indicated that the Chl a synthesized from the heterogeneous Chlide a fraction was also heterogeneous and was made up of at least two components. However, in contrast to the Chlide a fraction, the red-shifted component in the Chl a fractions occurred in higher concentrations than the blue-shifted one. Rough calculations indicated that the proportions of the long wavelength to the short wavelength component was about three to one.

Finally, the possible occurrence of heterogeneous Chl a and b in mature green tissues was also investigated. To this effect, the Chl of

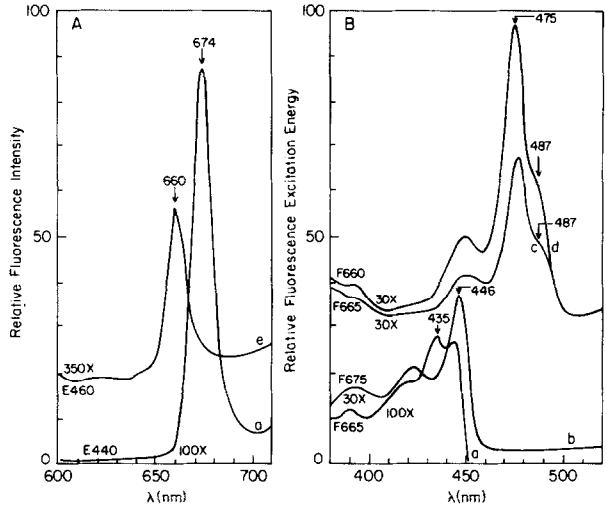

Figure 4. Fluorescence emission (A) and excitation (B) spectra of the purified Chl a (a,b) and b (c,d) fractions from green mature cucumber cotyledons, in ether at 77°K. All symbols are as in Fig. 1.

green fully expanded cucumber cotyledons from seedlings grown photoperiodically for 14 days (8) was extracted and purified on thin layers of cellulose. The emission spectra of the purified Chl a and b fractions in ether at 77°K are depicted in Fig. 4A. Chlorophyll a exhibited an emission maximum at 674 nm and Chl b a maximum at 660 nm. The Soret excitation spectrum of Chl a recorded at 666 nm exhibited both a blue-shifted maximum at about 475 nm and a red-shifted one (Fig. 4B). The Soret excitation spectrum recorded at 674 nm exhibited only the Soret of the red-shifted component (Fig. 4B). In this case too, the two Chl a components occurred in the same proportion as in the Chl a fraction that was formed from the heterogeneous Chlide a. Likewise, the Chl b fraction also appeared to be made up of two components with Soret excitation maxima at 475 nm and 487 nm, respectively. The detection of the red-shifted Chl b component at 487 nm was enhanced by recording the Soret excitation spectrum at the long wavelength tail of the Chl b band (Fig. 4B). In contrast to the Chl a fraction, however, the blue-shifted component of the Chl b fraction was the predominant Chl b species. The same Chl profile was also observed in an extract

of green spinach leaves. Altogether, the above results indicated that the Chl a and Chl b of a green tissue are also heterogeneous. It is therefore proposed that the Chl a and b of higher plants are each made up of at least two distinct chemical species.

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