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PHOTOCONVERTIBLE PROTOCHLOROPHYLL(IDE)$_{635/650}$ IN VIVO: A SINGLE SPECIES OR TWO SPECIES IN DYNAMIC EQUILIBRIUM?

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SUMMARY

The decreasing absorbances in vivo of protochlorophyll(ide) at 635 and 650 nm bear the same relationships to one another during photoconversion to chlorophyll(ide) *a* in the leaves of dark-grown barley seedlings, regardless of whether the actinic light is absorbed primarily at 630, 640 or 671 nm. Accordingly, the absorption bands at 635–637 and 650 nm of photoconvertible protochlorophyll(ide) are attributed to a single species of membrane-bound protochlorophyll(ide) molecule or, alternatively, to two species which are in dynamic equilibrium.

INTRODUCTION

From spectroscopy on the leaves of dark-grown seedlings, three states of protochlorophyll(ide) in vivo have been postulated. An absorption band at 628 nm has been assigned to non-photoconvertible protochlorophyll(ide). Additional absorption bands, at 635–637 and 650 nm have been attributed to two photoconvertible species, protochlorophyll(ide)$_{635-637}$ and protochlorophyll(ide)$_{650}$, respectively [1].

The possibility that protochlorophyll(ide)$_{650}$ represents aggregated pigment has been considered [2–5], but the 635–637- and 650-nm absorption bands have also been taken as evidence for two pools of protochlorophyll(ide) molecules which are bound to membrane protein in differing manners [1].

The notion that the 635–637- and 650-nm absorption bands of protochlorophyll(ide) belong to two transitions of a single kind of photoconvertible protochlorophyll(ide) molecule in vivo has not been considered earlier. Because of the efforts in this and other laboratories to gain insight into the binding states of protochlorophyll(ide) and chlorophyll(ide) and their relation to function, it was important to investigate this possibility. We took a negative approach and attempted to photoconvert preferentially either one or the other of the hypothesized species, protochlorophyll(ide)$_{635-637}$ or protochlorophyll(ide)$_{650}$.

MATERIALS AND METHODS

Barley seedlings (*Hordeum vulgare* L., cultivar Svarlöf's Bonus) were grown for 7 days in darkness, as described previously [6]. Dim green safelight was used for all operations requiring vision. Jigs guided the cutting of specific segments from the primary leaves. For spectrophotometry in vivo 7-mm segments from 17 to 24 and 24 to 31 mm (630- and 671-nm irradiations below) or 24 to 31 and 31 to 38 mm (640-nm irradiations) proximal to the leaf apices were cut with a razor blade. Five pairs of segments were placed in a single layer between glass plates of a holder, and the leaf material covered completely the area illuminated by the sample beam of the spectrophotometer. The leaf segments in the holder could alternatively be irradiated with actinic light outside the spectrophotometer and placed into the spectrophotometer for recording their spectra.

For pigment analyses following acetone extraction, 24–26 segments from 24 to 38 mm proximal to the leaf apices were cut. These segments were also mounted between glass plates before irradiation with actinic (640 nm) light. After illumination, the segments, weighing about 0.25 g, were ground with 5 ml of acetone–25% ammonia–water (85 : 1 : 14, by vol.), and the extract was clarified by centrifugation. The quantitative determination of protochlorophyll(ide) and chlorophyll(ide) in the acetone extracts has been described previously [7].

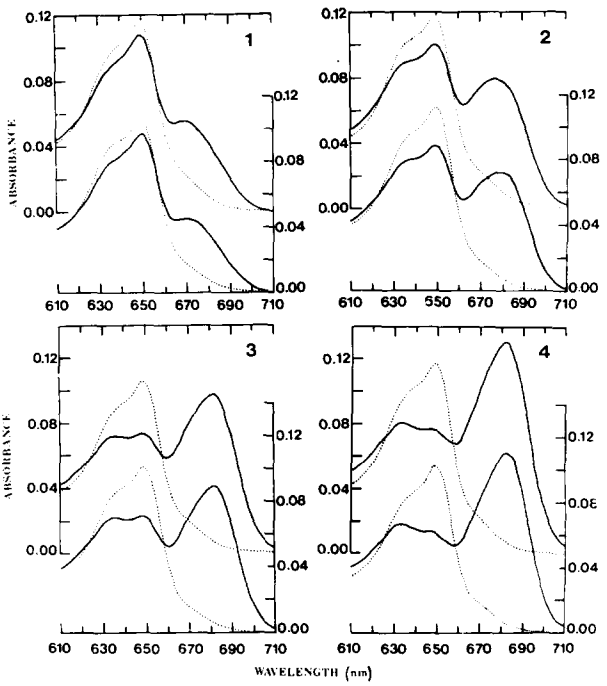
Irradiation of the leaf pieces was performed with a xenon lamp (Osram XBO 450 W/P) powered with stabilized direct current. The light beam passed through a 12.5-cm layer of water to remove infrared radiation, a 630- (No. 252273), 640- (No. 272442) or 671-nm (No. 454504) Depil interference filter, and finally an RG 610 red glass cut-off filter (all filters from Jenaer Glaswerk, Schott und Gen., Mainz, Germany) before shining on the leaf pieces. The intensities of the 630-, 640- and 671-nm light beams at the leaf pieces were $3.9 \cdot 10^{14}$, $2.1 \cdot 10^{14}$, and $1.3 \cdot 10^{15}$ quanta $\cdot$ cm$^{-2} \cdot$ s^{-1}, respectively. Measurements of light intensities were made as described earlier [7].

A Cary 17 spectrophotometer equipped with a scattered transmission accessory and an EMI 9659 QB multiplier phototube recorded all the spectra. A piece of double-layer tissue paper (Linella) placed in the reference beam served as a scatter blank during spectrophotometry of the living leaf pieces.

RESULTS AND DISCUSSION

We reasoned as follows: if 635–637-nm absorption is characteristic of one bound species and 650-nm absorption is characteristic of another bound species of protochlorophyll(ide) molecule, by choosing appropriate wavelengths of monochromatic photoconverting light it should be possible to elicit the photoreduction of one of the species at a greater rate than the other. Thus, in 630-nm light, 635-nm absorption should decrease more rapidly than 650-nm absorption, and in 671-nm light, 650-nm absorption should decrease more rapidly than 635-nm absorption, as protochlorophyll(ide) is converted to chlorophyll(ide) *a*.

Accordingly, by adjusting the intensity of 630- and 671-nm light to yield approximately equal absorbance by chlorophyll(ide) after any particular irradiation time, we produced four levels of photoconversion, each with 630- and 671-nm light.



Figs 1–4. Absorption spectra of sets of segments of dark-grown barley leaves before irradiation (···), and after various doses of light (—). In each figure the upper pair of spectra came from a set of segments that received 630-nm light and the lower pair from segments that received 671-nm light. Irradiation times and mean % of maximal photoconversion, respectively: Fig. 1, 8 s, 20 %; Fig. 2, 18 s, 36 %; Fig. 3, 35 s, 52 %; Fig. 4, 55 s, 67 %. Per cent photoconversion was calculated using chlorophyll(ide) absorbances in vivo and then transformed (multiplied by 0.85) to obtain the percentages given here. The reason for the transformation is given in the text.

No difference between the relationship of the residual 635- and 650-nm absorbances following photoconversion to any particular level with 630- versus 671-nm light was apparent (Figs 1–4). This indicated that whether photoconverting light is absorbed principally in the 635–637- or in the 650-nm absorption band of protochlorophyll(ide) does not affect the spectral character of the residual protochlorophyll(ide).

Attention is drawn to the absorption maxima of chlorophyll(ide) *a* in the spectra in Figs 1–4. While at high partial conversions (Figs 3 and 4) the maximum is at 682 nm, at 20% conversion (Fig. 1) the maximum is at 672–675 nm. Explanations of this difference have been given previously [5, 8, 9]. The present work tends to rule out a further possible explanation: Short wavelength chlorophyll(ide) comes from a short wavelength protochlorophyll(ide) species that is photoconverted before the bulk of the protochlorophyll(ide) absorbing at longer wavelength.

Absorbances determined from the spectra in Figs 1–4 and from similar sets of spectra can be treated quantitatively. If, as photoconversion proceeds, (1) the decline in absorbance by protochlorophyll(ide) at any particular wavelength is a function of its decreasing total quantity and independent of the quality of the photoconverting light; (2) the respective extinction coefficients of protochlorophyll(ide) and chloro-

phyll(ide) at 635- and 650-nm remain constant; (3) the absorbance increase of chlorophyll(ide) at its absorption maximum is directly related to chlorophyll(ide) quantity, and hence, to the decrease of protochlorophyll(ide) quantity; and (4) light scattering remains constant, the following relations should hold:

$$A_{635\,nm} = A_{635\,nm,\,0} - \left[\frac{\varepsilon Pchl_{635\,nm} - \varepsilon Chl_{635\,nm}}{\varepsilon Chl_{max}}\right] \Delta A_{max,\,chlorophyll(ide)} \quad (1)$$

$$A_{650\,nm} = A_{650\,nm,\,0} - \left[\frac{\varepsilon Pchl_{650\,nm} - \varepsilon Chl_{650\,nm}}{\varepsilon Chl_{max}}\right] \Delta A_{max,\,chlorophyll(ide)} \quad (2)$$

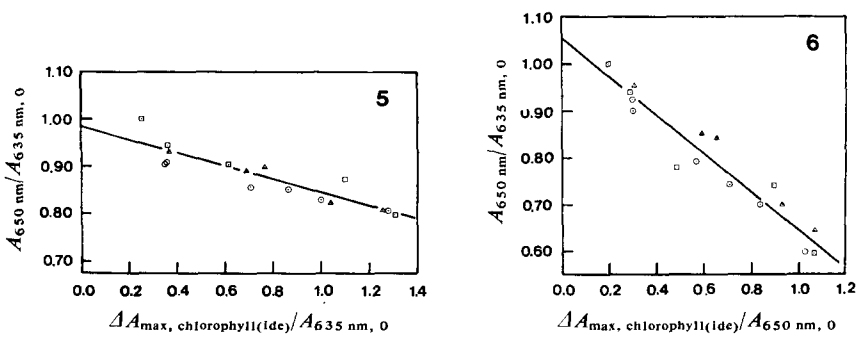
where $A_{635\,nm}$ and $A_{650\,nm}$ are total absorbances (specific absorbance of the pigments plus non-specific light loss from the measuring beam) at 635 and 650 nm, respectively ($A_{635\,nm,\,0}$ and $A_{650\,nm,\,0}$ are initial absorbances before illumination); $\Delta A_{max,\,chlorophyll(ide)}$ is the increase in absorbance at the wavelength of maximum absorption in the red region by the chlorophyll(ide) resulting from illumination of the leaf pieces; $\varepsilon Pchl_{635\,nm}$, $\varepsilon Pchl_{650\,nm}$, $\varepsilon Chl_{635\,nm}$ and $\varepsilon Chl_{650\,nm}$ are the molar extinction coefficients of protochlorophyll(ide) and chlorophyll(ide), respectively, at the wavelengths indicated; and εChl_{max} is the molar extinction coefficient of chlorophyll(ide) at the wavelength of its maximum absorption in the red region.

Since the sets of leaf segments irradiated with differing light doses and qualities did not all have identical initial absorption properties (cf. Figs 1–4), normalizations were appropriate, and these were gained readily by transforming Eqns 1 and 2 to:

$$\frac{A_{635\,nm}}{A_{635\,nm,\,0}} = 1 - \left[\frac{\varepsilon Pchl_{635\,nm} - \varepsilon Chl_{635\,nm}}{\varepsilon Chl_{max}}\right] \cdot \frac{\Delta A_{max,\,chlorophyll(ide)}}{A_{635\,nm,\,0}} \quad (3)$$

$$\frac{A_{650\,nm}}{A_{650\,nm,\,0}} = 1 - \left[\frac{\varepsilon Pchl_{650\,nm} - \varepsilon Chl_{650\,nm}}{\varepsilon Chl_{max}}\right] \cdot \frac{\Delta A_{max,\,chlorophyll(ide)}}{A_{650\,nm,\,0}} \quad (4)$$

Eqns 3 and 4 each describe a line with negative slope and intercepting the ordinate at 1.0. The spectra in Figs 1–4, together with other similar sets of spectra of leaf segments irradiated with various doses of 630-, 640- or 671-nm light, yielded the points plotted in Figs 5 and 6. The lines were determined by the method of least



Figs 5 and 6. Decreasing 635- and 650-nm absorbance of leaf segments during the photoconversion of protochlorophyll(ide) to chlorophyll(ide) in 630- (△), 640- (□), or 671-nm light (○). See text for definitions and rationale of the axes.

squares. Clearly, the absorbance decreases of protochlorophyll(ide) at 635 and 650 nm, as chlorophyll(ide) formation proceeded, were independent of whether the actinic light for the photoconversion was absorbed at 630, 640 or 671 nm.

The simplest interpretation of these observations is that each bound molecule of photoactive protochlorophyll(ide) in vivo has a 635–637- and a 650-nm absorption band, and consequently, the photoconversion of any single molecule leads to an instantaneous loss of absorption in both the 635–637- and 650-nm bands. An extension of this view and equivalent in the present context is the notion that protochlorophyll(ide) molecules, which occur in aggregates in membranes [1], are ordered and interact [2–5] to give an absorption band split. The relative intensities of the 635–637- and 650-nm absorptions vary from the tip toward the base of the 7-day-old etiolated barley leaf (Kahn, A. and Nielsen, O. F., unpublished), and the ratios of the intensities may depend on the ordering or the environments of the protochlorophyll(ide) molecules in the aggregates. An alternative explanation of the results is that the absorption bands at 635–637 and 650 nm belong to two, distinct classes of bound protochlorophyll(ide) molecule and that a dynamic equilibrium exists between them. Upon the removal of molecules from either of the pools, rapid replacement by molecules from the other pool occurs.

One of the demands of Eqns 3 and 4 is not met by the data in Figs 5 and 6; i.e. the intercepts of the lines with the ordinates depart somewhat from 1.0. These departures can be explained by the activation and subsequent photoconvertibility of the initially non-photoconvertible protochlorophyll(ide) [10–12]. Coincident with the activation, decreased absorption at 630–633 nm and increased absorption at 650 nm has been observed. Such activation would influence the intercepts in the directions found.

A second discrepancy (not shown) between our results and the Eqns 3 and 4 was that increases in chlorophyll(ide) absorbance failed to keep pace with decreases in protochlorophyll(ide) absorbance at 635 and 650 nm, when photoconversion approached completion. We found that the absorbances of chlorophyll(ide) in vivo were not related by a constant to the quantities of chlorophyll(ide) *a* present in the leaf segments throughout the course of photoconversion. The relative absorbance of chlorophyll(ide) in vivo rose linearly with the relative quantity of chlorophyll(ide) *a* (determined from acetone extracts) in the leaf segments up to 85% of maximal photoconversion, but then the absorption of chlorophyll(ide) in vivo did not increase during further photoconversion. There are two noteworthy consequences of these observations for our own work, when only the absorbance in vivo of chlorophyll(ide) *a* is monitored to ascertain the progress of photoconversion: (1) between 0 and 85% of maximal photoconversion, the degree of conversion will be overestimated by about 15%; and (2) beyond approx. 85% of maximal photoconversion, further conversion will remain undetected. We do not know that these consequences will apply equally to homologous measurements on other sorts of dark-grown leaves.

We emphasize that the present Results and Discussion concern pieces of dark-grown leaves that were essentially unmodified before the irradiations. Freezing and thawing or heating dark-grown leaves [2, 4, 14] or extraction of a soluble pigment–protein complex, protochlorophyll holochrome [1, 6, 13, 15–18], produce alterations whereby the 650-nm absorption band is reduced or eliminated and a band at shorter wavelength remains as the only prominent absorption band of photoconvertible

protochlorophyll(ide) in the red region.

While there are numerous spectral indications that require the close association and interaction of protochlorophyll(ide) molecules in vivo and in macromolecular preparations of protochlorophyll holochrome [1, 9, 16–19], there is no compelling demand for the universal occurrence of more than one photoconvertible species of bound protochlorophyll(ide) in vivo. Despite their complexity, the kinetics of protochlorophyll(ide) photoconversion [7] do not require the existence of more than one species of photoconvertible protochlorophyll(ide) in vivo. The single band of fluorescence emission at 655 nm from dark-grown leaves at 77 °K, although interpreted differently earlier [1], also is consistent with the presence of a single species of photoconvertible protochlorophyll(ide). Finally, we note that the present experiments cannot rule out the possible existence of two distinct species, protochlorophyll(ide)$_{635-637}$ and protochlorophyll(ide)$_{650}$, in dynamic equilibrium in vivo.

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