

# The light intensity dependence of protochlorophyllide photoconversion and its significance to the catalytic mechanism of protochlorophyllide reductase

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**Abstract** The light-dependent step in chlorophyll synthesis by higher plants involves hydrogen transfer from NADPH<sup>+</sup> to the porphyrin protochlorophyllide catalysed by the photoenzyme protochlorophyllide reductase. The light intensity dependence of the process has been studied *in vitro* using wheat etioplast membranes. The results suggest that a single photochemical event is involved in the photoconversion. In support of this conclusion we also demonstrate that illumination of these membranes with a train of ultrashort (150 fs) flashes resulted in chlorophyll accumulation. The significance of the findings in terms of possible mechanisms for the reductase are discussed.

**Key words:** Protochlorophyllide reductase;  
Light intensity dependence; Photoenzyme mechanism

## 1. Introduction

The enzyme protochlorophyllide reductase (EC 1.3.1.33) catalyses the light-dependent reduction of protochlorophyllide during chlorophyll synthesis in plants as a key step in the development of chloroplasts. In the reaction, NADPH serves as reductant and forms a dark-stable ternary complex with the enzyme and pigment. Light absorption by this complex induces the reduction resulting eventually in the *trans* addition of hydrogen to the C17 and C18 positions of the pigment to produce chlorophyllide (see [1,2] for reviews). Although this reaction has been extensively studied with a wide range of techniques over several decades, the mechanism of the process is still very poorly understood. In particular, the intriguing role of light in the reaction remains obscure. Interestingly, a light-independent system for protochlorophyllide reduction but involving at least three distinct gene products exists in photosynthetic bacteria and non-flowering plants but details of this process are again completely unknown [3].

Crucial to our understanding of the mechanism and role of light in protochlorophyllide reduction is the number of quanta or photochemical steps involved in the overall process. In the reaction, 2H atoms (2e<sup>-</sup> and 2H<sup>+</sup>) are transferred to the protochlorophyllide. Does this result from two distinct photo-induced single electron transfer reactions such as in the light reactions of photosynthesis or, alternatively, can a single photochemical event bring about direct transfer of 2 electrons (e.g. as a hydride), as occurs mechanistically in the conventional dehydrogenases? Evidence supporting both these possibilities is present in the extensive literature on photoconver-

sion. The ultra-fast rate of the process [4–6] suggests chlorophyll formation is possible from a single photoreaction whereas Litvin and co-workers maintain two successive [7] or parallel [8] photoreactions in the process.

In the current paper, we have studied the light dependence of protochlorophyllide photoconversion in isolated wheat etioplast membranes. The data on analysis suggest a single photoreaction in the process. It has also been demonstrated that such membranes are capable of accumulation of low, but finite levels of chlorophyllide when illuminated with a train of ultra-short femtosecond flashes. The significance of the data is discussed in terms of possible mechanisms of photoconversion.

## 2. Materials and methods

Etioplast membrane samples were isolated in Bristol from 7-day-old dark-grown wheat (*Triticum aestivum* var. Avalon) as previously described [9]. These were resuspended in buffer II [9]. Before illumination, this material was diluted in buffer II containing 1 mM NADPH and the mixture incubated in darkness in a 1 cm spectrophotometer cuvette on ice for at least 15 min to maximise the level of the photoactive reductase-substrates complex. Photoconversion was effected by 3 different procedures depending on the experiment. (1) With a 60 W tungsten lamp at a distance of approx. 10 cm for 30 s for maximum photoconversion. Extent was noted from the absorption spectra taken before and after illumination as previously described [9]. (2) Flash illumination with a xenon flash (4 μs/20 mJ). The transmission of a 2 μs probe beam was monitored immediately before and 45 ms after the actinic flash using a photodiode array detector as described [10,11]. The intensity of the actinic flash was varied by neutral density filters and the extent of the flash-induced photoconversion estimated from the series of light minus dark difference spectra as described previously [9]. (3) A 30 s train of 150 fs (200 μJ) pulses, wavelength 590 nm, repetition rate 540 Hz. During this treatment, the sample was manually mixed by a stirrer in the cuvette to achieve uniform illumination. Finally, the absorption spectrum of the sample was recorded on a conventional spectrophotometer.

## 3. Results

The effect of illumination (30 s from a 60 W lamp) on the absorption spectrum of wheat etioplast membranes is shown in Fig. 1. In the dark sample, the spectrum shows twin absorption peaks at around 635 and 652 nm which, on illumination, are replaced by a smaller peak at 632 nm and a more intense band at approx. 680 nm. This change is characteristic of the photoconversion of reductase-bound protochlorophyllide in the dark sample ( $\lambda_{\text{max}}$  at ~638 and 652 nm) to chlorophyllide ( $\lambda_{\text{max}}$  680 nm) on illumination. The small residual absorption at 632 nm seen in the latter (Fig. 1) represents non-photoactive pigment invariably found to a greater or lesser extent in such preparations. The extent of protochloro-

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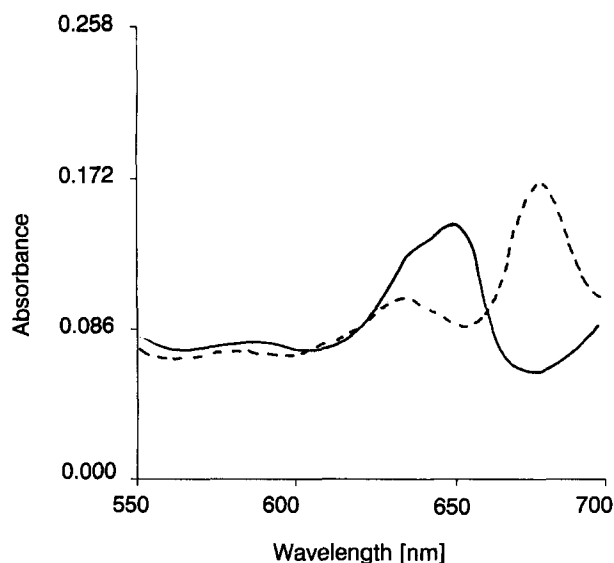


Fig. 1. Absorption spectra of dark and illuminated membranes. Wheat etioplast membranes (1.2 mg protein/3.0 ml buffer II) were incubated in darkness with 1 mM NADPH for 20 min and spectra recorded before (—) and after (---) illumination for 30 s with a 60 W tungsten lamp as previously described [9].

phyllide photoconversion in this preparation, as estimated from the spectra, is approx. 80%.

Fig. 2 shows an example of the light minus dark difference spectra obtained from the flash/diode array spectrometer. The actinic light in this case was from the unattenuated flash corresponding to the maximum light intensity. The flash-induced increase in absorbance at 680 nm was measured for different actinic light intensities ranging between 100 and 7.9% of the maximum.

The relationship between the light induced  $\Delta 680$  nm (as a measure of photoconversion) and actinic light intensity is plotted in Fig. 3. The data are overlaid by 2 theoretical plots derived from two different exponential equations describing one (solid line) and two photon (dashed line) models [12]. While the experimental data display a certain degree of scatter from both curves, they fit more closely over the entire light intensity range to the theoretical plot for the one photon model.

A photon of light is absorbed within  $10^{-15}$  s. The inference from Fig. 3 that protochlorophyllide photoconversion by protochlorophyllide reductase is a single photon process implies that an absorbed femtosecond pulse should be capable of forming chlorophyllide. Fig. 4 shows the spectrum resulting from illumination of etioplast membranes with a 30 s train of 150 fs 590 nm pulses at a repetition rate of 540 Hz. Under these conditions, extensive bleaching of the sample occurs. Despite this, the spectrum of the resulting sample, when compared with the unflashd membranes (Fig. 4) shows a distinct absorption at  $\sim 680$  nm indicating the accumulation of a small but definite amount of chlorophyllide under these conditions. The non-specific pigment bleaching observed, however (compare Figs. 1 and 4), makes it difficult to quantify the progress of photoconversion from the spectrum.

#### 4. Discussion

Extensive spectroscopic studies on the photoreduction of

protochlorophyllide in vitro have been carried out over many years and some biochemical properties of the system have been described [1]. Despite these, no consensus view on the molecular mechanism of the process has yet emerged. Recently, genetic studies have been described of heterologous expression of the active reductase to complement bacteriochlorophyll-deficient mutants of the photosynthetic bacterium *Rb. capsulatus*. [14,15]. Mutagenesis-based investigations of the reductase will be possible with these systems and they also offer possibilities of large-scale isolation of the enzyme for structural and mechanistic studies. Progress in such areas, however, will rely heavily on knowledge of the molecular mechanism of protochlorophyllide photoreduction. In this report, the fundamental basics of such a mechanism are discussed.

The photoconversion, which involves transfer of 2H atoms to protochlorophyllide, generates chlorophyllide very rapidly on illumination ( $t_{1/2} = 6-9$   $\mu$ s) [4]. Between absorption of light in  $10^{-15}$  s and this time, several spectroscopic intermediates have been detected. These include various metastable states of protochlorophyllide  $X_1$  and  $X_2$  absorbing, like protochlorophyllide, at around 640 nm and formed within approx. 2 ( $X_1$ ) and 250 ns ( $X_2$ ), respectively, and  $X_3$  or  $X_{690}$  formed within approx. 1  $\mu$ s.  $X_{690}$  decays within approx. 10 ms coincidental with the appearance of chlorophyllide [4,16]. While the rates of formation and decay of these forms show different temperature sensitivities, their precise chemical nature is unclear. The question of whether one or more photoreactions are involved in the process has not been seriously addressed. It is generally assumed that a single photochemical event drives the process despite the fact that it involves the transfer of 2H atoms. The experimental evidence that is available is contradictory, e.g. quantum yield measurements, though difficult to interpret, have produced values of approx. 0.5 [17]. Direct spectroscopic evidence in support of two successive photochemical reactions in the process have also been reported [7]. Again, from theoretical energetic considerations, it can be argued that the free energy available from a single quantum of red (650 nm) light and the oxidation of NADPH may be insufficient to drive the porphyrin to chlorin reduction.

Despite this, the evidence presented in this report, based largely upon studying the light intensity dependence of the photoconversion process in wheat etioplast membranes, sug-

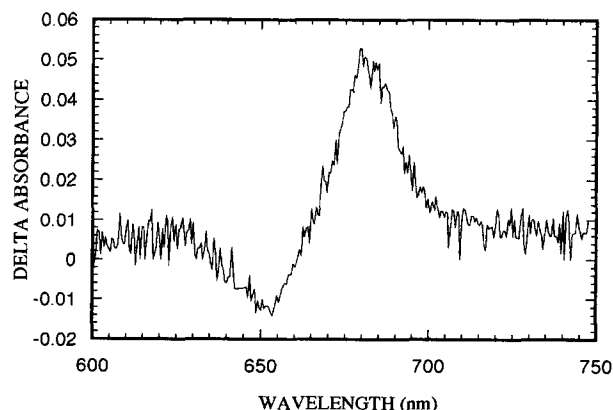


Fig. 2. Flash-induced absorbance changes in wheat etioplast membranes. The curve shows the change observed using the unattenuated flash i.e. 100% intensity. Samples, prior to illumination, were preincubated with 1 mM NADPH as described in Section 2.

gests it is driven by a single photochemical reaction. A plot of the light intensity dependence of the photoconversion coincides more closely with the theoretical plot derived for a single, rather than a two photon process (Fig. 3). Furthermore, the slope of a linearised plot of the low intensity data matches closely the corresponding value derived from a theoretical one photon model (data not presented). In support of this conclusion, a small but significant accumulation of chlorophyllide is observed after illuminating the sample with a sequence of femtosecond flashes spaced at 1.8 ms apart (Fig. 4). Such illumination would not be expected to produce a stable product if more than one photoreaction is involved in its formation, since the product of the initial light reaction would not survive the relatively long time between successive pulses.

This conclusion has important consequences regarding the mechanism of protochlorophyllide reductase. Considering the overall reaction catalysed by the enzyme, the data suggest that the absorbed quantum must effect the direct transfer of two electrons (as hydride?) from the co-enzyme to the pigment. Such an event could be initiated by the photogeneration of an electrophilic centre at C17 of protochlorophyllide. This could be formed as a consequence of light absorption by the pigment in the specialised environment of the active site leading to a splitting of one of the bonds between C17 and C18 to generate an intermediate biradical followed by electron transfer from C17 to C18. The resulting positive charge at C17 could constitute the site for nucleophilic attack by the hydride anion from the active site NADPH to generate the anion [Pchl<sub>id</sub>-H]<sup>-</sup> followed by neutralisation of the negative charge at C18 with a proton, from the protein or medium, to produce chlorophyllide. This mechanism is at odds with published models for the photoconversion process which maintain an anion radical of the pigment generated in a flavin-mediated

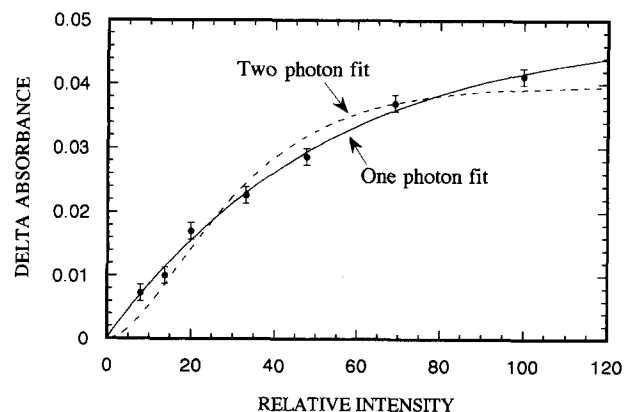


Fig. 3. Effect of light intensity on the absorbance change in isolated wheat etioplast membranes. Difference spectra were recorded as described in Fig. 2 but using a range of actinic light intensities, from 7.9 to 100%. Attenuation was by neutral density filters. The figure shows the actual data (circles) together with fits to the two different exponential equations [10,13] derived for the one (solid line) and two (dashed line) photon models. The equation used for the one photon model was  $Y = Y_{\max}[1 - \exp(-x)]$  where  $x$  is the product of the light intensity and a constant, reflecting the absorption cross-section and quantum efficiency. The term  $\exp(-x)$  denotes the probability that a centre will receive no hits. The equation used for the two photon model was  $Y = Y_{\max}[1 - \exp(-x) - x \exp(-x)]$ . The term  $x \exp(-x)$  denotes the probability that a centre will receive one hit. The two photon model assumes that the quantum efficiency and absorption cross-section for the two photochemical processes are the same.

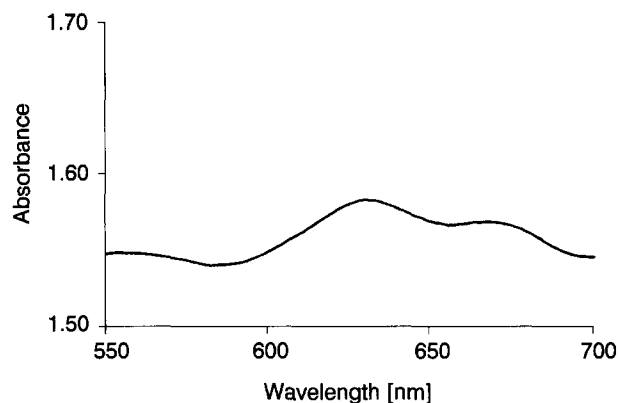


Fig. 4. Absorption spectrum of membranes after illumination with femtosecond flashes. Etioplast membranes, as described in Fig. 1 were flash illuminated with a 30 s train of 150 fs, 590 nm pulses at a repetition rate of 540 Hz. The absorption spectrum of the sample minus a scattering milk blank was finally recorded.

process as the initial photoproduct [8,18]. However, the recent demonstration of reductase activity in a protein purified from an *E. coli* transformant expressing the higher plant reductase gene in the absence of flavin contradicts such models (Wilks, H.M., Griffiths, W.T. and Teakle, G.R., in preparation).

Details of the true reaction mechanism will only be established following in depth characterisation of the experimentally observed transients that accompany the photoconversion process (see above). The ultimate precursor of protochlorophyllide observed experimentally is  $X_{690}$  [4] which, according to our mechanism above, equates with the [Pchl<sub>id</sub>-H]<sup>-</sup> anion. We have some indirect evidence for such an identity. The effect of pH, NADPD and D<sub>2</sub>O on the rate of conversion of  $X_{690}$  to chlorophyllide in etioplast membranes has been studied and appears compatible with this process as being a H<sup>+</sup> uptake thereby supporting identification of  $X_{690}$  as [Pchl<sub>id</sub>-H]<sup>-</sup> ([19]; Oliver, R.P., Griffiths, W.T. and Mathis, P., unpublished). The formation of  $X_{690}$  by nucleophilic attack of H<sup>-</sup> from the NADPH specifically at C<sub>17</sub> of the pigment [20] could be greatly facilitated by the special organisation of the two substrates at the enzymes' active site, with the actual transfer initiated by light absorption by the complex. Various protein-induced conformational/structural changes in the two substrates might be expected to mediate this transfer. Such changes could well explain some of the rapid spectral transients that have been reported to be associated with protochlorophyllide reduction (see above) the complete characterisation of which are anticipated in the near future.

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