

Spectroscopic and kinetic characterization of the light-dependent enzyme protochlorophyllide oxidoreductase (POR) using monovinyl and divinyl substrates

Derren J. HEYES^{*1}, Jerzy KRUK[†] and C. Neil HUNTER^{*}

^{*}Robert Hill Institute for Photosynthesis and Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, U.K., and [†]Faculty of Biotechnology, Jagiellonian University, ul. Gronostajowa 7, 30-387 Kraków, Poland

The enzyme POR [Pchlde (protochlorophyllide) oxidoreductase] catalyses the reduction of Pchlde to chlorophyllide, which is a key step in the chlorophyll biosynthesis pathway. This light-dependent reaction has previously been studied in great detail but recent reports suggest that a mixture of MV (monovinyl) and DV (divinyl) Pchldes may have influenced some of these properties of the reaction. Low-temperature absorbance and fluorescence spectroscopy have revealed several spectral differences between MV and DV Pchldes, which were purified from a *Rhodobacter capsulatus* strain that was shown to contain a mixture of the two pigments. A thorough steady-state kinetic characterization using both Pchlde forms demonstrates that neither pigment appears to affect the kinetic properties of the enzyme. The reaction has also been monitored following illumination at low temperatures and was shown to consist of an initial photochemical step fol-

lowed by four 'dark' steps for both pigments. However, minor differences were observed in the spectral properties of some of the intermediates, although the temperature dependency of each step was nearly identical for the two pigments. This work provides the first detailed kinetic and spectroscopic study of this unique enzyme using biologically important MV and DV substrate analogues. It also has significant implications for the DV reductase enzyme, which is responsible for converting DV pigments into their MV counterparts, and its position in the sequence of reactions that comprise the chlorophyll biosynthesis pathway.

Key words: chlorophyll biosynthesis, chlorophyllide, divinyl, low-temperature absorbance, monovinyl, protochlorophyllide oxidoreductase.

INTRODUCTION

The chlorophyll biosynthesis enzyme POR [Pchlde (protochlorophyllide) oxidoreductase; NADPH:protochlorophyllide oxidoreductase; EC 1.3.1.33] catalyses the reduction of the C-17–C-18 double bond of the *D*-ring of Pchlde to form Chlide (chlorophyllide; Scheme 1) [1,2]. The reaction is driven by light and consequently it is an important regulatory step in the chlorophyll biosynthetic pathway and in the subsequent assembly of the photosynthetic apparatus [2]. It is one of the only two enzymes known to require light for catalysis; the other is DNA photolyase [3]. In addition to POR, non-flowering land plants, algae and cyanobacteria possess a light-independent Pchlde reductase, consisting of three separate subunits, which is able to operate in the dark [4].

POR is a member of the 'RED' (Reductases, Epimerases, Dehydrogenases) superfamily of enzymes [5,6], which generally catalyse NADP(H)- or NAD(H)-dependent reactions involving hydride and proton transfers [7]. Indeed, a homology model of POR has been constructed using other family members as a structural template [8]. All members of this large protein family contain highly conserved Tyr and Lys residues, which have been shown to be essential for activity. In the reaction catalysed by POR, it has been proposed that the conserved Tyr residue donates a proton to the C-18 position with the Lys residue thought to be important for lowering the apparent pK_a of the Tyr, allowing deprotonation to occur [5]. The hydride is transferred from the *pro-S* face of NADPH to the C-17 position of the Pchlde molecule [9,10].

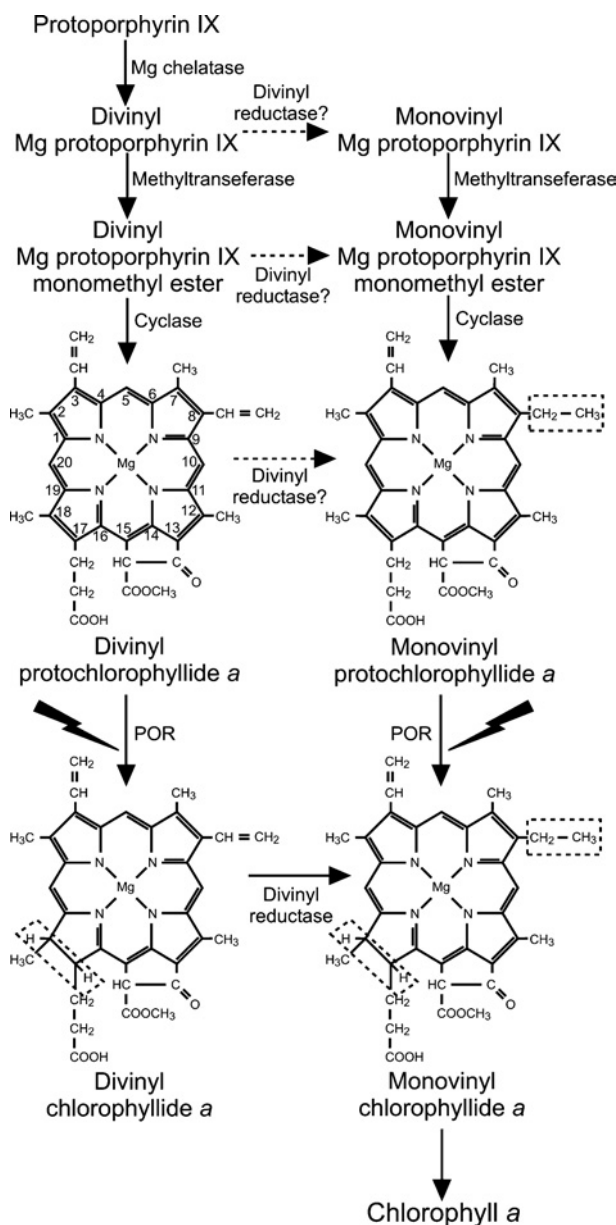
The fact that POR is light-activated means the enzyme–substrate complex can be formed in the dark, removing the diffusive

components out of the reaction. This has recently been exploited by studying Pchlde reduction at low temperatures to trap intermediates in the reaction pathway [11–13]. As a result, the reaction has been shown to consist of at least three distinct steps: an initial light-driven step, followed by a series of 'dark' reactions. An initial photochemical step can occur below 200 K [11], whereas two 'dark' steps were identified for *Synechocystis* POR, which can only occur close to or above the 'glass transition' temperature of proteins [12]. This implies a role for protein motions during these stages of the catalytic mechanism. A thermophilic form of the enzyme has been used to identify two additional 'dark' steps, which were shown to represent a series of ordered product release and cofactor binding events. First, NADP⁺ is released from the enzyme and then replaced by NADPH, before release of the Chlide product and subsequent binding of Pchlde have taken place [13]. Additionally, the reaction has been shown to proceed on an ultrafast timescale after catalysis was initiated with a 50 fs laser pulse. This suggests that the dynamic motions that accompany catalysis must therefore occur on similar short timescales [14].

By using a range of Pchlde analogues, it has been possible to gain information about the structural regions of the pigment that are essential for catalysis [15]. Pchlde analogues with different side chains at the C-17 position could not be used as substrates by POR from oat etioplasts, which suggests that the free carboxylic acid group (C-17) plays an important role in the binding of the pigment to the enzyme. Similarly, the central Mg atom and the structure of the isocyclic ring of the Pchlde molecule have also been shown to be crucial for activity. Conversely, the enzyme does not differentiate between vinyl and ethyl groups at the C-8 position of ring B as both MV (monovinyl) and DV (divinyl)

Abbreviations used: Chlide, chlorophyllide; DV, divinyl; MV, monovinyl; Pchlde, protochlorophyllide; POR, Pchlde oxidoreductase.

¹ To whom correspondence should be addressed at the present address: Faculty of Life Sciences, University of Manchester, Jackson's Mill, Manchester M60 1QD, U.K. (email derren.hey@manchester.ac.uk).



Scheme 1 Scheme of the chlorophyll biosynthesis pathway, illustrating the possible locations of the DV reductase enzyme

POR catalyses the reduction of the C-17–C-18 double bond of Pchl_{ide} and DV reductase catalyses the reduction of the vinyl group at the C-8 position of the tetrapyrrole molecule to an ethyl group. As MV and DV Pchl_{ides} are accepted equally as substrates by POR, we have shown that DV reductase can operate before or after the reduction of Pchl_{ide}. Therefore DV Chl_{ide} must act as a substrate for DV reductase (solid arrow), whereas the dashed arrows indicate DV reductase reactions that are still to be established. The dashed boxes indicate the groups that are reduced during each reaction and the IUPAC numbering system of the tetrapyrrole ring is shown.

forms of Pchl_{ide} can be converted into the respective Chl_{ides} [15]. However, there are currently no quantitative kinetic or spectroscopic data to establish the relative merits of the MV and DV forms of Pchl_{ide} as substrates for POR.

An additional reason for requiring such data arises from recent reports suggesting that impurities in the pigment preparations or the use of a mixed pool of MV and DV Pchl_{ides} may influence the kinetics and spectral properties of the POR-catalysed reaction [16,17]. Therefore it is important to analyse the reaction by using

highly purified preparations of MV and DV Pchl_{ide}. In addition, a preference for MV or DV Pchl_{ide} may be an important factor in determining the exact location in the pathway where the DV reductase enzyme [18,19] is able to operate. This enzyme has recently been identified in *Arabidopsis thaliana* and is proposed to catalyse the conversion of either DV Pchl_{ide} or DV Chl_{ide} into the respective MV pigments (Scheme 1) [18]. In the present study, MV and DV forms of Pchl_{ide} have been purified, allowing the first detailed kinetic and spectroscopic characterization of the reaction with both pigments.

MATERIALS AND METHODS

Preparation and separation of MV and DV Pchl_{ides}

Pchl_{ide} was isolated from *Rhodobacter capsulatus* ZY5 cultures as described previously [12]. MV and DV Pchl_{ides} were purified on a preparative scale by HPLC using C30 reverse-phase column (3 μm , 250 mm \times 4.6 mm; YMC Europe GmbH) as described previously [17]. For analytical purposes, MV and DV Pchl_{ides} were separated using the same column in methanol/acetonitrile/hexane (7:2:1, by vol.) at 0.8 or 1 ml \cdot min⁻¹ flow rate and absorption detection at 437 nm. The equipment used was a Jasco PU-980 Pump, a Jasco UV-970 UV/VIS detector and Borwin v. 1.5 software.

Steady-state measurements

POR from the cyanobacterium *Thermosynechococcus elongatus* BP-1 was overproduced and purified from *Escherichia coli* as described previously [13]. Activity assays were carried out as described previously [20] in the presence of 5 μM NADPH. The K_m for Pchl_{ide} and V_{max} values were obtained by fitting the initial rates of Chl_{ide} synthesis against the concentration of Pchl_{ide} using the following equation:

$$v = \{V_{max}(\text{app}) [\text{Pchl}_{ide}]\} / \{K_m(\text{app}) + [\text{Pchl}_{ide}]\} \quad (1)$$

where v is the initial rate, $V_{max}(\text{app})$ is the initial rate achieved as $[\text{Pchl}_{ide}]$ approaches infinity and $K_m(\text{app})$ is the apparent value of $[\text{Pchl}_{ide}]$ giving $V_{max}/2$. The data were fitted and standard errors calculated by nonlinear regression analysis using the Sigma Plot program (SPSS).

The binding of Pchl_{ide} to POR was measured by performing 77 K fluorescence emission spectra as described previously [11]. The apparent K_d values were obtained by fitting the fluorescence changes against the concentration of POR using the following equation:

$$\Delta F = (\Delta F_{max} [\text{POR}]) / \{ (K_d + [\text{POR}]) + F_0 \} \quad (2)$$

where ΔF is the change in the ratio of fluorescence emission at 631 nm/644 nm upon binding to Pchl_{ide}, ΔF_{max} is the apparent maximum change in the fluorescence ratio, K_d is the apparent dissociation constant for Pchl_{ide} binding to POR and F_0 is the initial ratio of fluorescence emission at 631 nm/644 nm. The data were fitted and standard errors were calculated by nonlinear regression analysis using the Sigma Plot program (SPSS).

Low-temperature absorbance and fluorescence spectroscopy measurements

All spectra were measured in 44% (v/v) glycerol, 20% (w/v) sucrose, 50 mM Tris/HCl (pH 7.5), 0.1% (v/v) Genapol X-080 and 0.1% (v/v) 2-mercaptoethanol and samples were maintained at the required temperature using an OpstatatDN nitrogen bath cryostat (Oxford Instruments). The temperature of the sample

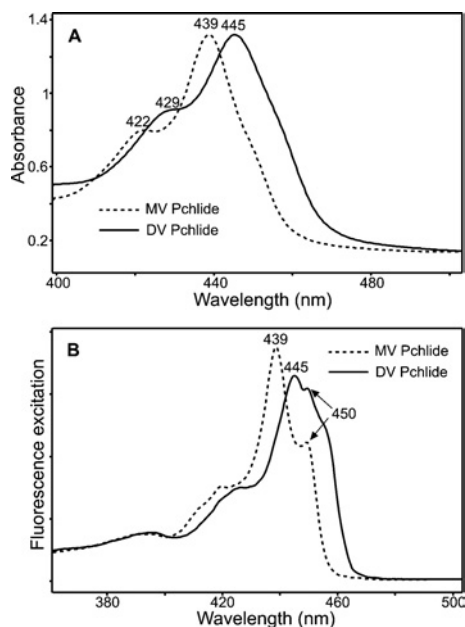


Figure 1 Low-temperature absorbance and fluorescence excitation spectra of MV and DV Pchlides

(A) The 77 K absorbance spectra of MV and DV Pchlides (6.5 μM). (B) The 77 K fluorescence excitation spectra of the 632 and 631 nm emission bands of MV and DV Pchlides (1.1 μM). The spectra are normalized for ease of clarification.

was monitored directly with a thermocouple sensor (Comark) and intermediates in the reaction pathway were isolated as described previously [12]. Fluorescence spectra were recorded using a SPEX FluoroLog spectrofluorimeter (Jobin Yvon) at 77 K. The exciting light was provided from a xenon light source, using excitation monochromator slit widths of 4.5 nm and emission monochromator slit widths of 3.6 nm. Absorbance spectra were recorded with a Cary 500 Scan UV–visible–NIR (Varian) spectrophotometer at 77 K. Normalization of the spectra, spectral deconvolutions and difference spectra were calculated using the Galactics software.

RESULTS

Analysis of Pchlide from *R. capsulatus*

Analysis of the Pchlide obtained from *R. capsulatus* cells showed that the pigment was a mixture of DV and MV Pchlides in a 5:1 molar ratio (results not shown). The MV and DV Pchlides were purified on a C30 reverse-phase column and the pigments that were used for all further experiments showed no cross-contamination.

Spectroscopic differences between MV and DV Pchlides

The MV and DV Pchlides were characterized by using 77 K absorbance and fluorescence spectroscopy. Low-temperature absorbance measurements revealed that MV Pchlide has an absorbance maximum at 629 nm and that this peak is slightly red-shifted to 630 nm for DV Pchlide (results not shown). However, in the Soret region, there are more considerable differences in the spectra of the two pigments. MV Pchlide has two main absorbance bands at 422 and 439 nm, whereas DV Pchlide has two major absorbance peaks at 429 and 445 nm (Figure 1A). A similar phenomenon is also observed for the low-temperature fluorescence spectra. MV Pchlide has a single fluorescence band at 631 nm, whereas DV Pchlide has a fluorescence maximum at 632 nm (results not

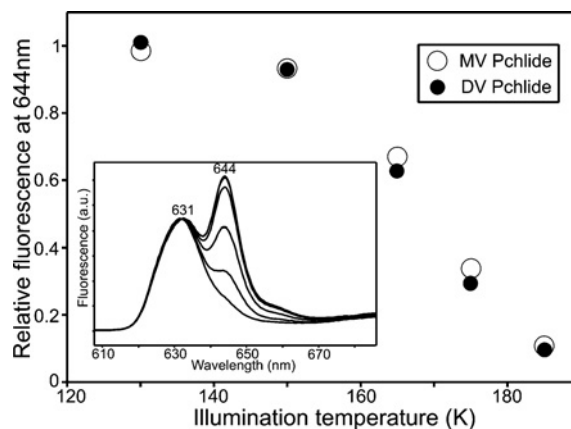


Figure 2 Characterization of the initial light-dependent step of the reaction for MV and DV Pchlides

The inset shows the 77 K fluorescence emission spectra of samples containing 1.1 μM DV Pchlide, 30 μM POR and 200 μM NADPH after illumination for 10 min at 130, 150, 165, 175 and 185 K. The intensity of the fluorescence band at 644 nm was measured at 77 K using an excitation wavelength of 450 nm. The temperature dependence of the initial photochemical step for MV and DV Pchlides, derived from these spectra, is shown. The decrease in fluorescence after illumination for 10 min at the respective temperatures was calculated relative to the largest decrease.

shown). Figure 1(B) shows the excitation spectra of these emission bands, which further emphasizes the distinctive properties of the two pigments. The fluorescence band at 631 nm, arising from MV Pchlide, has two main excitation peaks at 439 and 450 nm, whereas the excitation spectrum of the DV Pchlide band at 632 nm has two maxima at 445 and 450 nm and a shoulder at 457 nm.

Steady-state kinetic and binding measurements

The kinetic parameters for the reaction were measured over a range of MV and DV Pchlide concentrations with NADPH in excess. For both pigments, the dependence of the initial rate on Pchlide concentration followed Michaelis–Menten kinetics. The V_{max} was determined to be $0.53 \pm 0.05 \mu\text{M} \cdot \text{min}^{-1}$ for MV Pchlide and $0.61 \pm 0.05 \mu\text{M} \cdot \text{min}^{-1}$ for DV Pchlide. The K_{m} for MV Pchlide was calculated to be $1.36 \pm 0.34 \mu\text{M}$ and the K_{m} for DV Pchlide was $0.92 \pm 0.33 \mu\text{M}$.

The binding of both pigments to the enzyme has been monitored by measuring Pchlide fluorescence emission spectra at 77 K. The binding constant was determined by measuring the ratio of the bound (644 nm) and unbound (631 nm) Pchlide peaks at a range of enzyme concentrations. Under these conditions, the apparent dissociation constant was calculated to be $1.37 \pm 0.28 \mu\text{M}$ for the binding of MV Pchlide and $0.83 \pm 0.24 \mu\text{M}$ for the binding of DV Pchlide.

Initial light-driven step

Five distinct steps in the reaction pathway have previously been identified for the *T. elongatus* enzyme, an initial light-driven one followed by four ‘dark reactions’ [13]. In the present study, all of these steps have been analysed for both the MV and DV Pchlides by using low-temperature fluorescence measurements. The initial photochemistry, which involves the conversion of the POR–Pchlide–NADPH fluorescence band at 644 nm into a non-fluorescent intermediate (Figure 2, inset), was almost identical for the two pigments. A plot of the temperature dependence revealed that the light-driven step occurred over the same temperature range for both the MV and DV Pchlides (Figure 2).

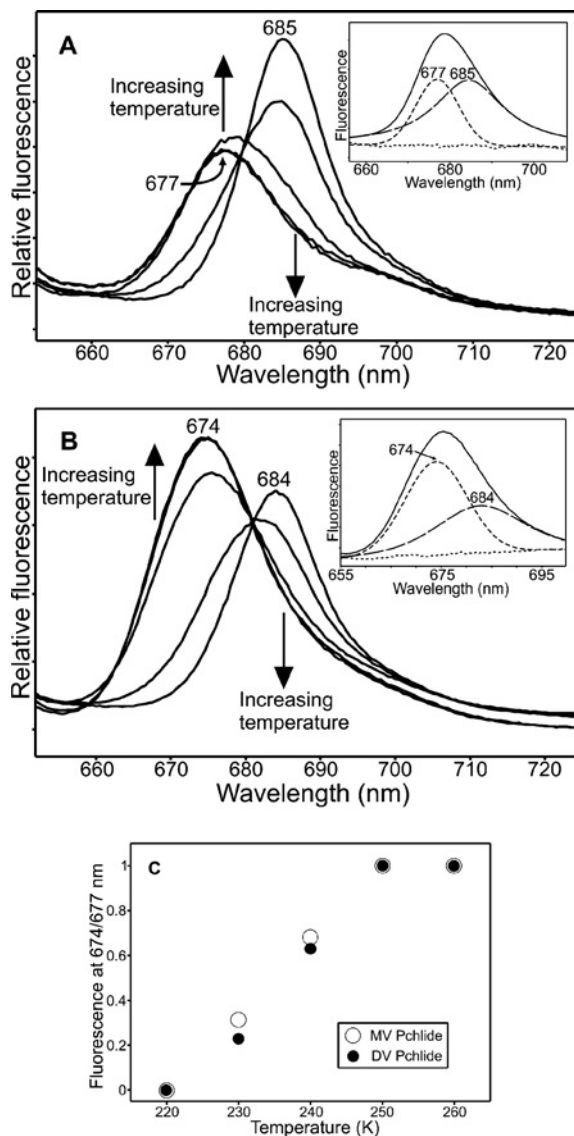


Figure 3 Spectroscopic characterization of the second 'dark' step of the reaction for MV and DV Pchlides

The 77 K fluorescence emission spectra of samples containing 1.1 μM MV Pchlides (A) or DV Pchlides (B), 30 μM POR and 200 μM NADPH after illumination for 10 min at 185 K and incubation in the dark for 10 min at 220, 230, 240, 250 and 260 K are shown. The arrows indicate the formation of a fluorescent band at either 677 nm (MV Pchlides) or 674 nm (DV Pchlides) and the disappearance of the fluorescent band at either 685 nm (MV Pchlides) or 684 nm (DV Pchlides) at increasing temperatures. Spectra were recorded with an excitation wavelength of 450 nm. The respective insets show deconvolutions of the 240 K spectra with the residuals represented by the small dotted lines. (C) The temperature dependence of the second 'dark' step was calculated by measuring the relative increase in fluorescence at either 677 nm (MV Pchlides) or 674 nm (DV Pchlides) over the temperature range 220–260 K.

The 'dark' steps

Any differences that may exist between the two pigments during the subsequent 'dark' reactions have also been explored. All samples were illuminated at 185 K for 10 min to trigger the initial photochemical step and were then warmed to progressively higher temperatures between 185 and 330 K in the dark. During the first 'dark' step, the non-fluorescent intermediate is converted into a new state that has previously been shown to represent a POR–Chlide–NADP⁺ complex. For the MV pigment, this new

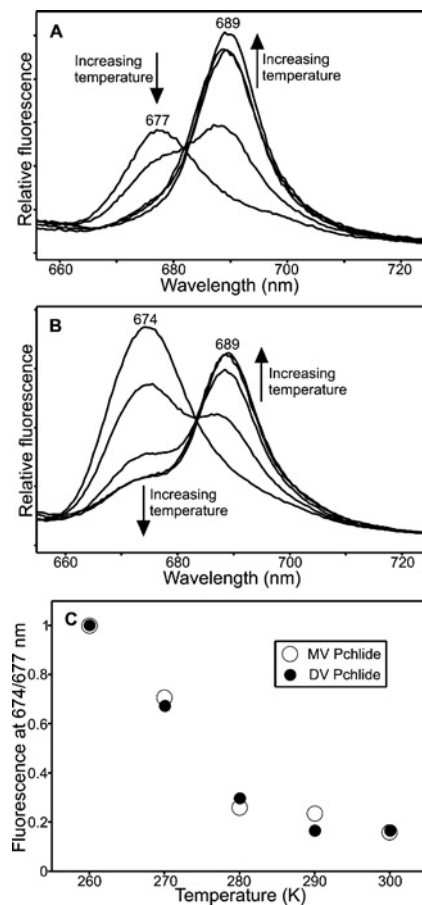


Figure 4 Spectroscopic characterization of the third 'dark' step of the reaction for MV and DV Pchlides

The 77 K fluorescence emission spectra of samples containing 1.1 μM MV Pchlides (A) or DV Pchlides (B), 30 μM POR and 200 μM NADPH after illumination for 10 min at 185 K and incubation in the dark for 10 min at increasing temperatures. Samples were incubated at 260, 270, 280, 290 and 300 K are shown. The formation of the fluorescence band at 689 nm and the simultaneous disappearance of the fluorescence band at either 677 nm (MV Pchlides) or 674 nm (DV Pchlides) at higher temperatures are indicated by the arrows. Spectra were recorded with an excitation wavelength of 450 nm. (C) The temperature dependence of the third 'dark' step was calculated by measuring the relative decrease in fluorescence at either 677 nm (MV Pchlides) or 674 nm (DV Pchlides) over the temperature range 260–300 K.

species fluoresces at 685 nm, but in the DV sample the fluorescent maximum is slightly blue-shifted at 684 nm (results not shown). The temperature dependence of this first 'dark' step, which is calculated by measuring the increase in fluorescence at 684 nm/685 nm, is very similar for both pigments (results not shown).

Conversely, significant differences between the two pigments are observed during the remaining 'dark' steps of the reaction. For both pigments, the second 'dark' step involves a decrease in the POR–Chlide–NADP⁺ fluorescence band at either 684 or 685 nm, but the states that are formed as a result exhibit differing spectral properties. In the MV samples, the new fluorescence band is centred at 677 nm with a half-bandwidth of 12 nm (Figure 3A), whereas in the DV samples the new species has a fluorescence peak at 674 nm with a half-bandwidth of 15 nm (Figure 3B). In addition, the fluorescence yield from the DV species at 674 nm is considerably greater than that from the MV species at 677 nm. The temperature dependence of this step, which is obtained by measuring the increase in fluorescence intensity at either 674 or

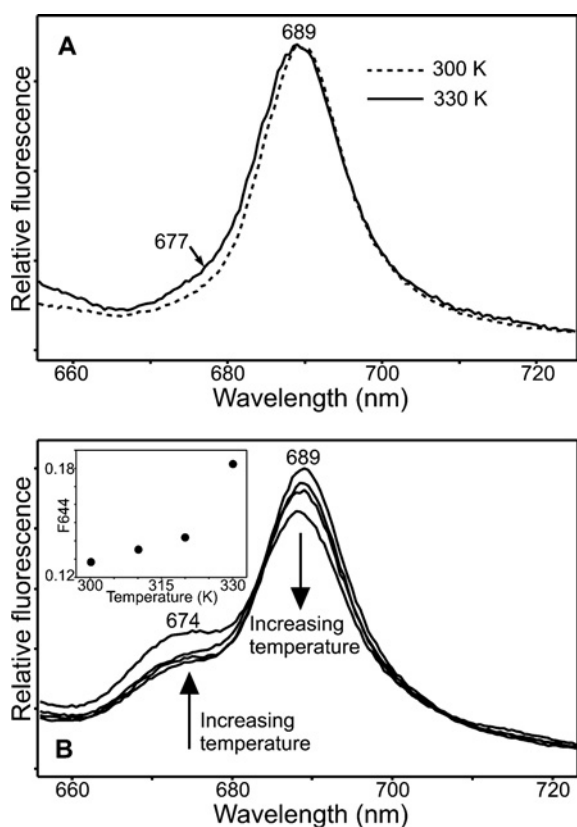


Figure 5 Spectroscopic characterization of the fourth 'dark' step of the reaction for MV and DV Pchlides

(A) The 77 K fluorescence emission spectra of samples containing $1.1 \mu\text{M}$ MV Pchlides, $30 \mu\text{M}$ POR and $200 \mu\text{M}$ NADPH after illumination for 10 min at 185 K and incubation in the dark for 10 min at either 300 or 330 K are shown. Spectra were recorded with an excitation wavelength of 450 nm. (B) The 77 K fluorescence emission spectra of samples containing $1.1 \mu\text{M}$ DV Pchlides, $30 \mu\text{M}$ POR and $200 \mu\text{M}$ NADPH after illumination for 10 min at 185 K and incubation in the dark for 10 min at 300, 310, 320 and 330 K are shown. The arrows indicate the formation of a fluorescence band at 674 nm and the disappearance of the fluorescence band at 689 nm at increasing temperatures. Spectra were recorded with an excitation wavelength of 450 nm. The inset shows the temperature dependence of the fourth 'dark' step for DV Pchlides, which was calculated by measuring the relative decrease in fluorescence at 674 nm over the temperature range 300–330 K.

677 nm, shows that it can occur between 220 and 260 K and is almost identical between the two pigments (Figure 3C).

For both pigment types, the third 'dark' step involves the disappearance of the fluorescence band at either 674 or 677 nm together with the formation of a new state, which fluoresces at 689 nm (Figures 4A and 4B). The temperature dependence of the third 'dark' step, which can be measured by plotting out the relative fluorescence at either 674 or 677 nm, revealed that it could occur over the same temperature range for both the MV and DV pigments (Figure 4C).

During the fourth 'dark' step, the state that fluoresces at 689 nm appears to be converted back into a species that fluoresces at either 674 or 677 nm (Figure 5). In the MV samples, this spectral change is rather subtle with only a small increase in fluorescence at 677 nm as the temperature is increased (Figure 5A). However, the conversion is much more apparent for the DV samples, allowing a temperature dependence of this fourth 'dark' step to be measured by plotting out the relative fluorescence at 674 nm over a range of temperatures (Figure 5B, inset).

DISCUSSION

The reduction of Pchlides to Chlides, catalysed by the light-dependent enzyme POR, is an important reaction in the chlorophyll biosynthetic pathway [2]. The steady-state kinetic properties of the enzyme from a range of organisms have previously been measured [11, 15, 20–25] and the reaction has also been analysed at low temperatures in order to trap intermediates in the reaction pathway [11–13]. Consequently, it was shown to consist of an initial light-dependent reaction [11] followed by a series of 'dark' reactions that involve a series of ordered product release and substrate re-binding events [12, 13]. However, it has been suggested that some of these properties of the reaction may be affected by using a mixture of MV and DV Pchlides [16, 17]. Therefore in the present study, both MV and DV forms of Pchlides have been purified and all aspects of the reaction have been studied using both pigment types.

Many previous studies on POR have used Pchlides preparations from a strain of *R. capsulatus* that has a mutation in one of the subunits of the light-independent Pchlides reductase and is unable to reduce Pchlides to Chlides [12]. The pigment content from this *R. capsulatus* strain has now been analysed and shown to consist of a mixture of MV and DV Pchlides with DV Pchlides present at approx. 5-fold higher levels than MV Pchlides. Therefore this implies that the DV reductase enzyme [18, 19], which is responsible for converting the vinyl group at the C-8 position of the tetrapyrrole macrocycle into an ethyl group, can operate either before or after the reduction of Pchlides to Chlides (Scheme 1). This is in agreement with previous hypotheses suggesting that the reduction of the 8-vinyl group may occur at various steps of the chlorophyll biosynthesis pathway [19].

In order to explore the effects that the two pigment types may have on the POR-catalysed reaction, purified MV and DV forms of Pchlides were successfully obtained. The spectral properties of both pigments have been analysed using low-temperature absorbance and fluorescence spectroscopy. The spectra exhibit similar features to those previously reported at room temperature [16, 26] and reveal several differences between the MV and DV Pchlides. Consequently, this may help us to understand the complex spectral diversity of Pchlides forms occurring *in vivo* [27–30].

Most previous steady-state kinetic measurements on POR have been carried out using pigment preparations that are a mixture of MV and DV Pchlides. These measurements have now been repeated for POR from the thermophilic cyanobacterium *T. elongatus* using either purified MV or DV Pchlides. Both Pchlides types appear to be accepted equally as substrates by POR with only minor discrepancies between the V_{max} and K_m values obtained for each pigment. These kinetic parameters are also in very close agreement with those that were recently calculated for the same enzyme [20]. In addition, the K_d values for the binding of each pigment are also very similar although the DV Pchlides appears to bind slightly more tightly than the MV Pchlides. All of these measurements suggest that it is not necessary for the vinyl group at the C-8 position to be reduced for POR activity and provides further evidence that the DV reductase enzyme can operate before or after POR (Scheme 1). This is significant because if POR had only been capable of reducing MV Pchlides, then vinyl reduction would have to precede Pchlides reduction, whereas if DV Pchlides had been the only accepted substrate, then vinyl reduction must follow Pchlides reduction.

As POR is a light-driven enzyme, it is possible to trap intermediates in the reaction pathway by illumination at low temperatures. As a result, it has previously been shown that the reaction for the *T. elongatus* enzyme consists of the initial photochemistry followed by four 'dark' steps [13]. Each of these steps has now

been characterized and the temperature dependence measured using either MV or DV Pchl_{ide}. The reaction for both pigments was shown to proceed via the same five steps with only subtle differences in the spectral properties of some of the intermediates that are formed. The POR–Chlide–NADP⁺ complex, which is formed after the first ‘dark’ step, is very slightly red-shifted for the MV samples (685 nm) compared with the DV samples (684 nm). Similarly, the MV Chlide–POR state (677 nm), formed after the second ‘dark’ step, and the ‘free’ MV Chlide (677 nm) are both red-shifted with respect to their DV counterparts (674 nm). Hence, these discrepancies may help to explain some of the different spectral intermediates and Chlide forms that are formed *in vivo* [27–30]. However, apart from these minor differences, the reaction pathway was remarkably similar for both pigments with virtually identical temperature dependencies.

In conclusion, we have shown that previous studies, which have used Pchl_{ide} preparations from *R. capsulatus*, have indeed used a mixture of MV and DV pigments. However, neither pigment appears to influence the enzyme kinetics or the steps in the reaction pathway as has been previously suggested. These results also have implications for the DV reductase enzyme, which we have shown can operate before or after the reduction of Pchl_{ide}.

This work was supported by the Biotechnology and Biological Sciences Research Council, U.K.

REFERENCES

- Griffiths, W. T. (1978) Reconstruction of chlorophyllide formation by isolated etioplast membranes. *Biochem. J.* **174**, 681–692
- Heyes, D. J. and Hunter, C. N. (2005) Making light work of enzyme catalysis: protochlorophyllide oxidoreductase. *Trends Biochem. Sci.* **30**, 642–649
- Aubert, C., Vos, M. H., Mathis, P., Eker, A. P. and Brettel, K. (2000) Intraprotein radical transfer during photoactivation of DNA photolyase. *Nature (London)* **405**, 586–590
- Fujita, Y. and Bauer, C. E. (2000) Reconstitution of light-independent protochlorophyllide reductase from purified Bchl and BchN–BchB subunits – *in vitro* confirmation of nitrogenase-like features of a bacteriochlorophyll biosynthesis enzyme. *J. Biol. Chem.* **275**, 23583–23588
- Wilks, H. M. and Timko, M. P. (1995) A light-dependent complementation system for analysis of NADPH:protochlorophyllide oxidoreductase. Identification and mutagenesis of two conserved residues that are essential for enzyme activity. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 724–728
- Baker, M. E. (1994) Protochlorophyllide reductase is homologous to human carbonyl reductase and pig 20- β -hydroxysteroid dehydrogenase. *Biochem. J.* **300**, 605–607
- Jörnvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J. and Ghosh, D. (1995) Short-chain dehydrogenases/reductases (SDR). *Biochemistry* **34**, 6003–6013
- Townley, H. E., Sessions, R. B., Clarke, A. R., Dafforn, T. R. and Griffiths, W. T. (2001) Protochlorophyllide oxidoreductase: a homology model examined by site-directed mutagenesis. *Proteins* **44**, 329–335
- Valera, V., Fung, M., Wessler, A. N. and Richards, W. R. (1987) Synthesis of 4R- and 4S-tritium labeled NADPH for the determination of the coenzyme stereospecificity of NADPH:protochlorophyllide oxidoreductase. *Biochem. Biophys. Res. Commun.* **148**, 515–520
- Begley, T. P. and Young, H. (1989) Protochlorophyllide reductase. 1. Determination of the regiochemistry and the stereochemistry of the reduction of protochlorophyllide to chlorophyllide. *J. Am. Chem. Soc.* **111**, 3095–3096
- Heyes, D. J., Ruban, A. V., Wilks, H. M. and Hunter, C. N. (2002) Enzymology below 200 K: the kinetics and thermodynamics of the photochemistry catalyzed by protochlorophyllide oxidoreductase. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 11145–11150
- Heyes, D. J., Ruban, A. V. and Hunter, C. N. (2003) Protochlorophyllide oxidoreductase: spectroscopic characterization of the ‘dark’ reactions. *Biochemistry* **42**, 523–528
- Heyes, D. J. and Hunter, C. N. (2004) Identification and characterization of the product release steps within the catalytic cycle of protochlorophyllide oxidoreductase. *Biochemistry* **43**, 8265–8271
- Heyes, D. J., Hunter, C. N., van Stokkum, I. H. M., van Grondelle, R. and Groot, M. L. (2003) Ultrafast enzymatic reaction dynamics in protochlorophyllide oxidoreductase. *Nat. Struct. Biol.* **10**, 491–492
- Klement, H., Helfrich, M., Oster, U., Schoch, S. and Rudiger, W. (1999) Pigment-free NADPH:protochlorophyllide oxidoreductase from *Avena sativa* L. *Eur. J. Biochem.* **265**, 862–874
- Lebedev, N., Karginova, O., Mclvor, W. and Timko, M. P. (2001) Tyr275 and Lys279 stabilize NADPH within the catalytic site of NADPH:protochlorophyllide oxidoreductase and are involved in the formation of the enzyme photoactive state. *Biochemistry* **40**, 12562–12574
- Kruk, J. and Mysliwa-Kurziel, B. M. (2004) Separation of monovinyl and divinyl protochlorophyllides using C-30 reverse phase high performance liquid chromatography column: Analytical and preparative applications. *Chromatographia* **60**, 117–123
- Parham, R. and Rebeiz, C. A. (1995) Chloroplast biogenesis 72: a [4-vinyl]chlorophyllide *a* as an exogenous substrate. *Anal. Biochem.* **231**, 164–169
- Nagata, N., Tanaka, R., Satoh, S. and Tanaka, A. (2005) Identification of a vinyl reductase gene for chlorophyll biosynthesis in *Arabidopsis thaliana* and implications for the evolution of *Prochlorococcus* species. *Plant Cell* **17**, 233–240
- McFarlane, M. J., Hunter, C. N. and Heyes, D. J. (2005) Kinetic characterisation of the light-driven protochlorophyllide oxidoreductase (POR) from *Thermosynechococcus elongatus*. *Photochem. Photobiol. Sci.* **12**, 1055–1059
- Martin, G. E. M., Timko, M. P. and Wilks, H. M. (1997) Purification and kinetic analysis of pea (*Pisum sativum* L.) NADPH:protochlorophyllide oxidoreductase expressed as a fusion with maltose-binding protein in *Escherichia coli*. *Biochem. J.* **325**, 139–145
- Townley, H. E., Griffiths, W. T. and Nugent, J. P. (1998) A reappraisal of the mechanism of the photoenzyme protochlorophyllide reductase based on studies with the heterologously expressed protein. *FEBS Lett.* **422**, 19–22
- Lebedev, N. and Timko, M. P. (1999) Protochlorophyllide oxidoreductase B-catalyzed protochlorophyllide photoreduction *in vitro*: insight into the mechanism of chlorophyll formation in light-adapted plants. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 9954–9959
- Heyes, D. J., Martin, G. E. M., Reid, R. J., Hunter, C. N. and Wilks, H. M. (2000) NADPH:protochlorophyllide oxidoreductase from *Synechocystis*: overexpression, purification and preliminary characterization. *FEBS Lett.* **483**, 47–51
- Urbig, T., Knaust, R. K. C., Schiller, H. and Senger, H. (1995) Solubilization and hydrophobicity test by Triton X-114-partitioning of NADPH:protochlorophyllide oxidoreductase from the unicellular alga *Scenedesmus obliquus*, mutant C-2A'. *Z. Naturforsch. C Biosci.* **50**, 775–780
- Mysliwa-Kurziel, B., Kruk, J. and Strzalka, K. (2004) Fluorescence lifetimes and spectral properties of protochlorophyllide in organic solvents in relation to the respective parameters *in vivo*. *Photochem. Photobiol.* **79**, 62–67
- Boddi, B., Ryberg, M. and Sundqvist, C. (1992) Identification of 4 universal protochlorophyllide forms in dark-grown leaves by analyses of the 77K fluorescence emission-spectra. *J. Photochem. Photobiol. B Biol.* **12**, 389–401
- Boddi, B., Ryberg, M. and Sundqvist, C. (1993) Analysis of the 77K fluorescence emission and excitation-spectra of isolated etioplast inner membranes. *J. Photochem. Photobiol. B Biol.* **21**, 125–133
- Boddi, B. and Franck, F. (1997) Room temperature fluorescence spectra of protochlorophyllide and chlorophyllide forms in etiolated bean leaves. *J. Photochem. Photobiol. B Biol.* **41**, 73–82
- Mysliwa-Kurziel, B., Amirjani, M. R., Strzalka, K. and Sundqvist, C. (2003) Fluorescence lifetimes of protochlorophyllide in plants with different proportions of short-wavelength and long-wavelength protochlorophyllide spectral forms. *Photochem. Photobiol.* **78**, 205–212

Received 6 October 2005/1 November 2005; accepted 7 November 2005

Published as BJ Immediate Publication 7 November 2005, doi:10.1042/BJ20051635