Protochlorophyllide reductase in photosynthetic prokaryotes and its role in chlorophyll synthesis

Jeremy D. ROWE and W. Trevor GRIFFITHS*

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

1. DNA sequences hybridizing with the wheat protochlorophyllide reductase gene have been detected during genomic Southern blots of various cyanobacterial DNA samples. No such hybridization was observed with DNA from photosynthetic bacteria. 2. A fragment amplified from *Phormidium laminosum* DNA has been characterized and shown to be 73 % similar to the corresponding wheat sequence. At the protein level the similarity is 91 %. When used as a probe for Southern blotting the *Phormidium* DNA fragment confirmed the authenticity of some of the original signals obtained with the wheat probe. 3. Peptides of molecular mass 36, 30 and 60 kDa are immunodetected by a

wheat reductase antibody during Western blotting of *Phormidium* preparations. These are purported to correspond to the cyanobacterial mature reductase protein, a stable proteolytic fragment and soluble dimeric forms of the latter respectively. 4. Adaptation of *Phormidium* to growth in red light ($\lambda > 670$ nm) or darkness led to no significant changes in the total level of immunodetected peptides or protochlorophyllide within the cells. 5. The specific activity of the reductase in *Phormidium* membranes has been tentatively estimated as 0.5 unit/mg of protein, a value comparable with that found in preparations from mature chloroplasts of higher plants.

INTRODUCTION

Chlorophyll *a* in O_2 -evolving photosynthetic organisms and bacteriochlorophyll in the photosynthetic bacteria are arguably the most important group of compounds for life on earth, because of their essential role in photosynthesis. Biosynthetically, all of the chlorophylls together with other cellular pyrroles such as the haems share a basic common pathway involving δ -aminolaevulinic acid as the first committed precursor, with the haem and chlorophyll pathways diverging at the tetrapyrrole protoporphyrin IX on chelation of Fe^{2+} and Mg^{2+} respectively.

Mg protoporphyrin IX is the first intermediate specific to the synthesis of chlorophyll, and it undergoes methylation and cyclization to generate protochlorophyllide (Pchlde) in reactions common to the synthesis of both chlorophyll *a* and bacteriochlorophyll. In oxygenic phototrophs the Pchlde is reduced to chlorophyllide (Chlide) *a*, from which chlorophyll *a* is formed by reduction of the 4-vinyl group and esterification of the C_{17} -propionate group with phytol. In bacteriochlorophyll synthesis the Chlide *a* is further modified around the periphery of the macrocycle to generate bacteriochlorophyllide *a*, the final esterification of which gives bacteriochlorophyll *a* as before (Scheme 1) (see [1,2] for reviews).

Despite sharing common intermediates for the synthesis of pyrroles, it has become apparent in recent years that various organisms may utilize different reactions to generate some of these intermediates. δ -Aminolaevulinic acid can thus be formed from either succinyl-CoA and glycine, as in non-phototrophic eukaryotes and some prokaryotes such as the purple photosynthetic bacteria, while plants and other bacteria, including *Escherichia coli*, can form δ -aminolaevulinic acid from glutamate via Glu-tRNA^{Glu} (see [3] for a review). Similarly, the formation of Chlide from Pchlde can be achieved by either of two distinct processes. These are a light-dependent reaction catalysed by the enzyme protochlorophyllide reductase (PchRed) [4], and a hitherto uncharacterized process involving at least three gene

products, designated *bch* B, L and N in bacteriochlorophyll-containing organisms and *chl* B, L and N in chlorophyll *a*-containing organisms. *bch* B, L and N are present in the genomic DNA of the bacteriochlorophyll-producing photosynthetic bacteria. Amongst chlorophyll *a*-containing organisms *chl* B, L and N are present in the genomic DNA of cyanobacteria and within the plastid genome of the non-flowering phototrophic eukaryotes. They are absent from flowering plants [5–7]. The presence of such genes confers on the organisms the potential for light-independent (bacterio)chlorophyll synthesis.

The light-dependent reductase was characterized long before the discovery of the light-independent system, yet its distribution is not so well established. This is probably due to the location of the gene within the complex nuclear fraction of eukaryotes, making its presence difficult to detect [8]. Physiologically, the involvement of the enzyme in higher plants is readily inferred from their failure to synthesize chlorophyll when deprived of light. In non-flowering plants and the photosynthetic prokaryotes, however, this test obviously cannot be applied due to the dark Pchlde-reducing system. Despite this, the reductase has been identified in the green alga *Chlamydomonas reinhardtii* [9]; J. Li and M. P. Timko, personal communication) and in some gymnosperms [10,11]. When the present work was initiated there had been no studies reported of the reductase in prokaryotes apart from genetic deletion studies in *Plectonema* which indirectly implied the presence of a functional light-dependent reductase in this organism [12]. One opinion expressed at that time suggested that PchRed was exclusively a eukaryotic product [13]. This was based upon the nuclear location of the gene in photosynthetic eukaryotes [8], with the light-independent (*bchl* genes *bch* B, L and N and *chl* B, L and N taking over this role in phototrophic prokaryotic cells. The latter survive in non-flowering photosynthetic eukaryotes (see above) within the prokaryotic-type plastid genome.

In the present paper we report studies on the status of PchRed in some common phototrophic prokaryotes. Evidence is

Abbreviations used: Pchlde, protochlorophyllide; Chlide, chlorophyllide; PchRed, protochlorophyllide reductase; ECL, enhanced chemiluminescence.

* To whom correspondence should be addressed.

Scheme 1 Intermediates of chlorophyll and bacteriochlorophyll synthesis

The intermediates indicated by * can participate as either the 8-vinyl or 8-ethyl derivatives. Abbreviations: ALA, δ -aminolaevulinic acid; (Mg) proto IX, (Mg) protoporphyrin IX; BChlide, bacteriochlorophyllide; Chl, chlorophyll; BChl, bacteriochlorophyll.

presented for the expression of enzymically active reductase in cyanobacteria, but we could find no suggestion of its occurrence in photosynthetic bacteria.

A brief account of this work was originally presented to the International Symposium on Photosynthetic Prokaryotes, Urbino, Italy; 10–15 September 1994.

MATERIALS AND METHODS

Biological samples

Genomic DNA from *Rhodobacter sphaeroides* and *Synechocystis* PCC6803 was kindly supplied by Professor C. N. Hunter, University of Sheffield, U.K. DNA and cultures of *Phormidium laminosum* and *Anacystis nidulans* R2 DNA were supplied by Dr. C. Howe, Department of Biochemistry, University of Cambridge, U.K. The construct pWP24, containing the *Marchantia polymorpha frxC* gene, was supplied by Dr. M. Merrick, AFRC Unit of Nitrogen Fixation, University of Sussex, Brighton, U.K.

Enzymes and reagents

All restriction enzymes, λ HindIII markers, '123' ladder and DNA ligase were obtained from Gibco BRL, Life Technologies (Renfrewshire, Scotland, U.K.). The random primed DNA labelling kit was from Boehringer Mannheim (Lewes, W. Sussex, U.K.). NICK columns were purchased from Pharmacia LKB Biotechnology Ltd. (Milton Keynes, U.K.), and Genescreen Plus was from NEN-DuPont (Stevenage, U.K.). Radionucleotides and enhanced chemiluminescence (ECL) chemicals were obtained from Amersham International (Little Chalfont, Bucks., U.K.). Taq polymerase was Amplitaq from Perkin-Elmer Cetus. Magic DNA clean-up kit was purchased from Promega (Southampton, U.K.). The Sequenase DNA sequencing kit was purchased from United States Biosciences (Cambridge Bioscience, Cambridge, U.K.).

Growth of *Phormidium laminosum* cultures

Cells were grown shaking under aerobic conditions in the presence of 5% CO₂ at 45 °C in 'medium D' of Kratz and Myers [14]. Cells were normally grown under constant illumination, supplied by 5 × 60 W fluorescent tubes, and subcultured at approx. 10 day intervals. New cultures were seeded with 1/10 volume of established exponential-phase cells. Red light treatment was generated using a 60 W tungsten bulb in conjunction

with Lee celluloid filters nos. 106 and 120 (obtained from Stage Electrics, Avonmouth, U.K.) to exclude light of less than 670 nm.

Southern blotting

Restriction digestion of genomic DNA was carried out using a range of enzymes in accordance with the manufacturers' instructions. Reactions were carried out overnight in a volume of 100 μ l using 10 units of enzyme per μ g of DNA. DNA was separated by agarose gel electrophoresis as described in [15]. This was then transferred on to Genescreen Plus using the capillary blot method of Reed and Mann [16]. Template DNA for radioactive labelling was obtained by excision from a low-melting-point agarose gel and subsequent dilution with greater than 1.5 vol. of water. Random primed labelling was carried out following the manufacturer's instructions (Boehringer-Mannheim), and labelled probes were cleaned on NICK columns (Pharmacia). Hybridizations were carried out overnight in 10 ml of 0.25 M Na₂HPO₄/NaH₂PO₄/7% (w/v) SDS [17], containing 125 ng of radiolabelled probe. Hybridization temperatures were optimized for each sample, and subsequent washes were also carried out at this temperature as follows: 2 × 15 min in 100 ml of 2 × SSC/1% SDS; 2 × 15 min in 100 ml of 2 × SSC/0.1% SDS; 10 min in 100 ml of 0.1 × SSC (2 × SSC is 0.3 M NaCl/30 mM trisodium citrate).

PCRs

PCRs were carried out in 50 μ l under mineral oil with 20 μ M degenerate primers (see the Results section), 2.5 mM each of the four dNTPs and 1 unit of Amplitaq polymerase, using the reaction buffer supplied. Reactions were carried out on 1 μ g of genomic DNA using a Perkin-Elmer Cetus thermal cycler using the following program. Initial step at 72 °C for 10 min; one cycle at 94 °C for 3 min, 58 °C for 1 min, 72 °C for 1 min; one cycle at 94 °C for 40 s, 58 °C for 1 min, 72 °C for 1 min; two cycles at 94 °C for 40 s, 57 °C for 1 min, 72 °C for 1 min; two cycles at 94 °C for 40 s, 56 °C for 1 min, 72 °C for 1 min; two cycles at 94 °C for 40 s, 55 °C for 1 min, 72 °C for 1 min; two cycles at 94 °C for 40 s, 54 °C for 1 min, 72 °C for 1 min; 25 cycles at 94 °C for 40 s, 53 °C for 1 min, 72 °C for 1 min; final step at 72 °C for 10 min.

Cloning of PCR products

DNA bands excised from an agarose gel were extracted twice with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) and ethanol-precipitated. This DNA was then digested with *Hind*III and *Xba*I and cleaned using the magic DNA kit (Promega) prior to ligation into *Hind*III/*Xba*I-cut pBluescript (KS).

Generation of a polyclonal antibody against PchRed fusion protein

The plasmid pUEX-PCR previously described [18] expresses full-length wheat PchRed as a fusion protein with β -galactosidase. Inclusion bodies containing the fusion product were isolated from expressing *E. coli* cultures as already described [15]. Inclusion body proteins were separated on large-scale SDS/PAGE gels using the discontinuous buffer system of Laemmli [19]. PchRed fusion protein running at approx. 120 kDa was excised from the gel and used to prepare a slurry for immunization of a rabbit as described in [20].

Preparation of membrane samples from *Phormidium laminosum*

Large-scale purification of total cell membranes was carried out by lysis in a French Press. A 250 ml volume of culture was pelleted by centrifugation at 6000 *g* for 15 min. Cells were then washed in 30 ml of Buffer I (0.5 M sucrose, 50 mM Hepes, 50 mM Tes, 2 mM MgCl₂, 1 mM EDTA, pH 7.5), pelleted and finally resuspended in 30 ml of Buffer I [4] to which the following were added immediately prior to lysis: 2 mM NADPH [as a regenerating system consisting of glucose 6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (0.5 unit) and NADPH (2 mM)], 1 mM protease inhibitors antipain (Sigma) and PMSF (Calbiochem), 0.6 mg of DNase I (Sigma) and 5 mM cysteine.

All subsequent steps were carried out under a green safe light and the samples were kept on ice throughout. The cell suspension was passed twice through a pre-cooled French pressure cell at a pressure of 90–120 MPa (6–8 tons/in²). Cell debris was pelleted by centrifugation at 6000 *g* for 10 min at 4 °C and the supernatant was retained. Membranes were then pelleted by a further centrifugation step at 100 000 *g* for 40 min at 4 °C.

Extraction of total membranes of *Phormidium laminosum* by sonication

Sonication was used in the small-scale preparation of cell lysates for analysis using SDS/PAGE. *P. laminosum* cells were pelleted from the culture medium by centrifugation at 6000 *g* for 10 min, resuspended in denaturing sample loading buffer, sonicated for 2 × 30 s (3 mm probe; Rapido Ultrasonics Ltd., Shipley, Yorks., U.K.) and finally boiled for 5 min.

Western blotting

Western blotting of protein samples was carried out as previously described [21]. Blots were visualized using the ECL Western blotting detection kit and Hyperfilm-MP from Amersham International.

Extraction of pigments from *P. laminosum* for spectroscopic analysis

Cells were pelleted from 1.5 ml of fresh culture and resuspended in 200 μ l of water. Pigments were extracted under green safe light conditions by sonication as before followed by immediate addition of 800 μ l of acetone. Finally the mixture was centrifuged

for 15 s at 13 000 *g* in a microcentrifuge and the absorption spectrum of the supernatant was obtained using a Unicam UV/VIS Spectrophotometer (ATL Unicam, Cambridge, U.K.).

RESULTS

The genomic Southern blots shown in Figure 1 record attempts at identifying DNA sequences similar to that of the wheat reductase gene in chromosomal DNA from cells of the cyanobacteria *Anacystis nidulans* R2, *Phormidium laminosum* and *Synechocystis* PCC6803. Restriction enzyme digestion of 10 μ g of genomic DNA generated a wide spectrum of differently sized fragments, as revealed by ethidium bromide staining (lanes 1–3). On hybridizing with the ³²P-labelled wheat reductase gene at 64 °C (Figure 1b), some non-specific background labelling was observed together with more intense bands, notably at 2.5 kb amongst the *Anacystis* fragments (lane 4), bands at 4, 8 and 11 kb in the *Phormidium* digests (lane 5), with a sharp band at 6.5 kb and a diffuse region centred at approx. 3.5 kb in the *Synechocystis* digests (lane 6). Similar blots carried out on *Eco*RI-digested DNA from *Rhodobacter sphaeroides* gave no specific signals when probed with the wheat gene over a range of hybridization temperatures (results not shown). Similar tracks of *A. nidulans* and *P. laminosum* digests were probed at 64 °C with the *Marchantia frxC* gene (equivalent to the *bchl* L and *chl* L genes associated with Pchl reduction in *R. capsulatus* and *Chlamydomonas reinhardtii* respectively) (Figure 1c). Clear bands can be seen at 4 and 1.5 kb in *Anacystis* and *Phormidium* respectively (lanes 7 and 8).

Figure 1 highlights the problems associated with attempting Southern blotting using non-homologous probes. Blots probed with the wheat PchRed gene gave only weak, ill-defined signals (lanes 4–6), whereas probing with *frxC* gave unambiguous clear bands, as expected for a gene which is known to be highly conserved among a wide range of photosynthetic organisms. Despite the apparent lack of specificity of hybridization observed when using the wheat PchRed gene, Figure 1 could still indicate that homologues of this gene may be present in these cyanobacterial species. In view of this, a series of degenerate primers was designed to attempt to amplify regions of such a gene by the

Figure 1 Southern blot analysis of cyanobacterial genomic DNA

The DNA samples from *Anacystis* and *Phormidium* were digested with the restriction enzyme *Hinc*II and the *Synechocystis* samples with *Hind*III. Fragments were separated by agarose gel electrophoresis and stained with ethidium bromide (a). After transfer the filters were probed with the radiolabelled wheat reductase clone pWR5 (b) or the *Marchantia frxC* clone pWP24 (c) at a hybridization temperature of 64 °C, as described in the Materials and methods section. Lanes 1, 4 and 7, *Anacystis* digests; lanes 2, 5 and 8, *Phormidium* digests; lanes 3 and 6, *Synechocystis* digests.

Figure 2 Degenerate primers used in PCRs

The wheat PchRed protein sequence was back-translated using the codon usage table of *Anacystis nidulans* R2 with the program BACKTRANSLATE of the Wisconsin GCG package. Areas of minimum redundancy were chosen as primer annealing sites in the region around Cys-296, a residue previously implicated in substrate binding [22]. Restriction sites were included in these primers for use during subsequent cloning.

Wheat	GASGSAMIDG	DESFDGAKAY	KDSKVCNMLT	MQEFHRRYHE	ETGITFSSLY	PGCIATTGLF
Oat	GASGSAMIDG	DESFDGAKAY	KDSKVCNMLT	MQEFHRRYHE	DTGITFSSLY	PGCIATTGLF
Barley	GASGSAMIDG	DESFDGAKAY	KDSKVCNMLT	MQEFHRRYHE	ETGITFSSLY	PGCIATTGLF
Pea	GLNSSAMIDG	GD.FDGAKAY	KDSKVCNMLT	MQEFHRRYHE	ETGITFASLY	PGCIATTGLF
Pine	GVNISPMDG	GE.FDGAKAY	KDSKVCNMLT	MQEFHRRYHE	ETGITFASLY	PGCIATTGLF
<i>Arabidopsis</i>	GLNSSAMIDG	GD.FDGAKAY	KDSKVCNMLT	MQEFHRRFHE	ETGVTFFASLY	PGCIATTGLF
<i>Phormidium</i>	AMIDG	DE.FKAGKAY	KDSKLCNMIT	VRELHRRYHD	TAGITFSSLY	PGCIATS...

Figure 3 Sequence alignment of *Phormidium lamosum* PchRed fragment with those of higher-plant reductases

Alignments were carried out using the program PILEUP (Genetics Computer Group, University of Wisconsin).

PCR. These primers (Figure 2) cover regions of the gene encoding a segment of the protein that is highly conserved amongst the reductase sequences already published, and include a cysteine residue (Cys-296) thought to be involved in the active site of the enzyme [22]. The codon preference data of *Anacystis* R2 were incorporated into the design of the partially degenerate primers, and flanking restriction sites, *Bam*HI and *Xba*I, were also included (Figure 2) to aid subsequent cloning. Amplification reactions were carried out on genomic DNA samples from *Phormidium lamosum*, *Anacystis nidulans*, *Synechocystis* PCC 6803 and *Rhodobacter spaeroides* under the conditions described in the Materials and methods section and the products were analysed by electrophoresis on agarose gels. A product of the expected size (170 bp) was only obtained when using DNA from *Phormidium*. Under these conditions no product was formed in control reactions lacking template DNA.

The amplified *Phormidium* product was excised from the gel and re-amplified in a second round of PCR, and the product was extracted, digested with *Bam*HI and *Xba*I, ligated into pBluescript and sequenced as described in the Materials and methods section. The technique of PCR is susceptible to contamination artefacts and, as the wheat gene is used extensively in this laboratory, it is reassuring that the product amplified from *Phormidium* was shown on sequencing to be only 73 % similar to that of wheat. After translation the inferred protein sequence of the *Phormidium* fragment is 91 % similar to the wheat protein

sequence, as estimated using the Bestfit program of the GCG suite (Genetics Computer Group Inc., University of Wisconsin). Alignments of the protein sequence of the *Phormidium* fragment with the corresponding regions from published PchRed sequences are shown in Figure 3.

Figure 4 shows the result of probing *Anacystis* and *Phormidium* genomic blots identical to those used in Figure 1 with the labelled PCR-amplified *Phormidium* fragment at 64 °C. Under these conditions clear single bands were obtained in both the *Anacystis* and *Phormidium* digests (Figure 4), but with only a much weaker signal at 6 kb in a *Synechocystis* *Hind*III digest (results not shown). Such differential hybridization patterns probably reflect variations in the codon usage by these species. No detectable hybridization was observed in an *Eco*RI digest of *R. spaeroides* (results not shown). The problems associated with the use of a non-homologous probe (Figure 1) have been largely eliminated in the *Anacystis* and *Phormidium* Southern blots by using the *Phormidium* probe (Figure 4), where the much greater specificity of hybridization is immediately apparent. Comparing the hybridization patterns observed in Figures 1 and 4, the single sharp band at 2.4 kb highlighted by the *Phormidium* probe in the *Anacystis* DNA (Figure 4, lane 3) matches that already obtained when probing with the wheat gene (Figure 1, lane 4). Of the three bands at 11, 8 and 4 kb detected during probing of the *Phormidium* digest with the wheat probe (Figure 1, lane 5), only the 11 kb fragment is highlighted using the *Phormidium* probe

Figure 4 Southern blotting of cyanobacterial DNA probed with the *Phormidium* PchRed fragment

Genomic DNA of *Anacystis* and *Phormidium* was digested with *HincII* and separated as described in Figure 1. These were blotted as before and probed with radiolabelled *Phormidium* PchRed fragment at 64 °C as described in the Materials and methods section. Lanes 1 and 3, *Anacystis*; lanes 2 and 4, *Phormidium*.

Figure 5 Western blot analysis of *Phormidium lamosum* preparations

French press homogenates of *Phormidium* cells were fractionated into membrane and soluble fractions and the protein was extracted, separated by SDS/PAGE, transferred to nitrocellulose and challenged with an antibody against the wheat reductase. Decorated peptides were detected by the ECL technique as described in the text. (a) Coomassie Blue-stained gel; (b) ECL-highlighted Western blot. Lane 1, markers; lanes 2 and 6, homogenates (~1.5 µg of protein); lanes 3 and 7, membranes (~15 µg of protein); lanes 4 and 8, supernatants (~10 µg of protein); lanes 5 and 9, wheat etioplast membranes (~0.5 µg of protein).

(Figure 4, lane 4). The 8 and 4 kb bands detected using the full-length wheat clone (Figure 1) could therefore be due to non-specific hybridization of the full-length wheat probe. One of these additional bands could also have arisen as a consequence of internal digestion of the gene in the *Phormidium* DNA. This could generate a fragment lacking the region covered by the *Phormidium* probe, and as such would only hybridize with the full-length wheat probe. Likewise the failure of the *Phormidium* fragment to label one of the two bands (at 3.5 kb; see above)

Figure 6 PchRed activity in *Phormidium* membrane preparations

Phormidium membrane samples (~140 µg of protein) or wheat etioplasts (~88 µg of protein) were supplemented with Pchlde and NADPH, and chlorophyll synthesis was initiated by flashing light and monitored as ΔA (672 minus 710 nm) using a dual-wavelength spectrophotometer as previously described [4]. (a) Wheat membranes; (b) *Phormidium* membranes; (c) continued flashing of (b) after 10 min rest.

previously highlighted by the wheat probe on the *Synechocystis* blot (Figure 1, lane 6) again probably reflects the internal digestion of the PchRed gene by *HindIII* in this organism.

Having detected a DNA fragment from *Phormidium* with sequence identity to the higher-plant reductase gene, evidence was sought for the expression of this putative gene in *Phormidium*. Proteins were extracted from cell samples and subjected to Western blot analysis using antibodies generated to a β -galactosidase fusion protein of the wheat reductase (as described in the Materials and methods section). Figure 5 shows the Coomassie Blue-stained peptide profiles of different *Phormidium* cell fractions and the corresponding patterns of reductase antibody tagging as revealed by Western blotting. The total lysate contains a large number of Coomassie Blue-stained peptides (lane 2). On separation into membrane and soluble phases the former (lane 3) is dominated by peptides of approx. 50 and 30 kDa, while the major band visible in the soluble fraction (lane 4) is at approx. 60 kDa. All fractions also contained a diffuse band of approx. 18 kDa.

When incubated with the antibody against the wheat reductase, three bands were highlighted in the total lysate (lane 6); two major bands of approx. molecular masses 36 and 30 kDa and a minor one of approx. 60 kDa. On fractionation, the predominant signal in the membrane sample was at 30 kDa (lane 7), whereas the supernatant fraction (lane 8) contained exclusively the 60 kDa band. The fractionation procedure was surprisingly accompanied by a considerable decrease in the overall 36 kDa signal, resulting in only a trace of this band surviving in the membrane fraction (lane 7). None of these bands showed any reaction with the preimmune serum (results not shown). The reductase in wheat etioplast membranes was immunoblotted as an intense band of 36 kDa (lane 9) at a protein loading which was barely detectable by Coomassie Blue staining (lane 5), endorsing the specificity of the antibody for PchRed.

The enzymic activity of the wheat reductase can readily be assayed spectrophotometrically in plastid membranes from higher plants, especially if prepared from dark-grown plants [4] which are free from interfering chlorophyll. Figure 6 records our

Figure 7 Absorption spectra of *Phormidium lamosum* cultures transferred from white to red light

Spectra were recorded of 8-day-old white-light-grown cultures before (1) and after (2) transfer to red light ($\lambda > 670$ nm) for 48 h.

attempts at monitoring the activity of the enzyme in cell-free preparations from *Phormidium*. The problem of endogenous chlorophyll in the preparation interfering with the assay is immediately apparent in the considerably reduced signal-to-noise ratio (Figure 6b) compared with etioplast membranes (Figure 6a). The linear light-induced increase in absorbance at 672 nm in the etioplast membranes equates with a PchRed activity of 3.53 units/mg of protein (1 unit corresponding to the formation of 1 nmol of Chlide/min). In the *Phormidium* cell membrane preparations, isolated after cell breakage in the French press, a flash-induced absorbance change at 672 nm was observed under the same conditions (Figure 6b). However, the extent of the change was considerably decreased compared with that seen in etioplasts (Figure 6a) and furthermore was not sustained, decreasing to practically zero after six flashes. After a 'resting' period of about 10 min in darkness, complete recovery of the flash-induced changes was seen (Figure 6c). These *Phormidium* traces are different from those of wheat etioplasts, and this may reflect the difficulties associated with the high level of endogenous

chlorophyll in the sample. The absorbance changes are, however, completely reproducible and entirely dependent on added substrates. Furthermore, the spectrum of the change shows a maximum at 672 nm, as expected for the formation of Chlide resulting from the activity of PchRed. From the 'initial' rate of the flash-induced absorbance change an estimated rate of reductase activity can be obtained, giving a specific activity for the preparation of approx. 0.5 unit/mg of protein. This estimate must be considered as preliminary, however, until optimal assay conditions for these preparations have been fully established.

In higher plants the level of PchRed responds remarkably to light with high concentrations occurring in the dark, whereas only a trace exists in illuminated tissues [23]. This response is largely a consequence of stabilization of the enzyme by its substrates Pchlide and NADPH, which accumulate in darkness in the absence of enzymic turnover [24]. It is well known that it is difficult to grow cyanobacteria heterotrophically in complete darkness, though the reasons for this are not clear. When *Phormidium* was dark-adapted for 2 days no Pchlide accumulation or increase in PchRed levels was observed (results not shown). This is perhaps not surprising since cyanobacteria possess a light-independent alternative to PchRed. Therefore, in cyanobacteria, darkness cannot be exploited for reductase studies as in higher plants. Interestingly, however, it has been reported [25] that *Anacystis nidulans* Tx20 and *Agmenellum quadruplicatum* when cultured under far-red light accumulated Pchlide within 24 h, a situation reminiscent of dark-grown higher plants. It was therefore considered of some interest to adapt *Phormidium* to far-red light to see if Pchlide or, more significantly, PchRed accumulated in such cells.

To test the effect of light quality on pigment accumulation by *Phormidium*, 7-day-old light-grown cultures were transferred to either darkness or red light ($\lambda > 670$ nm) or left in white light. After various periods of adaptation, aliquots were removed for pigment and protein analysis. The cells, despite showing differences in growth rates in the different light regimes, generated pigment extracts, all of which after normalization appeared spectroscopically very similar. To illustrate this, Figure 7 shows the pigment extracted from the initial culture in white light (curve 1) and the extract from the same culture after adaptation to red light for 48 h (curve 2). There is no evidence of enhanced absorption at approx. 630 nm, which would be indicative of accumulated Pchlide in the latter. The protein extracts prepared from the various samples were analysed by Western blotting, and again no significant differences were apparent between cells taken from the various light regimes. Figure 8 illustrates this with

Figure 8 Western blot analysis of *Phormidium lamosum* cells during adaptation to red light

Proteins were extracted from aliquots of the red-light-adapting cultures from Figure 7 and subjected to Western blot analysis by challenging with the wheat reductase antibody as described in Figure 5. (a) Coomassie Blue-stained gel; (b) Western blot. Lanes 1–5 and 7–11 correspond to proteins ($\sim 8 \mu\text{g}$) from cells adapted to red light for 0, 6, 12, 24 and 48 h respectively. Lanes 6 and 12 represent 0.5 μg of wheat etioplast membranes.

a time course of protein profiles observed in cells from white-light-grown cultures on transfer to red light. As before (Figure 5), major groups of peptides of approx. 50 kDa and 36 kDa were identified in the cell extracts by Coomassie Blue staining, with their relative intensities remaining unchanged after adaptation to light for 24 h (cf. lane 2 with lanes 3–6, Figure 8). The Western blots of the adapting samples highlight the same three bands approx. 36 kDa, 30 kDa and 60 kDa as before (Figure 5), and again no differences in their relative intensities was observed on adaptation (compare lane 8 with lanes 9–11, Figure 8). It is interesting, however, that the relative contribution of the 60 kDa signal is greater in Figure 8 than was observed in Figure 5. The reason for this is not clear, but may reflect variation in the samples or experimental technique.

DISCUSSION

It is well established that chlorophyll synthesis in higher plants requires light to satisfy the photoenzyme PchRed. This enzyme catalyses the reduction of Pchl_{ide} to Chl_{ide} as a late step in the chlorophyll *a* biosynthetic pathway. Photosynthetic prokaryotes and non-flowering plants are also capable of light-independent synthesis of chlorophyll, or bacteriochlorophyll in the case of the photosynthetic bacteria, and as such possess enzyme(s) other than the reductase for Pchl_{ide} reduction. Recently, three genes encoding components of this light-independent system have been identified within the photosynthetic gene cluster of photosynthetic bacteria and designated *bch* B, L and N. Disruption of any of these results in non-photosynthetic phenotypes lacking bacteriochlorophyll and accumulating Pchl_{ide} [26–28]. Related genes designated *chl* B, *chl* L and *chl* N have also been identified in cyanobacteria and non-flowering plants that are capable of light-independent chlorophyll *a* synthesis [7,12,29–31]. Interestingly, in non-flowering plants these genes reside within the plastid genome but, as expected, they have not been detected in the chloroplast DNA or total DNA from flowering plants.

In contrast to our knowledge on the distribution of genes for the light-independent Pchl_{ide} reductase, less information is available on the distribution of PchRed. Although ubiquitous amongst flowering plants, the only other reports of its presence in eukaryotes is in the alga *Chlamydomonas* [9]; J. Li and M. P. Timko, personal communication) and in gymnosperms (*Pinus* spp. [10,11]). No direct report of its occurrence in the photosynthetic prokaryotes has been made (see [12]). This has not been considered surprising in view of the nuclear location of the gene in eukaryotes [8] and the now well documented presence of the light-independent system in the photosynthetic prokaryotes. Contrary to expectation, however, we report here evidence supporting the expression of active PchRed in the filamentous cyanobacterium *Phormidium laminosum*. Such evidence has been obtained by Southern blotting, PCR and Western blotting. These techniques rely heavily on sequence identity, and this has created considerable problems when investigating phototrophic prokaryotes using probes derived from a higher-plant species. Furthermore, spectroscopic assay of PchRed, which is straightforward in higher-plant extracts, is much more difficult in cyanobacteria due to interfering pigments and possibly a lower level of enzyme.

By careful optimization of experimental conditions, tentative positive signals were observed during Southern blotting of DNA from three cyanobacteria probed with the wheat reductase gene (Figure 1). No such specific hybridization was observed with DNA isolated from the photosynthetic bacterium *R. sphaeroides* despite attempts over a wide range of stringency conditions (results not shown), implying the absence of any DNA fragments

with identity to the wheat reductase in the *R. sphaeroides* genome. Support for the initial Southern blotting data was provided by the signals obtained when probing with the *Phormidium* PchRed fragment (Figure 4). The alignment of some bands in Figures 1 and 4 confirms that at least some of the signals obtained during the initial wheat probing were indeed due to hybridization to PchRed sequences in these cyanobacteria.

A series of degenerate primers against flanking regions of a highly conserved segment of the reductase generated products of the expected size during amplification using the *Phormidium* DNA as templates. Cloning and sequencing of the reamplified *Phormidium* fragment showed it to be 73 % identical to the wheat sequence at the DNA level and 91 % similar at the protein level. A missing codon in the *Phormidium* fragment results in the deletion of a serine residue, corresponding to Ser-256 in wheat. Interestingly, while this serine is conserved in all the cereal sequences, its deletion is not unique to *Phormidium*, as it is also absent from the pea, pine and *Arabidopsis* sequences (Figure 3). The full significance of such differences may become more apparent when we eventually characterize a full-length reductase clone from cyanobacteria. Recently a cosmid library for *Synechocystis* PCC 6803 was made available to us [32]. This library yielded a strong positive signal when probed with the wheat PchRed gene, and as a result a single cosmid was isolated containing approx. 30 kb of genomic DNA. This is currently being characterized.

The DNA analysis data are further supported by evidence suggesting expression of the reductase gene and generation of the active enzyme in *Phormidium* cells. Protein extracts of various *Phormidium* samples contain three peptides, of 60, 36 and 30 kDa, which are decorated by the antibody against the wheat reductase fusion protein during Western blot analysis, with the relative intensity of each band varying between different fractions (Figure 5). This pattern bears a strong resemblance to the appearance of the reductase in higher plants where a principal 36 kDa peptide, representing the mature enzyme, is very often accompanied by a peptide of approx. 70 kDa [33] and a minor one of approx. 30 kDa. These have been provisionally characterized by sequence analysis and immunoblotting as a dimer (70 kDa) and a transient proteolysis product (30 kDa) of the mature reductase (36 kDa) (N. L. Lebedev and W. T. Griffiths, unpublished work). Our interpretation of the *Phormidium* Western blotting data is therefore that the reductase in *Phormidium* behaves in a similar fashion to the wheat protein. Proteolysis of a 36 kDa protein (Figure 5) generates a specific stable product of molecular mass 30 kDa. This, however, can aggregate like the mature higher-plant enzyme to generate a dimer of molecular mass 60 kDa (Figure 5). Confirmation of this suggestion awaits further characterization of these various forms. Whether these changes are of any physiological significance or are merely artefacts of the experimental procedures also remains to be established.

It is interesting to consider the distribution of these three forms in the different fractions derived from the *Phormidium* cells. The initial cell-free homogenate shows evidence that all three forms are present. The isolated membrane fraction is enriched in the 30 kDa breakdown fragment together with a trace of the residual 36 kDa protein (Figure 5), whereas the supernatant fraction contains almost exclusively the 60 kDa aggregate form. Higher-plant reductases are very hydrophilic proteins but are membrane-associated in plastids, probably via interaction with the membrane-localized hydrophobic pigment substrate molecule [22]. By analogy, the 36 and 30 kDa forms of the membrane-bound *Phormidium* enzyme are expected to be associated with Pchl_{ide} and the membrane via hydrophobic 'patches' on the protein. Our inability to find Pchl_{ide} on such

membranes (Figure 7) probably reflects the low level present coupled with difficulties in its detection due to the endogenous high concentration of other pigments. Dissociation of the apo-protein from the pigment generates free soluble proteins which could interact through the exposed hydrophobic patches to generate soluble dimers as observed (Figure 5). Although feasible, direct evidence for such a proposal must await further characterization of the enzyme from this source.

In higher plants it is possible, within certain limits, to correlate the level of reductase in tissues with the amount of Pchl_{ide} since the pigment, together with NADPH, results in the stabilization of the enzyme [24,34]. Plants grown in darkness accumulate PchRed in parallel with Pchl_{ide}. On transfer to light, both rapidly decline to reach a new low steady-state level compatible with the flux through the chlorophyll biosynthetic pathway. On redarkening, the process is reversed with a reaccumulation of the enzyme and its substrate. It has been reported [25] that some cyanobacteria accumulate Pchl_{ide} when grown in far-red light. Light of such quality is ineffective in promoting turnover of PchRed. If control mechanisms related to the higher-plant system are operational in cyanobacteria it might be expected that such pigment accumulation would be accompanied by the parallel build-up of the reductase. However, no build-up of either the putative reductase peptide or Pchl_{ide} is observed when *Phormidium* is transferred into red light (Figures 7 and 8), suggesting that this species shows different regulation to those studied by Myers et al. [25].

Our attempts at assaying PchRed activity in *Phormidium* preparations (Figure 6) provide further support for the DNA and protein analysis regarding the enzyme's presence in this organism. The calculated activity in isolated *Phormidium* membranes, even though much lower than that found in etioplast membranes, is comparable with the activity detected in higher-plant chloroplast preparations [34]. However, the non-linear profiles of the assays using the *Phormidium* preparations (Figure 6) suggests that these are being carried out under suboptimal conditions. This may not be very surprising bearing in mind the difficulties associated with PchRed assays on this material. In addition, the presence of the light-independent system in this organism may introduce further complications. Current work is aimed at refining the conditions for assaying the enzyme in *Phormidium* extracts.

In conclusion we feel that, taken together, the data presented in this paper suggest the presence of a functional light-dependent PchRed in the cyanobacterium *Phormidium lamosum*. This appears to be closely related to the nuclear-encoded higher-plant enzyme, indicating transfer of a prokaryotic PchRed to the nucleus during evolution. In support of our findings we cite recent work on *Plectonema boryanum*, where it has been demonstrated that genetic deletion of *chl L* resulted in mutants in which chlorophyll synthesis is completely light-dependent, indirectly suggesting the presence of a functional reductase in this species [12]. Furthermore, the complete sequence of a full-length light-dependent PchRed gene from *Synechocystis* PCC 6803 that

shows identity to the higher-plant reductase has recently been obtained [35]. The presence of PchRed in cyanobacteria, and its apparent absence from the photosynthetic bacteria, is of interest when considering the evolution of the photosynthetic apparatus.

We are grateful to Professor C. N. Hunter (University of Sheffield, U.K.) for the provision of DNA from *Rb. sphaeroides* and *Synechocystis* PCC 6803, Dr. Chris Howe (University of Cambridge, U.K.) for DNA samples from *Anacystis nidulans* R28L and *Phormidium lamosum* and for cultures of *P. lamosum*, and Dr. Carl Bauer (University of Indiana, Bloomington, IN, U.S.A.) for a preview of his paper with Dr. Jon Suzuki on the *Synechocystis* reductase gene. J.D.R. thanks the SERC for financial support by the award of a postgraduate research studentship.

REFERENCES

- Smith, A. G. and Griffiths, W. T. (1993) *Methods Plant Biochem.* **9**, 299–343
- Senge, M. O. (1993) *Photochem. Photobiol.* **57**, 189–206
- Beale, S. I. and Weinstein, J. D. (1991) *New Compr. Biochem.* **19**, 155–235
- Griffiths, W. T. (1978) *Biochem. J.* **174**, 681–692
- Suzuki, J. Y. and Bauer, C. E. (1992) *Plant Cell* **4**, 929–940
- Liu, X.-Q., Xu, H. and Huang, C. (1993) *Plant Mol. Biol.* **23**, 297–308
- Richard, M., Tremblay, C. and Bellamare, G. (1994) *Curr. Genet.* **26**, 159–165
- Batschauer, A., Santel, H. J. and Apel, K. (1982) *Planta* **154**, 459–464
- Ford, C., Mitchell, S. and Wang, W.-Y. (1981) *Mol. Gen. Genet.* **184**, 460–464
- Forreiter, C. and Apel, K. (1993) *Planta* **180**, 536–549
- Spano, A. J., He, Z. and Timko, M. P. (1992) *Mol. Gen. Genet.* **236**, 86–95
- Fujita, Y., Takahashi, Y., Shuganji, M. and Matsubara, H. (1992) *Plant Cell Physiol.* **33**, 81–92
- Griffiths, W. T. (1991) in *Chlorophylls* (Scheer, H., ed.), pp. 433–450, CRC Press, Boca Raton, FL
- Kratz, W. A. and Myers, J. (1955) *Am. J. Bot.* **42**, 282–287
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Reed, K. C. and Mann, D. A. (1985) *Nucleic Acids Res.* **13**, 7207–7221
- Church, G. M. and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991–1995
- Teakle, G. R. (1991) Ph.D. Thesis, University of Bristol
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Harlow, E. and Lane, D. (1988) in *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Gorlach, A., Holtermann, G., Jeltmann, W., Hancock, J. T., Jones, S. A., Jones, O. T. G. and Acker, H. (1993) *Biochem. J.* **290**, 771–776
- Teakle, G. R. and Griffiths, W. T. (1993) *Biochem. J.* **296**, 225–230
- Mapleston, R. E. and Griffiths, W. T. (1977) *Biochem. Soc. Trans.* **5**, 319–321
- Kay, S. A. and Griffiths, W. T. (1983) *Plant Physiol.* **72**, 229–237
- Myers, J., Graham, J. R. and Wang, W. T. (1982) *Plant Physiol.* **69**, 549–550
- Burke, D. M., Alberti, M. and Hearst, J. E. (1993) *J. Bacteriol.* **175**, 2414–2422
- Bollivar, D. W., Suzuki, J. Y., Beatty, J. T., Dobrowolski, J. M. and Bauer, C. E. (1994) *J. Mol. Biol.* **237**, 622–640
- Coomber, S. A., Chandri, M., Connor, A., Britton, G. and Hunter, C. N. (1990) *Mol. Microbiol.* **4**, 977–989
- Li, J., Goldschmidt-Clermont, M. and Timko, M. P. (1993) *Plant Cell* **5**, 1817–1829
- Fujita, Y., Matsumoto, H., Takahashi, Y. and Matsubara, H. (1993) *Plant Cell Physiol.* **34**, 305–314
- Lidholm, J. and Gustafsson, P. (1991) *Plant Mol. Biol.* **17**, 787–798
- Shestopalov, V. I., Nashchokina, O. O., Shestakov, S. V. and Yankovskii, N. K. (1994) *Genetika* **30**, 452–455
- Boddi, B., McEwen, B., Ryberg, M. and Sunqvist, C. (1994) *Physiol. Plant.* **92**, 160–170
- Griffiths, W. T., Kay, S. A. and Oliver, R. P. (1985) *Plant Mol. Biol.* **4**, 13–22
- Suzuki, J. and Bauer, C. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3749–3753