Expression of the Synechocystis PCC6803 Ferredoxin-NADP+ oxidoreductase: Involvement of a novel type of divergent operator

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Characterization and transcriptional regulation of the *Synechocystis* PCC 6803 *petH* gene, encoding ferredoxin-NADP$^+$ oxidoreductase: involvement of a novel type of divergent operator

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Abstract

The *petH* gene, encoding ferredoxin-NADP$^+$ oxidoreductase (FNR), has been characterised in the unicellular cyanobacterium *Synechocystis* PCC 6803. Its product, FNR, was heterologously produced and functionally characterized. The start-site of the monocystronic *petH* transcript was mapped 523 bp upstream of the predicted PetH initiation codon, resulting in an unusually large 5'-untranslated region. The 5' end of the *petH* transcript is situated within the open reading frame of phosphoribulokinase (encoded by *prk*), which is transcribed in opposite orientation with respect to *petH*. The transcription start site of the *prk* transcript was mapped 219 bp upstream of the initiation codon, resulting in a 223 bp antisense region between both transcripts.

Under many conditions the expression of both genes (i.e. *petH* and *prk*) is co-regulated symmetrically at the transcriptional level, as was concluded from both northern hybridization experiments and from primer extension analyses; it became uncoupled, however, when specifically *petH* expression was stimulated, independent of *prk* expression, by stressing the *Synechocystis* cells with high salt concentrations. A model for a new type of bidirectional operator, regulating the expression of *petH* and *prk*, is proposed.

Abbreviations: FNR, ferredoxin-NADP$^+$ oxidoreductase; PSI, photosystem I; Prk, Phosphoribulokinase; PCR, polymerase chain reaction

Introduction

In chloroplasts and cyanobacteria, ferredoxin-NADP$^+$ oxidoreductase (FNR) is described as the enzyme that catalyses the terminal step in light-driven electron transfer from water to NADP$^+$ in oxygenic photosynthesis. Cyanobacterial thylakoid membranes, however, carry photosynthetic, as well as respiratory-chain components. Surprisingly, and in contrast to respiratory electron transfer pathways in bacteria and mitochondria, cyanobacteria preferentially respire NADPH over NADH. This has first been demonstrated in *Anacystis nidulans* [1], and more recently in *Aphanocapsa* PCC 6714 (or *Synechocystis* PCC 6714) [2] and in *Plectonema boryanum* [3]. In thylakoid membranes of these organisms, the maximal capacity of NADPH oxidation is higher than the capacity of NADH oxidation, although the ratio of both activities is variable between these species [4]. Alpes *et al.* [5] have concluded that NADPH and NADH are oxidized by separate dehydrogenases in *Anabaena variabilis*, from the observation that NAD$^+$ does not inhibit respiratory oxygen uptake, whereas NADP$^+$ does. Also, the NADPH oxidizing activity could be separated from the NADH oxidizing activity via chromatography

[6]. Subsequently, these authors tentatively identified ferredoxin-NADP$^+$ oxidoreductase as the NADPH-specific respiratory dehydrogenase. *In vitro*, reconstitution of respiratory NADPH oxidation was demonstrated by adding purified FNR to washed *Spirulina maxima* thylakoid membranes [7]. Notwithstanding these results, a functional role of FNR in respiratory electron transfer in cyanobacteria is not yet generally accepted in the literature. Sequence analysis of the cyanobacterial genome has revealed that in addition to the well characterized typeI NADH dehydrogenase [1–4] also genes homologous to type II NADH dehydrogenases are present in *Synechocystis* PCC 6803 [8].

It has been demonstrated previously that a domain homologous to the CpcD phycobilisome linker polypeptide is recognizable at the N-terminus of both the *Synechococcus* PCC 7002 [9] and the *Anabaena variabilis* PCC 7119 [10] *petH* sequences. Analysis of the *petH* sequence of a third cyanobacterium should reveal whether this domain organization is indeed conserved among the majority of, or all, cyanobacterial species.

The chloroplast FNR enzyme functions primarily in linear oxygenic photosynthesis. However, the enzyme has also been implicated to function in cyclic electron transfer around photosystem I [11, 12]. Membrane association may be an important prerequisite for the participation of the chloroplast FNR in this process. It has been suggested that a binding protein is involved in the binding of the enzyme to the thylakoid membrane [13]. The expression of chloroplast FNR is regulated at the transcriptional level. The promoter region of the spinach *petH* gene contains light-responsive elements. The 5′-untranslated region of the transcript is essential for the expression of the gene. In the dark, the spinach FNR is down-regulated [14, 15]. Light-dependent transcriptional control has been demonstrated in *Synechocystis* PCC 6803 for the *glnA* gene, encoding glutamine synthetase [16]. Transcription of *psbA* genes, encoding D1 reaction center proteins, is known to be responsive to the presence and intensity of light in cyanobacteria [17, 18]. Other well studied examples are genes encoding phycobilisome components [19, 20, 21].

As discussed above, cyanobacterial FNR may function in multiple electron transfer pathways (in the dark and/or upon illumination). As a consequence, its expression may be regulated in an even more complex way than expression of its homologue in plants. Here we report the characterization of *petH* from *Synechocystis* PCC6803 and its gene product, as well as the transcriptional regulation of its expression, as a first step in elucidating all the facets of the role of FNR in the physiology of cyanobacteria.

Materials and methods

Media and culture conditions

Synechocystis PCC 6803 was cultured in BG11 medium [22] in an orbital shaker at 28 °C under continuous illumination using two TL tubes, which yielded an average light intensity of 70 μE m^{-2} s^{-1}. A 'dark' culture was obtained by growing a 100 ml culture to an OD$_{750}$ of 0.8 under continuous illumination and wrapping the Erlenmeyer flask in foil, whereafter the culture was incubated with shaking for an additional 14 h. Transformation of *Synechocystis* was performed essentially as described [23]. Solid medium for the growth of kanamycin-resistant transformants was prepared from BG11 medium, supplemented with 1.5% (w/v) agar (Difco), 10 mM *N*-tris[hydroxymethyl]-2-aminoethanesulfonic acid (TES)/KOH (pH 8.2), 5 mM glucose, 20 μg/ml kanamycin and 0.3% (w/v) sodium thiosulfate. For the growth of transformants in liquid culture, 20 μg/ml kanamycin was added to the medium.

Recombinant DNA techniques

Chromosomal DNA was isolated by a procedure adapted from Joset [24]. Cell pellets were resuspended in 1/10 volume STET buffer (8% sucrose, 50 mm EDTA, 50 mm Tris pH 8.0 and 5% Triton X-100). An incubation of 15 min at 65 °C was followed by the addition of 0.5 M sodium perchlorate. DNA was recovered by phenol/chloroform extractions, RNAse treatment and ethanol/acetate precipitation. Heterologous Southern hybridization was performed with a randomly primed [^{32}P]-labelled probe, which spanned the entire open reading frame of the *Anabaena variabilis* ATCC 29413 *petH*. This probe was produced by PCR, with the oligonucleotides NPETH and CPETH (see Oligonucleotides, below). All cloning procedures were performed in the *Escherichia coli* strain XLI-Blue.

The *petH* probe hybridized to a 2.9 kb *Bam*HI/*Hin*dIII chromosomal DNA fragment. A *Bam*HI- and *Hin*dIII double digest of genomic DNA was fractionated on a gel, and approximately 3 kb fragments were excised. These DNA fragments were ligated to *Bam*HI/*Hin*dIII-digested and purified pBluescript II SK+. Transformants were selected on

plates containing ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal) and grown in pools of 5 clones. Miniprep plasmid DNA preparations of these pooled cultures were digested with *Bam*HI and *Hin*dIII and run on an agarose gel. Southern hybridization revealed a single positive clone in one of these pools, with a *Bam*HI/*Hin*dIII insert of the predicted size. The presence of a *Xba*I restriction site, at ca. 900 bp from the *Bam*HI site, confirmed the identity of the cloned insert in one of the clones from this pool. This clone was designated pSP35-9.

The 2.0 kb *Xba*I/*Hin*dIII fragment of pSP35-9 was subcloned into pBluescript II SK+ in the *Xba*I/*Hin*dIII sites, yielding plasmid pSP35-1KS. pSP35-1KS was linearized with *Sac*I and *Xba*I, and digested with Exonuclease III. After S1 nuclease digestion, re-ligation and transformation to *E. coli* XLI-Blue, a set of nested deletions was obtained. pSP35-9 was linearized with *Kpn*I and *Hin*dIII, in order to obtain a set of nested deletions in the opposite strand. Both sets of nested deletions were sequenced using the '–40' universal pUC/M13 primer. The 931 bp *Bam*HI/*Xba*I fragment was subcloned in both pBluescript II SK+ and in pBluescript II KS+ to yield pSP35-900SK and pSP35-900KS respectively. Both constructs were sequenced in the same manner.

Nucleotide sequences were determined by the dideoxy-chain-termination method [25] (U.S. Biochemical). Clones in phagemid pBluescript were coînfected with phage M13KO7 for template isolation and were sequenced using the '–40' universal pUC/M13 primer. The sequence data were analysed with PC/Gene (version 6.5; Intelligenetics, California) and the University of Winsconsin Genetics Computer Group (UWGCG) software (version 7.0). Sequence data were obtained from the SwissProt and PIR data libraries using the sequence comparison program FASTA [26]. All additional recombinant DNA techniques were performed as described [27].

Oligonucleotides

Primer sequences are given from the 5′ to 3′ direction. The following primers were used: NPETH, ATGTCTAATCAAGGTGC;
CPETH, CTAATATGTTTCTACGTGC;
PETEXT1, TTACCGGCATCGCTCTGGCG;
PETEXT2, GAGGGTCAGGCTATATCTCTGGC;
PRKEXT, CACGCGGTCTAGCTGTGTGG;
SPETBAM,GCGGATCCGATGACGATGACAAAAT

GTACAGTCCCGGTTACG

Purification of the recombinant FNR

The FNR overexpression strain *E. coli* M15 (pREP4)(pSHISPET1; see Results) was grown overnight in Luria Bertani (LB) medium containing 2% glucose, 100 μg/ml ampicillin and 20 μg/ml kanamycin. This culture was diluted 1:100 in LB medium, containing 100 μg/ml ampicillin. FNR expression was induced at OD_{600} 0.7 by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h of induction the culture was harvested at an OD_{600} of 1.5. Overexpression was monitored by SDS-PAGE analysis of whole-cell extracts, followed by Coomassie Brilliant Blue staining. After centrifugation, the cells were resuspended in 1/10 volume of phosphate/salt buffer (50 mM NaH_2PO_4, 300 mM NaCl; pH 7.8) and lysed by five subsequent sonications of 1 min at a duty cycle of 50%. Soluble material was separated from the insoluble fraction by 20 min centrifugation at 30 000 $\times$ g in a Sorvall centrifuge at 4 °C. The supernatant was filtered through a 0.2 μm filter and directly applied to a Ni^{2+}-Agarose column (Qiagen). Recombinant FNR was eluted from the column with a linear gradient of 0 to 400 mM imidazole in phosphate/salt buffer. The isolated material, usually 70–80% pure as estimated by SDS-PAGE and by measurement of the absorption of the flavin prosthetic group at 459 nm versus the absorption at 280 nm [7], was dialysed against phosphate/salt buffer before freezing to remove the imidazole, or against glycine buffer (50 mM glycine-KOH, 50 mM KCl, pH 8.0) for the subsequent purification on Red Sepharose (Pharmacia). The protein was adsorbed to Red Sepharose (Pharmacia) in glycine buffer, and was eluted from the column with a linear gradient of 0 to 1 M KCl in glycine buffer. Recombinant FNR elutes at ca. 300 mM KCl, yielding pure enzyme that was dialysed against glycine buffer before storage at –80 °C.

SDS-PAGE was performed as described [28]. Proteins were stained with Coomassie Brilliant Blue G250. Protein concentration was assayed as described [29], or from the absorbance at 459 nm, assuming an extinction coefficient of $E_{459}=10$ mM cm^{-1}.

RNA isolation and characterization

Total cellular RNA was isolated from fresh cultures, at an OD_{750} of 0.8, by a method modified from [30].

Cells were resuspended in a buffer containing 4.5 M guanidine thiocyanate (Fluka), 2% N-lauryl sarcosine, 50 mM Tris, pH 7.0. This suspension was extracted with phenol/chloroform at 65 °C. After ethanol precipitation of the nucleic acids, DNA was digested by RNAase-free DNAase (Promega), using 200 Units for each 100 ml cell suspension. The RNA was recovered by phenol/chloroform extractions and precipitated by the addition of 2.2 M lithium chloride.

For northern hybridization 10 μg of total RNA, as determined by measurement of the A_{260}, was run on a 1% formaldehyde/agarose gel. The size of the mRNAs were estimated by comparing it with the mobility of an RNA size marker (Gibco). Hybridization was performed in a buffer containing 50% formamide at 42 °C with randomly primed [^{32}P]-labelled DNA probes. Hybridizing bands were quantified with a Molecular Dynamics phospho-imager.

For primer extension analysis, oligonucleotides were end-labelled using T4 kinase (Pharmacia) with [^{32}P]-γ-ATP. After heat inactivation of T4 kinase, 50 fmol of labelled primer was annealed to 10 μg of RNA, and extended for 1 h at 37 °C using 200 units of M-MLV Reverse Transcriptase (Promega), using the recommended reaction buffer, with the addition of 2 mM of dNTPs (Pharmacia). Products were ethanol/acetone precipitated and denatured in 90% formamide before loading on a sequencing gel. DNA sequencing ladders that were used to measure the length of the primer extension products, were produced with 1 pmol of [^{32}P]-end-labelled primer.

Immunological techniques

Immunological detection of the phosphoribulokinase protein (Prk) was performed both with western blot and by immuno-electrophoresis. Proteins resolved on SDS-PAGE were blotted onto nitrocellulose and subsequently immunolabelled as described [31]. The purified rabbit anti-spinach phosphoribulokinase antibody was used at a 1:1500-fold dilution for the specific detection of the *Synechocystis* PCC 6803 phosphoribulokinase in western blot, and the anti-*Chlorogloeopsis fritschii* phosphoribulokinase antibody at a dilution of 1:150. The secondary antibody, horseradish peroxidase conjugated goat anti-rabbit IgG (BioRad), was used at a dilution of 1:3000. Rocket immuno-electrophoresis was performed as described [32] in a Multiphor 2117 apparatus (Pharmacia).

Diaphorase assays

The diaphorase activity of FNR was measured essentially as described [13]. Each assay contained 50 mM N-tris[hydroxymethyl]methyl-3-amino-propanesulfonic acid (TAPS) pH 8.5, 2 mM $K_3Fe(CN)_6$, 200 μM NADPH (except when stated otherwise), 10 mM glucose 6-phosphate and 15 μg/ml yeast glucose 6-phosphate dehydrogenase (Boehringer). The reaction was allowed to proceed at 25 °C and monitored by following the absorption at 410 nm. Reaction rates were measured with this assay at 2.5, 10, 15, 25, 50, 100 and 200 μM NADPH in triplo. Data were plotted in a Lineweaver-Burke plot (not shown), to determine K_m and V_{max} values.

For the measurement of diaphorase activity in cell homogenates, cells were centrifuged and resuspended in a buffer containing 50 mM TAPS pH 8.5, 50 mM KCl, 0.1% Triton X-100, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was sonicated 5 times 60 s with a Branson sonifier 250 at a duty cycle of 50%, with cooling on ice. As two additional minutes of sonication did not increase the measured FNR activity, it was concluded that 5 times 60 s sonication is sufficient for complete disruption of the cells.

Results

Cloning and nucleotide sequence analysis: identification of the petH *ORF*

The chromosome in the vicinity of *petH* was mapped by Southern hybridization with an *Anabaena variabilis* ATCC 29413 *petH* probe [33]. Single hybridising bands were observed in *Kpn*I (11 kb), *Bam*HI (7 kb), *Hin*dIII (5.5 kb), *Xba*I (2.3 kb), *Xba*I/*Hin*dIII (2.0 kb) and *Bam*HI/*Hin*dIII (2.9 kb) digests. Electrophoretic separation of chromosomal and endogenous plasmid DNA followed by Southern hybridization showed that the *petH* gene is encoded on the chromosome.

The *petH* gene of *Synechocystis* PCC 6803 was cloned on a restriction fragment from a genomic DNA digest. Analysis of the nucleotide sequence of the resulting clone pSP35-9 revealed the presence of a single, complete putative open reading frame, designated *petH*, on the 2.9 kb *Bam*HI/*Hin*dIII fragment (Figure 1). The initiation codon ATG is situated at position 711 and the open reading frame is predicted to terminate at position 1949. This ORF encodes the
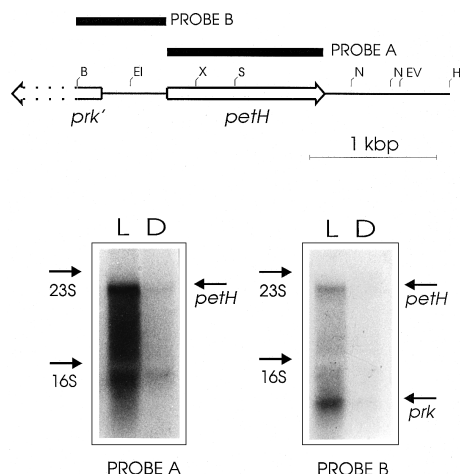
Figure 1. Mapping of the *petH* and *prk* transcript 5′ ends. Primer extension analysis of the *petH* transcript (left panel) and the *prk* transcript (right panel). Oligonucleotides used in these reactions were PETEXT2 and PRKEXT respectively. Reactions were performed in either case with 10 μg of RNA extracted from light-grown (L) and dark-adapted (D) samples. Sequencing reactions were produced with [^{32}P]-end-labelled oligonucleotides.

Synechocystis PCC 6803 ferredoxin-NADP$^+$ oxidoreductase, a protein of 413 amino acids with a size of 46 168 Da. The initiation codon is preceded by a putative Shine-Delgarno sequence (^{679}GGAG; Figure 2). A search in the sequence database showed that the predicted open reading frame has a high similarity to both eukaryotic and to prokaryotic ferredoxin-NADP$^+$ oxidoreductase sequences. The highest overall similarity (63.9% identity) of the predicted *Synechocystis* PCC 6803 PetH sequence was found with the *Synechococcus* PCC 7002 PetH sequence, but the highest score (73,9% identity) was found with the *Spirulina* PetH sequence over a domain of 291 residues. The corresponding *petH* sequence in Cyanobase has the accession number slr1643 and is 98.5% identical to the sequence determined from clone pSP35-9 [8]. The N-terminal domain of FNR has a high similarity (58.8% identity over 68 residues) with the *Synechococcus* PCC 7002 9 kDa rod linker polypeptide CpcD [34], and is 48.2% identical to the native CpcD sequence over a domain of 83 residues [8].

Interestingly, sequence comparison further revealed that the first 413 bp of this sequence (*Bam*HI-*Eco*RI fragment of pSP35-9) has already been cloned and sequenced [35]. In this latter study a chromosomal DNA fragment was cloned that encoded the *Synechocystis* PCC 6803 phosphoribulokinase (Figure 1). The first 187 bp of pSP35-9 codes for the N-terminus of

Prk. This latter nucleotide sequence has been deposited by Su and Bogorad under EMBL accession number M77134.

Overproduction, purification and characterization of the recombinant Synechocystis *PCC 6803 ferredoxin-NADP$^+$ oxidoreductase*

The *petH* open reading frame was amplified in 20 cycles using the DeepVent thermostable proofreading DNA polymerase (New England Biolabs) with the oligonucleotides SPETBAM and the M13/pUC universal '–40' primer using the *Synechocystis* PCC 6803 *petH* clone pSP35-9 as a template. From this PCR product a 346 bp *Bam*HI/*Xba*I DNA fragment was cloned in pBluescript SK+ and sequenced to confirm that no mutations were introduced by PCR. The overexpression plasmid pSHISPET1 was constructed by ligating the 346 bp *Bam*HI/*Xba*I fragment together with a 2009 bp *Xba*I/*Hin*dIII fragment from pSP35-9 to *Bam*HI/*Hin*dIII digested pQE30 (Quiagen).

The expression strain *E. coli* M15(pREP4)(pHISPE T1) was used for the overproduction of FNR as a fusion protein with 6 histidine residues fused to the N-terminus of the protein, which subsequently can be cleaved by addition of enterokinase. This enzyme recognizes the sequence Asp-Asp-Asp-Asp-Lys, encoded by SPETBAM. Purification of the recombinant enzyme with Ni^{2+}-Agarose routinely yielded 5–10 mg protein from 500 ml cultures, with an estimated purity of 80%. A second affinity purification step, using Red Sepharose, resulted in pure enzyme preparations as judged from Coomassie Brilliant Blue-stained SDS-PAA gels. In comparison with native enzyme preparations, that are prone to proteolytic attack at the N-terminus [36], the recombinant enzyme was less sensitive to proteolytic degradation. Prolonged incubation at room temperature of purified recombinant enzyme gave rise to the formation of a proteolytically cleaved product, ca. 10 kDa smaller in size, that retains high affinity for the Ni^{2+} column, indicating that cleavage took place near the C-terminus.

Activity of the recombinant enzyme was measured by using the NADPH/K$_3$Fe(CN)$_6$ diaphorase assay, performed at pH 8.5 [13]. Substrate-concentration dependent reaction rates were measured by using yeast glucose 6-phosphate dehydrogenase as a substrate regenerating system, which allows the continuous measurement of the reaction rate at a fixed substrate concentration. The measured kinetic characteristics of recombinant untruncated FNR were: K_m (NADPH) =

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141                                              ← prk   GAATCCCCGG CAACACCAAT

                                 ↓
161   AAGAACCACG CGGTCTAGCT GTGTGGTCAT AACATCCCTC AAAAATGCAA AACTGATTGT
221   TAACCCGGTT TCCTAGAAAA TTTCTCCTCA CCCCAGATGG CGTGTTGGAG AGACAAACCA
281   GAGCCTATGG GCTTGTTAAC CCCCTGTCAA GGGCCGAAGG AAAACTAATC CTGAGATCTA
341   GTCATCCTGG TCAACCCCAG ATAACGCTCT TCGGTTCGAG CCAGAGATAT AGCCTGACCC
             ↓
401   TCTGAATACT GAGTATGAAT TCTATCAGAA TTGTATTCCC TTCTACAAGA TTGACCGATT
461   CAAGGGGGGC GCGCCCTATG ACTAGACTTA ACTTAGACTA GTGGAGGTTC ATTCCAGGGT
521   TAATCGTCCG GTAAAGTGAT ACATGGGTTA CATTTTATCA AGGTTAACTT TGTTAGGGTG
581   GTAGGACTAA ATATCCGCTT CTGACCCCAC TGCTCCTCCC GGCCACGCGA TCGCCTGACG
641   ATGGTAGGCT TGATAATACT GCGATAAATT TCTGTCAGTA TTATTTGATT GAGTAGGGAG
701   CAATTAACCC ATGTACAGTC CC  petH →
```
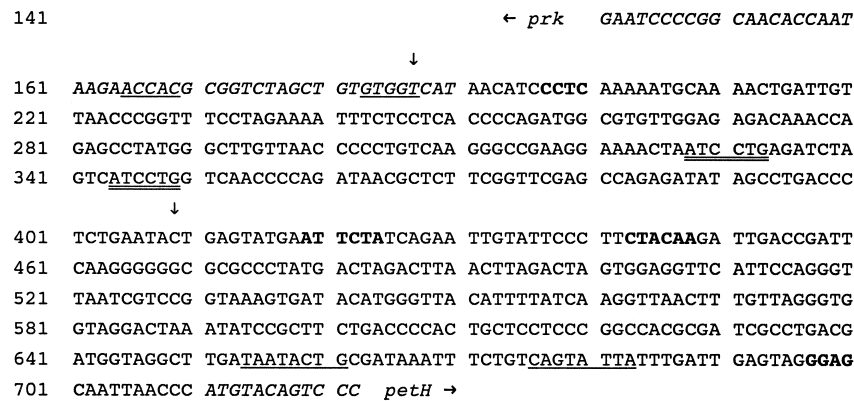
Figure 2. Northern analysis of *petH* and *prk* transcripts. Northern hybridization of equal amounts of RNA, extracted from light-grown (L) and dark-adapted (D) cells with double-stranded DNA probes that span the PetH open reading frame (Probe A), or the 5′-UTR region of the *petH* transcript, including part of the Prk open reading frame (Probe B). The map is drawn to scale. Some restriction sites are indicated: B, *Bam*HI; EI, *Eco*RI; X, *Xba*I; S, *Sma*I; N, *Nco*I; EV, *Eco*RV; H, *Hin*dIII.

21 μm and $V_{\mathrm{max}} = 110$ U/mg. These values are comparable with published kinetic data of the *Anabaena variabilis* ferredoxin-NADP$^+$ oxidoreductase [36]. The spectral properties of the recombinant FNR are typical of a flavo enzyme. Three maxima are present in its UV/VIS absorption spectrum: at 280 nm, 392 nm and 459 nm, with relative intensities of 9.2: 0.94: 1.0 for the pure protein. The maxima at 392 nm and 459 nm are due to absorption by the flavin cofactor. These data show that FNR acts as a functional reductase with its CpcD-like N-terminal domain attached.

Transcriptional organisation of petH

In order to study the regulation of transcription of *petH*, total RNA was isolated from cultures of *Synechocystis* PCC 6803, grown in two ways: The first type of cells were grown with continuous illumination, to an OD$_{750}$ of 0.8. As an alternative, light-grown cells (OD$_{750}$ = 0.8) were subsequently incubated in darkness for a period of 14 h, before harvesting and extraction of total RNA. These latter cells will be referred to as dark-adapted cells.

Primer extension reactions with oligo 'PETEXT1', which anneals close to the initiation codon inside the predicted open reading frame, generated a large product (>400 bp) when RNA from either type of cells was used. Therefore, a second oligonucleotide, 'PETEXT2', was designed to hybridize 308 bp upstream of the initiation codon on the transcript. With this latter primer a single 5′ end of the transcript was mapped, both with RNA extracted from cells grown in

continuous light, as well as from cells adapted to darkness (Figure 3). The reactions with both 'PETEXT1' and 'PETEXT2' revealed that in the RNA sample extracted from dark-adapted cells, the *petH* transcript levels were significantly decreased, when compared to light-grown cultures. This observation is based on an evaluation of the yield of primer extension products with the use of a phospho-imager. The transcription start site was mapped 523 bp upstream of the predicted initiation codon, in transcripts from both cell types.

In the large region upstream of *petH*, no significant open reading frames could be identified by computer analysis. Therefore, this region is referred to as a 5′-untranslated region (5′-UTR).

Total RNA of both light-grown- and dark-adapted cultures was subjected to northern analysis, using two different double stranded DNA probes (Figure 1). A single hybridizing band could be detected in both samples when the entire open reading frame of *petH* was used as a probe (Probe A). The low-molecular weight smear visible in this figure indicates an active turnover of the transcript. Quantification of these transcript levels, by phospho-imaging, revealed a ratio of 5:1 between samples from light-grown and dark-adapted cells, when the intensity of the entire lane was integrated. The level of the full-length transcript, however, was only marginally above the detection level, in samples from dark-adapted cells.

Probe B spans the 5′-UTR as well as the N-terminal part of the *prk* gene. Northern hybridization with this probe therefore results in detection of transcripts from both *petH* and *prk*. The *prk* transcript is estimated to
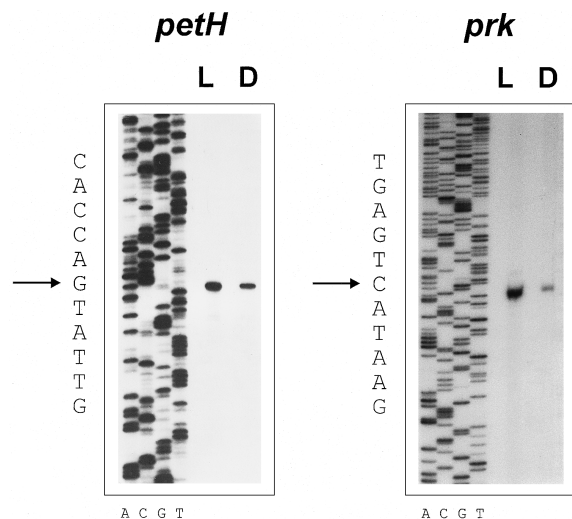
Figure 3. Nucleotide sequence of the *petH* and *prk* 5′-untranslated regions. Positions are given relative to the *Bam*HI site inside the *prk* open reading frame. The start of the *prk* open reading frame, printed in italics, is position 190. The start of the *petH* open reading frame, also printed in italics, is at position 711. The arrow at position 187 indicates the mapped transcription start site of the *petH* mRNA. The arrow at position 409 indicates the mapped transcription start site of the *prk* mRNA. The proposed *prk* '–10' and '–35' promoter sequences are printed in bold at positions 419–424 and 443–448. Two inverted repeats are depicted by the underlined sequences 165–169/183–187 and 654–661/676–683. One direct repeat, doubly underlined, is indicated at positions 328–333/344–349. The putative Shine-Dalgarno sequence of *petH*, GGAG at position 697, and the Shine-Dalgarno sequence of *prk*, GAGG at position 199, is printed in bold.
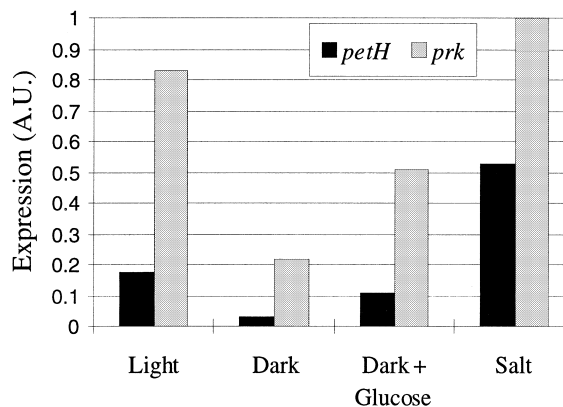

Figure 4. Relative *petH* and *prk* transcript levels. Full-length transcripts were quantified by phospho-imaging. Normalization was performed by densitometric measurement of the ethidium bromide-stained total RNA in an agarose gel. A.U.= arbitrary units. Glucose was added to a final concentration of 5 mM, and NaCl to a final concentration of 550 mM.

be ca. 1 kb in size, which corresponds to the size of its predicted open reading frame [35]. No additional transcripts were detected with probe B. This adds to the evidence that the assigned 5′-UTR of *petH* is non-coding.

The approximate size of the *petH* transcript was estimated to be 2.3 kb, by comparing its mobility with the 23S and the 16S rRNA, as well as with an RNA size marker. Since the 5′-UTR is 523 bp long and the *petH* open reading frame contains 1239 bp, some 400–500 bp remained un-accounted for. Therefore, northern hybridization with a 315 bp *Nco*I fragment, situated between 212 bp and 527 bp downstream of the open reading frame (Figure 1), was performed on the same samples. Hybridization with this probe yielded essentially the same result as obtained with probe A (data not shown). In this latter hybridization experiment the difference in intensity between samples from light-grown and dark-adapted cells was ca. 5:1 too. No significant reading frames could be identified in the 3′ region (3′-

UTR) of the transcript; neither could other transcripts be detected using this probe.

To further reveal details of the regulation of transcription of *petH* and *prk*, by the bidirectional promoter that we identified in the intergenic region between the two open reading frames, cultures of *Synechocystis* PCC6803 were subjected to salt stress through the addition of 0.5 M NaCl to cells incubated in growth medium in the light [37]. Surprisingly, this treatment gave rise to a significant increase in the *petH* mRNA level, with little corresponding change in *prk* mRNA, i.e. salt stress – in contrast to illumination and assuming no gross changes in stability of specific mRNAs – stimulates this bidirectional promoter very asymmetrically (Figure 4). Upon a transition to the dark *prk* and *petH* transcript levels were down-regulated by a comparable ratio, 3.6 and 5.6 respectively. The level of both transcripts was partially restored when glucose was added to a final concentration of 5 mM when cells were transferred to the dark, resulting in levels 0.6 and 0.5 relative to levels in continuous light (Figure 4). Similarly, northern analysis of the expression of *petH* and *prk* under additional culture conditions, such as photoheterotrophic growth (data not shown), showed a conserved ratio between the level of both transcripts, comparable with the results presented in Figure 4, except for the sample from salt-stressed cells.

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Mapping of the prk *transcription start site*

As noted above, the transcription start site of *petH* was mapped inside the open reading frame of phosphoribulokinase (Prk; Figures 3 and 2). Therefore, both transcripts must overlap to some extent. Su and Bogorad [35] postulated a sequence 95 bp upstream of the initiation codon of *prk* to be the promoter of this gene. This indicates that there must be a 99 bp overlap between both transcripts. To verify this, the actual transcription start site of *prk* was mapped by primer extension analysis, using the oligonucleotide PRKEXT. This primer was designed to anneal inside the *prk* gene close to the initiation codon. Primer extension reactions were again performed using RNA samples from both light-grown- and from dark-adapted cells, to verify the specificity of the reaction by making use of the downregulation of transcription observed in dark-adapted cells. One discrete primer extension product, ending 219 bp upstream of the *prk* initiation codon, was observed in both reactions, representing the transcription start site of *prk* (Figure 3). The yield of primer extension product was significantly lowered in the RNA sample from dark-adapted cells, which is in line with the assumption that the reaction is specific for *prk* mRNA.

Insertional mutagenesis of petH

The *petH* open reading frame was interrupted by insertion of the kanamycin resistance cartridge from pUC4K in a unique *Sma*I site (Figure 1). The resulting construct was transformed into *Synechocystis* 6803. After re-streaking on plates containing kanamycin, several resistant strains were grown in liquid medium and assayed for segregation by Southern hybridization. The transformants did not segregate, not even after many generations of growth. Characterization of the chromosomal DNA of one of the transformants showed the mutant genotype to be present only in low copy numbers, or even a single-copy (data not shown), indicating that *petH* is essential for growth of the cells under these conditions.

Expression levels of ferredoxin-NADP$^+$
oxidoreductase and phosphoribulokinase proteins

The transcriptional repression in the dark, and the induction of the *petH* mRNA during salt stress, presumably reflect the physiological requirements for the FNR enzyme in the metabolic activities of the cell. To verify this, FNR activity was measured in culture

Table 1. Diaphorase (NADPH → $K_3Fe(CN)_6$) activities in homogenates of various wild-type cultures.

Type of culture	Activity (U/mg Chl a)	Standard deviation
Continuous light	0.439	0.051
Dark-adapted	0.213	0.032
Salt-stressed	0.845	0.134

Activities are expressed on the basis of the amount of chlorophyll *a*. Culture homogenates were prepared at densities of ca. 150 μg/ml Chl *a*. 50 μl samples were used in an assay volume of 1 ml.

homogenates by the NADPH/$K_3Fe(CN)_6$ diaphorase enzyme assay [13]. The results presented in Table 1 confirm that the repression in the dark, and the induction during salt stress, of the *petH* transcript levels, resulted in decreased and increased levels of PetH enzyme activity, respectively.

The 5′-UTR of the *petH* mRNA is antisense to the initiation codon and the putative Shine-Dalgarno sequence of the phosphoribulokinase open reading frame. During salt stress the relative level of the *prk* mRNA is hardly affected while the *petH* mRNA is induced, resulting in a substantial level of mRNA antisense to the *prk* translational signals (Figure 2). The relative protein level of phosphoribulokinase in a reference culture and in a salt-stressed culture, was quantified with immunochemical techniques, to establish whether the bi-directional *petH*/*prk* operon functions in translational control on expression of the kinase. The rabbit-anti-*Chlorogloeopsis fritschii* phosphoribulokinase antiserum, when used at a 1:150 dilution, and the rabbit-anti-spinach phosphoribulokinase antibody, when used in a 1:1250 dilution [35], both resulted in specific detecion of the *Synechocystis* PCC 6803 Prk protein. The latter antibody was used in rocket immunoelectrophoresis to specifically quantify the amount of phosphoribulokinase protein in cell homogenates. No differences could be detected in the relative Prk protein levels in both samples (i.e. from cells that had and had not been subjected to salt stress, respectively) when homogenates with the same chlorofyll *a* content were used (data not shown).

Discussion

The fact that expression of the *petH* gene is regulated by light suggests that the function of its gene product is similar in cyanobacteria and chloroplasts, with respect to the energetic demands of the organism. It was

demonstrated by northern analysis and by measurement of the diaphorase activity of ferredoxin-NADP$^+$ oxidoreductase in homogenates, that the expression level of *petH* decreases as a result of prolonged incubation of cells in the dark. However, this is partially restored when 5 mM glucose is added to dark-incubated cells as a substrate for respiratory electron transfer.

A clear induction of *petH* transcription is seen when cells are stressed by the addition of a high concentration of salt, a condition known to increase the rate of PSI-dependent cyclic electron transport in *Synechocystis* PCC 6803 [37]. The redox carriers shared by cyclic electron transfer and respiratory activity are the PQ-pool, the cytochrome $b_6 f$ complex and the soluble electron carriers cytochrome *c* and plastocyanine. Therefore, it may well be that the expression of *petH* and *prk* is controlled by the redox state of any of these components. This type of redox sensing has been proposed to control gene expression in other cyanobacteria as well [38].

The Southern data indicates that *petH* is a single-copy gene located on the chromosome. A *Bam*HI/*Hin*dIII restriction fragment has been cloned that contains all the regulatory and the coding information of the gene. The *petH* gene that was subsequently identified in the *Synechocystis* PCC 6803 genome project is almost, but not completely identical to the one described here. The neighbouring sequences and putative open reading frames that were found on clone pSP35-9 do match the gene structure found in the genome project, except from a single basepare difference in the *prk* sequence. The *petH* gene does not appear to be located in a region of the chromosome enriched in photosynthesis genes. As shown in this paper, *petH* seems to be linked transcriptionally to the gene encoding phosphoribulose kinase. Remarkably, the promoter region of both the *prk* and the *petH* transcript overlap to some extent. The 5′ end of the *petH* transcript was mapped just inside the *prk* open reading frame. The promoter of the *prk* gene has been postulated to be located 95 bp upstream of the initiation codon [35]. If so, 99 bp of overlap would exist between the *petH* and *prk* transcripts. The actual transcription start site was mapped 219 bp upstream of the translation initiation codon, resulting in an overlap of 223 bp of both transcripts. Interestingly a direct repeat at position 349 is present within this region of overlap (Figure 2). The results presented in Figure 4 show that the transcriptional unit of *petH* and *prk* can be regulated symmetrically, and stimulated asymmetric-

ally. A transcriptional repressor is likely to regulate the transcription of both genes in a bidirectional fashion, whereas binding of a specific transcriptional activator could be responsible for the selective induction of *petH* transcription. The inverted repeat (ACCAC..13 bp.. GTGGT)[187], overlapping with the *petH* transcription start site, is proposed to be involved in the transcriptional stimulation seen in salt stressed cultures, where transcription of *prk* is unaffected. A proposed binding site for a repressor is the direct repeat (ATCCTG ..10 bp.. ATCCTG)[349] present in the sequence of the overlapping region between both 5′-UTR's shown in Figure 2.

Bidirectional promoters have already been identified in bacteria, such as the *malX* promoter [39], the *crp* promoter [40] and the *fepB/entC* promoter [41], all from the enterobacteria. In eukaryotes this type of promoter organization has also been identified. Transcription of the human collagen genes is mediated by a well-known divergent promoter [42]. Overlap between divergent mRNAs in which the promoters are positioned in a 'face-to-face' fashion, however, has rarely been reported [43]. Formation of antisense RNA is usually a regulatory event, where the production of a non-coding antisense RNA results in the downregulation of the sense transcript by inducing premature termination of transcription [44]. Furthermore, when antisense mRNAs are coding, transcription of the antisense mRNA generally inhibits transcription of the sense mRNA. The observation made here, that transcription of the *prk* mRNA does not interfere with transcription of *petH*, is even rarer. A precedent is found in the fact that expression from the *E. coli trp* promoter is not affected by the *lacUV5* promoter, when positioned in a face-to-face fashion that leads to the production of antisense RNAs [45]. Regulatory sequences that control transcription of divergently transcribed genes are often centrally located [43, 46]. Both the *petH* and the *prk* transcript are expressed from their own promoter, but transcriptional down-regulation affects the abundance of both genes. Therefore, it is a bidirectional operator, rather than a bidirectional promoter, that regulates the expression of both genes. In this respect the *petH/prk* operator appears unique in its organisation.

The function of the 3′ region in the *petH* transcript, that is believed not to be translated, still has to be determined. Our results with the probe in the 3′ region indicates that an open reading frame in this region, located on the strand opposite to *petH*, that has some homology with an *E. coli* hypothetical protein is not expressed above detectable levels. A low-molecular-

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weight smear is present when probes are used that span either the 5′ region, the coding region, or the 3′ region. Apparently this mRNA is actively degraded by endonucleases. It was not an artifact from the RNA isolation procedure since the *prk* mRNA did not show a similar smear (Figure 1). In cells grown in the light, the *prk* gene is heavily expressed compared to *petH*, but in a salt-stressed culture there is a significant amount of the sense 5′-UTR of *petH* present compared to the amount of antisense region of the *prk* transcript, that contains the translational signals for Prk. However, quantification of the amount of phosphoribulose kinase showed no obvious change in the salt-stressed culture. This eliminates a function of the *petH* 5′-UTR in translational control of the kinase. In contrast, the 5′-UTR of the *Synechocystis petH* transcript may have a similar function as the spinach *petH* 5′-untranslated leader sequence, which has been shown to contain 'gene-specific control elements' that apparently regulate at the transcriptional level [14].

An extension of the amino-acid sequence at the N-terminus of the cyanobacterial FNR enzyme, homologous to the CpcD phycobilisome linker polypeptides, has already been reported for *Synechococcus* PCC 7002 [9] and *Anabaena variabilis* PCC 7119 [10]. The association of FNR with the phycobilisomes as a result of this N-terminal sequence has been investigated to the extent that phycobilisome preparations seem to contain moderate amounts of FNR [9, 47]. This could result from the fact that the N-terminal sequence of FNR, like the CpcD polypeptides are very basic peptides [48]. The isoelectric point of the N-terminal sequence of the deduced aminoacid sequence of the *Synechocystis* FNR is calculated to be 10.8. Both the CpcD polypeptides and the N-terminal FNR extensions may interact with the phycobillin proteins, generally acidic in nature, by means of electrostatic interactions. It remains to be determined whether the attachment of FNR to the peripheral rods of the phycobilisomes has a physiological function.

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