Molecular Characterization of Ferredoxin–NADP$^+$ Oxidoreductase in Cyanobacteria: Cloning and Sequence of the *petH* Gene of *Synechococcus* sp. PCC 7002 and Studies on the Gene Product[†,‡]

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ABSTRACT: The *petH* gene encoding ferredoxin–NADP$^+$ oxidoreductase (FNR) was cloned and sequenced from the cyanobacterium *Synechococcus* sp. PCC 7002. The deduced amino acid sequence of the FNR protein (402 amino acids) is approximately 110 amino acids longer at the N-terminus than FNR proteins which have been characterized from other cyanobacteria. N-Terminal amino acid sequence analysis of the protein confirms the assigned translational start codon and shows that the initiator methionine is not removed. Mapping of the *petH* transcript by primer extension demonstrates that transcription initiates 112–114 bp upstream from this translational initiation site. Analyses of the mature protein from whole-cell extracts by polyacrylamide gel electrophoresis and subsequent immunoblot analysis with anti-spinach FNR antibodies revealed two distinct forms of the mature protein; both had masses of approximately 45 kDa, corresponding to the predicted molecular mass deduced from the nucleotide sequence data. Analyses by Triton X-114 phase-partitioning indicate that one form of the protein is found exclusively in the cytosol and is hydrophilic when extracts are made at low ionic strength while the second form of the protein is hydrophobic and is tightly associated with the total membrane fraction. Hydroxylamine treatment converted a portion of the membrane-associated, hydrophobic form into a protein which then behaved like the hydrophilic form. These results suggest that a portion of the FNR pool may be acylated via an ester linkage to aid in attachment of the protein to the membranes. A computer database search revealed that the N-terminal extension of the FNR protein was 78% similar to the 9-kDa phycocyanin-associated linker protein CpcD, a structural component of the phycobilisomes. It is hypothesized that the N-terminal domain of FNR serves to localize the protein near the thylakoid membrane by docking FNR at the extremities of the peripheral rods of the phycobilisomes. Consistent with this notion, FNR is present in the phycobilisomes of *Synechococcus* sp. PCC 7002. Immunoblotting analyses of other cyanobacterial species showed that in all cases the major proteins recognized by the spinach FNR antibodies had masses of 42–55 kDa and were much larger than previously reported. Smaller cross-reactive species in the mass range 24–35 kDa appear to be proteolytic degradation products.

As the terminal step in electron transport associated with photosystem I, ferredoxin–NADP$^+$ oxidoreductase (EC 1.18.1.2; hereafter referred to as FNR[1]) catalyzes the transfer of electrons from reduced ferredoxin to NADP$^+$, thus forming NADPH. The active form of the spinach enzyme contains one noncovalently bound molecule of FAD and occurs as a monomeric species with a mass of about 35 kDa. The structure of spinach chloroplast FNR has recently been determined by X-ray diffraction at nearly atomic resolution (2.6 Å) by Karplus and co-workers (Karplus et al., 1991). Although dimerization has been shown to occur, this is believed to be a nonphysiological artifact of purification (Zanetti & Arosio, 1980; Rowell et al., 1981). Additional properties of the enzyme are summarized in several recent review articles (Carrillo & Vallejos, 1987; Pschorn et al., 1988; Knaff & Hirasawa, 1991).

In higher plants the gene encoding FNR, denoted *petH*, is found in the nucleus (Newman & Gray, 1988; Jansen et al., 1988; Michalowski et al., 1989), and this is also true for the phylogenetically ambiguous biflagellated protozoan *Cyanophora paradoxa* (Bayer et al., 1990). FNR is synthesized in the cytoplasm as a precursor protein of 43 kDa which carries a leader sequence targeting the protein for import into the chloroplast stroma (Grossman et al., 1982). During transit into the chloroplast, the transit sequence is proteolytically removed to produce the mature protein (Smeekens et al., 1990). In higher plants FNR has been reported to be tightly associated with the thylakoid membranes via a trimer of a 17.5-kDa intrinsic binding protein (Carrillo & Vallejos, 1982; Vallejos et al., 1984; Ceccarelli et al., 1985). A 10-kDa protein ("connectin") may also be involved in the attachment of FNR to the thylakoids (Nosaki et al., 1985; Shin et al., 1986). In contrast to these views, Møller and co-workers have recently found that some FNR can be found associated with the photosystem I holocomplex in barley and that this FNR can be covalently cross-linked to the *psaE* gene product by the zero-length cross-linking agent EDC (Andersen et al., 1991). FNR from higher plants exhibits considerable microheterogeneity due to the susceptibility of the N-terminus of the protein

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[1] Abbreviations: bp, base pair(s); Da, dalton; DCPIP, 2,6-dichlorophenolindophenol; EDC, *N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; FNR, ferredoxin–NADP$^+$ oxidoreductase; PAGE, polyacrylamide gel electrophoresis; PCC, Pasteur Culture Collection; SDS, sodium dodecyl sulfate.

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to proteolysis (Newman & Gray, 1988; Gadda et al., 1990; Shin et al., 1990). From the crystal structure of the spinach protein, it is clear that the N-terminal region of the protein is highly exposed to solvent, a fact which could explain this sensitivity. The proteolytic degradation products generally retain full enzymatic activity, however (Gadda et al., 1990).

FNR has also been isolated from a variety of cyanobacterial species, and the molecular mass of the enzyme has consistently been reported to be about 31.5–36 kDa (Hutber et al., 1978; Masaki et al., 1979; Koike & Katoh, 1980; Rowell et al., 1981; Sancho et al., 1988; Scherer et al., 1988). The complete amino acid sequence of the FNR of *Spirulina platensis* has been reported by Yao et al. (1984), and the nucleotide sequence of the *petH* gene of *Anabaena* sp. PCC 7119 has also been determined (Fillat et al., 1990).

We are interested in structural and functional aspects of photosystem I in cyanobacteria. We report here the isolation and characterization of the *petH* gene from the unicellular, marine cyanobacterium *Synechococcus* sp. PCC 7002 and demonstrate that this gene encodes an FNR protein which is much larger (approximately 110 amino acids) than previously characterized cyanobacterial proteins. Immunoblotting experiments demonstrate that diverse cyanobacteria contain FNR protein in the 43–55-kDa size range but that these proteins are quite sensitive to proteolytic degradation. Additional characterization of the *Synechococcus* sp. PCC 7002 FNR protein suggests that the protein occurs as two forms, one of which is found in the cytosol when cells are broken in low-ionic-strength buffers and one which is tightly associated with cellular membranes. A preliminary report of this data was made at the VIIth International Symposium on Photosynthetic Prokaryotes in Amherst, MA (Schluchter & Bryant, 1991).

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. Cyanobacterial strains used in this study were *Synechococcus* sp. PCC 7002 (formerly *Agmenellum quadruplicatum* strain PR-6), *Pseudanabaena* sp. PCC 7409, *Anabaena* sp. PCC 7120, *Nostoc* sp. PCC 8009, and *Synechocystis* sp. PCC 6803 and 6714. All strains were originally obtained from the Pasteur Culture Collection (Rippka et al., 1979). All cyanobacterial strains were grown in liquid medium B-HEPES (Dubbs & Bryant, 1991) with the exception of the marine strain *Synechococcus* sp. PCC 7002 which was maintained on medium A containing 1 mg mL^{-1} sodium nitrate (Stevens & van Baalen, 1973).

Escherichia coli strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD) was used for all recombinant DNA manipulations. All cloning and sequencing procedures were performed with the plasmid vector pUC19 (Yanisch-Perron et al., 1985).

Cloning, Transformation, and Hybridization Procedures. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, MD), New England BioLabs (MA), and Boehringer Mannheim Biochemicals (Indianapolis, IN) and were used according to recommendations of the manufacturer. DNA fragment isolations were performed after agarose gel electrophoresis of DNA fragments in Tris–acetate buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). DNA fragments of interest were excised from the gel, and the DNA was purified using Geneclean (Bio 101, La Jolla, CA) according to the instructions of the manufacturer. DNA ligations were performed by standard procedures. Transformation of *E. coli* was performed according to the supplier's instructions (Bethesda Research Laboratories, Gaithersburg, MD), and transformants were selected on LB (Luria–Bertani)–agar plates containing 100 mg mL^{-1} ampicillin. Transformations of *Synechococcus* sp. PCC 7002 were performed as previously described (Buzby et al., 1983). Southern hybridizations were performed as described by Bryant and Tandeau de Marsac (1988). Labeling of DNA probes with [α-^{32}P]dATP (New England Nuclear, Boston, MA) using the random priming method was performed using a DNA-labeling kit as detailed by the manufacturer (Boehringer Mannheim, Indianapolis, IN). RNA for Northern hybridizations was fractionated on formaldehyde-containing agarose gels (Damerval et al., 1987) and transferred to Hybond-N nylon filter membranes (Amersham, Arlington Heights, IL) as previously described (Dubbs & Bryant, 1991).

DNA and RNA Isolation and DNA Sequencing. Small-scale *E. coli* plasmid isolations were done using the rapid boiling procedure of Holmes and Quigley (1981). Large-scale plasmid isolations were performed by the alkaline extraction method of Birnboim and Doly (1979); the resultant plasmids were purified by CsCl–ethidium bromide equilibrium density-gradient ultracentrifugation (Sambrook et al., 1989). Cyanobacterial chromosomal DNA was extracted as described previously (de Lorimier et al., 1984). RNA extraction from *Synechococcus* sp. PCC 7002 was performed as described (Golden et al., 1987).

Double-stranded DNA sequence analyses were performed using the dideoxy chain-termination method on base-denatured templates as previously described (Cantrell & Bryant, 1987). DNA fragments were labeled with [α-^{35}S]thio-dATP with Sequenase (U.S. Biochemicals, Cleveland, OH) as recommended by the manufacturer. The 5′ end point of the *petH* mRNA was mapped using the primer extension protocol previously described (Ausubel et al., 1987) except that 200 μg of total RNA was used in the extension reaction. The primer extension product was denatured and electrophoresed on a standard DNA sequencing gel alongside DNA sequencing ladders.

Cyanobacterial Extract Preparation, Phycobilisome Isolation, and Enzyme Assays. Cyanobacterial whole-cell extracts were prepared from 20-mL cultures in the midexponential phase of growth. Cells were either pelleted by centrifugation or collected on filter paper by vacuum filtration, washed in 50 mM Tris-HCl buffer, pH 7.5, and 10 mM EDTA, and resuspended in 1.0 mL of the same buffer. Cells were disrupted by sonication with the microtip of an Artek sonic dismembrator (Artek Systems, Inc., Farmingdale, NY). Unbroken cells were pelleted by centrifugation for 10 min in a bench-top microcentrifuge at 12000*g*. Phycobilisomes from *Synechococcus* sp. PCC 7002 strains were purified as described previously (Bryant et al., 1990; de Lorimier et al., 1990b).

FNR enzymatic activity was measured as NADPH diaphorase activity using DCPIP as an electron acceptor (Masaki et al., 1979) or by the cytochrome *c* reduction method described by Zanetti and Curti (1980).

Cell Fractionation Studies. Synechococcus sp. PCC 7002 cells (150-mL culture) were grown to midexponential phase. Cells were harvested by centrifugation, washed, resuspended in 5 mL of 50 mM Tris-HCl buffer, pH 7.5, and 10 mM EDTA, and broken in a French pressure cell operated at 4 °C at 20 000 psi. The lysate was centrifuged at 6000*g* for 10 min to pellet any unbroken cells. Half of the extract was left untreated as the "whole-cell extract". Streptomycin sulfate was added to the second half to a final concentration of 1% (w/v) to precipitate membranes; the solution was incubated on ice for 10 min and centrifuged at 6000*g* for 10 min to pellet

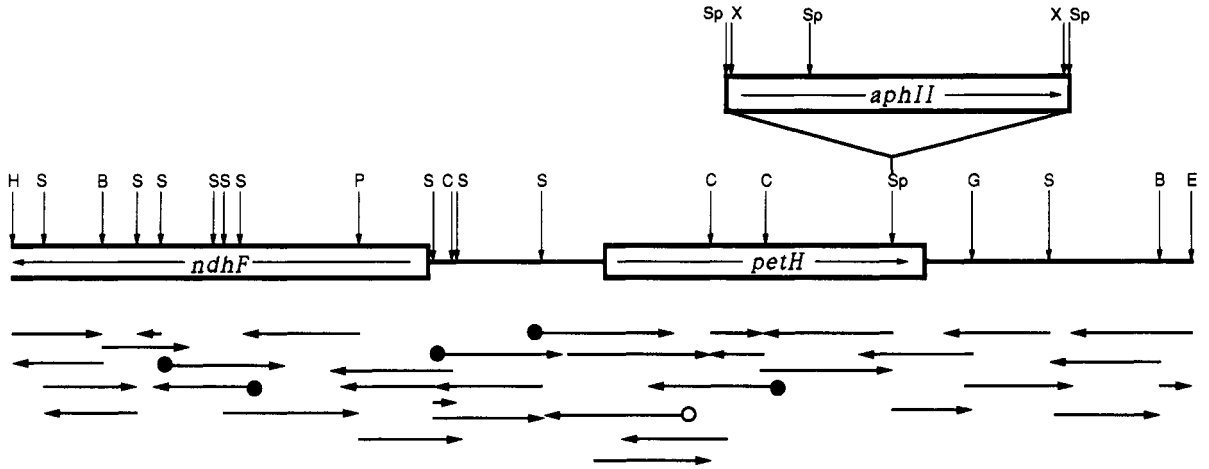

FIGURE 1: Physical map of the insert in plasmid pWS1 which contains a 4.5-kbp *Hin*dIII–*Eco*RI fragment cloned from *Synechococcus* sp. PCC 7002. The positions of the *petH* and *ndhF* genes are indicated. Arrows within the boxes representing *ndhF*, *petH*, and *aphII* indicate the direction of transcription. The *aphII* gene was inserted into the *Sph*I site of the *petH* gene in a mutational study (see text). Arrows beneath the diagram indicate the sequencing strategy; plain arrows indicate sequences obtained from subclones with either the M13 forward or M13 reverse sequencing primers. Closed circles represent synthesized oligonucleotide primers while the open circle represents the position of a synthetic oligonucleotide used in both sequence analysis and the primer extension experiment (see text). Restriction enzyme abbreviations: H = *Hin*dIII; S = *Sty*I; B = *Bam*HI; P = *Pst*I; C = *Hin*CII; Sp = *Sph*I; X = *Xba*I; G = *Bgl*II; and E = *Eco*RI.

the membranes. The supernatant, which was cleared of virtually all chlorophyll, was collected as the "cytosolic fraction", and the pelleted "total membranes" were resuspended in 2 mL of 50 mM Tris-HCl buffer, pH 7.5, and 10 mM EDTA. All extracts were stored with 5% (v/v) glycerol at −80 °C until used.

Membrane-associated proteins were further differentiated from cytosolic proteins by phase-partitioning with the detergent Triton X-114 (Bordier, 1981; Bricker & Sherman, 1982). Aliquots (200 μL) of whole-cell extract, cytosolic fraction, or total membranes were added to an equal volume of ice-cold detergent-containing buffer [20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1.0% (v/v) Triton X-114]. The solutions were mixed thoroughly and brought to room temperature for 10 min to allow phase separation to occur. The detergent phase was collected by centrifugation for 5 min in a bench-top microcentrifuge at 12000*g*. The supernatant phase containing hydrophilic proteins was removed and combined with an equal volume of ice-cold acetone; after 15 min on ice, the precipitated proteins were harvested by centrifugation at 4 °C for 15 min in a bench-top microcentrifuge at 12000*g*. The Triton X-114 detergent pellet was resuspended in ice-cold 50 mM Tris buffer, pH 7.5, and 10 mM EDTA and combined with an equal volume of ice-cold acetone, and the precipitated proteins were harvested as above. Protein pellets were allowed to air dry, and each pellet was resuspended in SDS dissociation buffer for analysis by polyacrylamide gel electrophoresis in the presence of SDS.

To test for the presence of an ester-linked acyl group, 200 μL of either cytosolic proteins or total membranes was subjected to phase-partitioning as described above. Prior to separation of the phases, 400 μL of an ice-cold 1.0 M NH_2OH, pH 10.0 solution was added, and the samples were incubated on ice for 20 min and at room temperature for an additional 40 min. The soluble and detergent phases were collected as described above and analyzed by electrophoresis.

Polyacrylamide Gel Electrophoresis and Immunoblotting. Polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE) was performed as described by Laemmli (1970) on 12.5% (w/v) polyacrylamide slab gels (30.0:0.8 acrylamide/bisacrylamide). Proteins separated by electrophoresis were electrophoretically transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH) for 2 h at 200 mA. Protein transfer was verified by monitoring the transfer of prestained molecular mass standards (Bio-Rad, Richmond, CA). The resulting blot was incubated for 30 min in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) plus 1.0% (w/v) bovine serum albumin. Primary rabbit anti-spinach FNR antibodies (IgG fraction) were added at a 1:6667 dilution, and the incubation was continued for an additional 30 min at room temperature. After three washes in TBST buffer, goat anti-rabbit alkaline phosphatase conjugated antibodies (Sigma Chemical Co., St. Louis, MO) were added at a 1:1000 dilution in TBST and incubated for 30 min. After three washes in TBST, the blot was developed in 50 mL of alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 0.33 mL of a 50 mg mL^{-1} solution of nitroblue tetrazolium in 70% (v/v) *N*,*N*-dimethylformamide and 0.165 mL of a 50 mg mL^{-1} solution of bromochloroindolyl phosphate in water. After color development was complete, the blots were rinsed in distilled water, air dried, and stored in the dark.

Results

Molecular Cloning and Nucleotide Sequence Analysis. The *petH* gene of *Synechococcus* sp. PCC 7002 was detected and cloned by heterologous hybridization with a partial pea cDNA insert derived from plasmid clone FNR144 (Newman & Gray, 1988). Genomic Southern hybridizations at reduced stringency indicated that only one *petH* gene was present in this cyanobacterial genome (see below) and that the gene was encoded on a *Hin*dIII–*Eco*RI fragment of approximately 4.5 kbp. This fragment was cloned from a size-fractionated library of *Hin*dIII–*Eco*RI fragments in plasmid pUC19 to produce plasmid pWS1. Figure 1 shows the physical map and nucleotide sequencing strategy employed in the characterization of this clone.

The complete nucleotide sequence of the insert in plasmid pWS1 has been determined on both strands, and Figure 2 shows a 3062-bp portion of this sequence. As indicated in Figures 1 and 2, analyses of the sequence data revealed the presence of four open reading frames. The first of these, including nucleotides 60–1 in Figure 2, corresponds to the *ndhF* (*ndh5*) gene (Ohyama et al., 1986; Shinozaki et al., 1986; Matsubayashi et al., 1987; Kohchi et al., 1988) which encodes a subunit of the NAD(P)H dehydrogenase complex

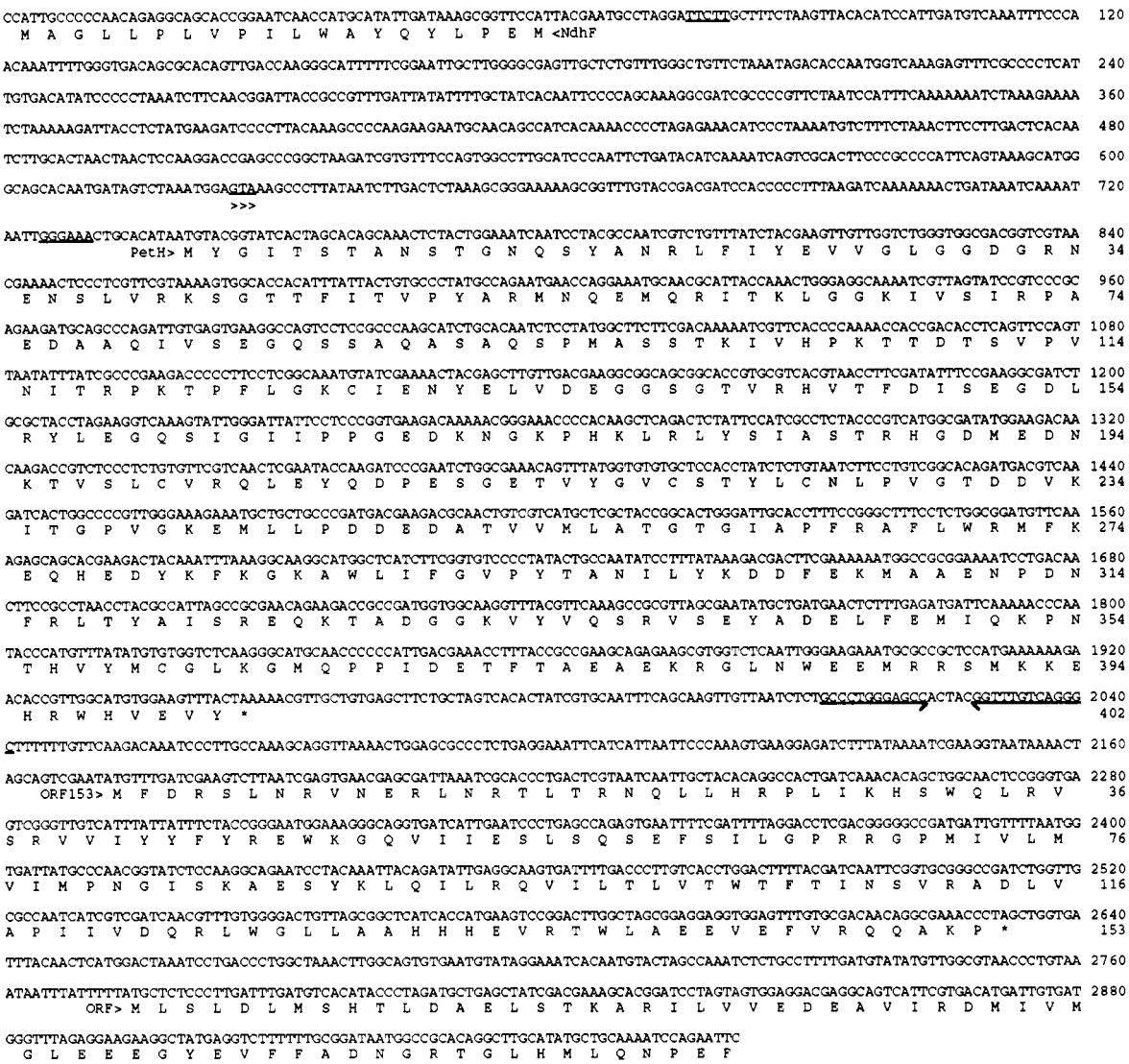

FIGURE 2: Nucleotide sequence of a 3062-bp region of the insert in plasmid pWS1 and the deduced amino acid sequences for the *petH* gene, for a portion of the *ndhF* gene, and for two unidentified open reading frames (ORF153 and the N-terminal portion of another ORF) of *Synechococcus* sp PCC 7002. Putative ribosome binding sites upstream from *petH* and *ndhF* are underlined. The direction of transcription is indicated by the arrowhead in front of the start codon for each ORF. The mapped 5′ end point for the *petH* mRNA is indicated by three arrowheads at nucleotides 626–628. An imperfect inverted repeat which could play a role in transcription termination or mRNA stabilization is shown by inverted arrows downstream of the *petH* gene.

of this cyanobacterium. The characterization, complete nucleotide sequence, and mutagenesis of this gene will be described elsewhere (W. M. Schluchter and D. A. Bryant, manuscript in preparation).

The *petH* gene was located by its homology to previously determined sequences and is shown in Figure 2 as nucleotides 740–1948. As shown in Figures 2 and 3, the gene predicts a protein of 402 amino acids with a mass of 44 950 Da and an isoelectric point of 5.66. The assigned start codon has been confirmed by amino acid sequence analysis of the protein (see below). A purine-rich motif (5′-GGGAAA), which could play a role in ribosome binding, is found from 10 to 15 bp upstream from the translational start codon. An imperfect inverted repeat, occurring from nucleotides 2012 to 2041 and followed by a T-rich sequence motif, could act as a transcriptional termination signal or mRNA stabilizer. Figure 3 presents a comparison of the deduced amino acid sequence of the FNR of *Synechococcus* sp. PCC 7002 with those of two other cyanobacteria and with those of four eukaryotes. As shown in Figure 3, all of these sequences share high degrees of identity (54–70%) to the *Synechococcus* sp. PCC 7002 FNR beginning at about amino acid residue 110 and continuing to the C-terminal tyrosine residue. The *S. platensis* sequence was determined by protein sequence analysis, while the *Anabaena* sp. PCC 7119 sequence was deduced from a nucleotide sequence. It is interesting to note that the N-terminal sequence of the *Anabaena* sp. PCC 7119 sequence does not begin near the same position as the *S. platensis* sequence (see Discussion). The four eukaryotic sequences are nuclear-encoded as precursor proteins, but the chloroplast-targeting sequences of these proteins are not similar in sequence to the N-terminal region of the *Synechococcus* sp. PCC 7002 protein.

Computer-assisted database searches revealed an unexpected origin for the N-terminal domain of the *Synechococcus* sp. PCC 7002 FNR protein. As shown in Figure 4, the N-terminal amino acids 1–85 are 78% similar to the CpcD protein of *Synechococcus* sp. PCC 7002 (de Lorimier et al., 1990a). This protein is found in phycobilisomes where it is a phycocyanin-associated linker protein whose function is to limit peripheral rod-length heterogeneity and which is associated with the core-distal phycocyanin trimer of the peripheral rods (de Lorimier et al., 1990a,b). The N-terminal domain of *Synechococcus* sp. PCC 7002 FNR is as similar to the *Synechococcus* sp. PCC 7002 CpcD protein as the homologous

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                        10        20        30        40        50        60        70        80        90       100
Synechococcus 7002:  MYGITSTANSTGNQSYANRLFIYEVVGLGGDGRNENSLVRKSGTTFITVPYARMNQEMQRITKLGGKIVSIRPAEDAAQIVSEGQSSAQASAQSPMASST
Spirulina platensis:
Anabaena 7119:
Cyanophora paradoxa:                MAFVAS PV-FANASGLKTEA VCQKPALKNSFFRGE VTSRSFFASQAVSAK  TTFEVDTTIRAQAV----------
Spinach:                            MTTAVTAAVSFPSTKTTS SAR SSVISPDKISYK--KVPL--YYRNVSATGK------MGP-IRAQI SDVEAP P PAK
Pea:                                MAAAVTAAVSLPYS STS PIRTS-IVAPERLVFK--KVSL----NNVSI G ------VGT-IRAQVTT--EAP----AK
Mesembryanthenum:                   MAAAVTAAVSFPSTKSTP STRTSSVITHEKINFN--KVPL--YYRNVSVGGK------VGT-IRA-V SDVEAPV---AK

                       110       120       130       140       150       160       170       180       190       200
Synechococcus 7002:  KIVHPKTTDTSVPVNIYRPKTPFLGKCIENYELVDEGGSGTVRHVTFDISEGDLRYLEGQSIGIIPPGEDKNGKPHKLRLYSIASTRHGDMEDNKTVSLC
Spirulina platensis:        /AK DI     K  N YI   LS E   R   T     LI    G                T N                   HV D
Anabaena 7119:       /MTQK AGHAD    L   NA  I  V S EP  K   I I Q IK  LTG N K I           V                     DV D  I
Cyanophora paradoxa: ---DA KKGD-I L LF  AN YI    Y ERI G  AP ETK II T-H  KVP             T  D                  FG D     S
Spinach:             VEK S KMEEGIT  KFK     YV R LL TKITGDDAP ETW MV S-H  EIP R    V V  D                 SAL  FG A S
Pea:                 VVK S KQDENIV  KFK  E YV R LL TKITGDDAP ETW MV S-T  EVP R       V D I                 SAI  FG S T
Mesembryanthenum:    VEK S KMEEGVI  K K  NPYT R LL TKITGDDAP ETW MV S-H  EIP R    V V  E I                 RPL  FG S T

                       210       220       230       240       250       260       270       280       290       300
Synechococcus 7002:  VRQLEYQDPESGETVYGVCSTYLCNLPVGTDDVKITGPVGKEMLLPDDEDATVVMLATGTGIAPFRAFLWRMFKEQ----HEDYKFKGKAWLIFGVPYTA
Spirulina platensis:        KHPET              EA AD-              E     II M              I    ----        L   F  I  SP
Anabaena 7119:              KHPE             THIEP SE-               PE N I          M TY      DAERAANPE Q   FS  V    TTP
Cyanophora paradoxa:  KR    T -AN NLVK    N   D KP DE- M      TT  M E QS  II            S  R   E T---- A    N L   FL   TSS
Spinach:              KR I TN-DA   IK    NF  D KP AE-  L        M K PN  II  G          S   K  F K---- D    N L   FL   TSS
Pea:                  KR V TN-DA   VVK   NF  D KP SE-           M K PN   I  G          S   K  F K----    Q N L   FL   TSS
Mesembryanthenum:     KR I TN-DN   IVK   NF  D KP SE- VL        M K PN  II             S   K  F K---- D    N L   FL   TSS

                       310       320       330       340       350       360       370       380       390       400
Synechococcus 7002:  NILYKDDFEKMAAENPDNFRLTYQISREQKTADGGKVYVQSRVSEYADELFEMIQKPNTHVYMCGLKGMQPPIDETFTAEAEKRGLNWEEMRRSMKKEHRWHVEVY
Spirulina platensis:       QQEL ELQE F E     LA     QNPE   M I D IK N  Q W L       T I      EGG  EGMS A G FDVD SDYQKEL  K      T
Anabaena 7119:             EEL EIQQKY        A      NPQ  RM I D  A H  E WQL KNQK  T I   R  EEG  AALS A A E VT SDYQKDL  AG     T
Cyanophora paradoxa: TL    REEL   QKA  N     D A    TDSK E M I N IA    N FWN       F      R  EDG  QQCMEDI KAN TT DAVVKGL   K     T
Spinach:             SL    EE     KEKA       DFAV   TNEK E M I T MAQ   V  W  LK D  YF       EKG  DIMVSL AAE ID I YK QL  AEQ N
Pea:                 SL    EE     KEKA E     DFAV   VNDK E M I T MAQ   E  W LLK D  F        EKG  DIMVSL A D ID I YK TL  AEQ N
Mesembryanthenum:    SL    EE     KEKA E     DFAV   TNEK E M I T MAQ DR  W LLK D  Y        EKG  DIMVSL AED ID FDYKKQL  AEQ N
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FIGURE 3: Comparison of the deduced amino acid sequences for the FNRs of *Synechococcus* sp. PCC 7002 (this work), *S. platensis* (sequence from the protein; Yao et al., 1984), *Anabaena* sp. PCC 7119 (Fillat et al., 1990), *C. paradoxa* (Wolfgang Löffelhardt, personal communication), spinach (Jansen et al., 1988), pea (Newman & Gray, 1988), and *Mesembryanthenum crystallinum* (common ice plant; Michalowski et al., 1989). Only the residues which differ from those of the *Synechococcus* sp. PCC 7002 FNR sequence are shown. Dashes denote insertions or deletions introduced to maximize the homology. The beginning of the two cyanobacterial sequences (*S. platensis* and *Anabaena* sp. PCC 7119) are indicated by the slash before the first residue. The eukaryotic sequences contain leader sequences which target the protein to the chloroplast or cyanelle and which are not homologous to the N-terminus of FNR from *Synechococcus* sp. PCC 7002.

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                        10        20        30        40        50        60        70        80        90
Synechococcus sp. 7002 PetH:  MYGITSTANSTGNQSYANRLFIYEVVGL--GGDGRNENSLLRKSGTTFITVPYARMNQEMQRITKLGGKIVSIRPAEDAAQIVSEGQSSAQA...
Synechococcus sp. 7002 CpcD:   LSQFANGTEAAS----- V T   Q  RQTEETD QEYAF R  SV  N            LR        K YTG TASDE *
Anabaena sp. 7120 CpcD:        F Q - LGAGSVSSS S V R      RQSSETDKNKYNI N  SV      S   E Y    R      K EQLVS EA*
Calothrix sp. 7601 CpcD1:      L SVL RR SSG- D-  V V   E  RQNEQTD NRYQI N S IE Q   S   E DR   R   R N    GENPTEDASEN*
Mastigocladus laminosus CpcD:  F Q - LGIDSVSSS S V RF     MRQNEENDKNKYNI R  SVY     N  SE     HR      K E LTR  G*
Calothrix sp. 7601 CpcD3:      VYQSRSFQVEVSGLHQ -----  --------TNQN YPI S  SVF  I FS F E L   NR      N Q LNQINEN*
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FIGURE 4: Amino acid sequence alignment of the N-terminus (amino acids 1–92) of the *Synechococcus* sp. PCC 7002 FNR protein with CpcD gene products from *Synechococcus* sp. PCC 7002 (de Lorimier et al., 1990a), *Anabaena* sp. PCC 7120 (Belknap & Haselkorn, 1987), *Calothrix* sp. PCC 7601 (Mazel et al., 1988; Mazel & Marlière, 1989), and *Mastigocladus laminosus* (Füglistaller et al., 1986). CpcD is the 9-kDa phycocyanin-associated linker polypeptide which functions to limit the length of phycobilisome peripheral rods (de Lorimier et al., 1990a). Only those amino acids which differ from the *Synechococcus* sp. PCC 7002 FNR N-terminal sequence are shown.

CpcD proteins found in phylogenetically diverse unicellular and filamentous cyanobacteria.

Two additional open reading frames could be identified downstream from the *petH* gene. The first of these, predicting a protein of 153 amino acids with a mass of 18 084 Da and an isoelectric point of 10.73, occurs from nucleotides 2172 to 2633 (see Figure 2). Database searches failed to identify any proteins with significant amino acid sequence homology to this potential protein. The second open reading frame is not complete but begins at nucleotide 2774 and continues to the end of the fragment. This open reading frame was 32–42% similar to the two-component, prokaryotic transcription regulators RscC, PhoB, the dye-resistance protein, and SprA of *E. coli* and showed greatest similarity to SprA and the dye-resistance protein (data not shown).

Northern Blot Hybridizations and Primer Extension Analysis. Total RNA was prepared from exponentially growing *Synechococcus* sp. PCC 7002 cells, separated by electrophoresis on formaldehyde–agarose gels, and transferred to nylon filters by capillary transfer. The resultant blot was probed with a radiolabeled probe corresponding to the 486-bp *Hin*cII–*Sph*I fragment of the *petH* gene. This hybridization revealed a single transcript approximately 1350 nucleotides in length (Figure 5A). This result strongly suggests that the *petH* gene is transcribed as a monocistronic transcript. The 5′ end point of the *petH* mRNA was mapped by primer extension using a synthetic oligonucleotide of 20 bases (5′-GAAGCCATAGGAGATTGTGC-3′) which corresponds to the complement of nucleotides 1032–1013 in Figure 2. The primer extension product was compared to two different sequencing ladders to determine its length; one of these is shown in Figure 5B. These studies indicate that transcription initiates 112–114 bp upstream from the translational start codon of the *petH* gene. Although a sequence (5′-TAGTCT-3′) weakly resembling the *E. coli* consensus promoter (5′-TATAAT-3′) occurs upstream from the transcription start in the −10 region, no sequencing resembling the −35 motif of consensus *E. coli* promoters could be found (see Figure 2).

Attempted Interposon Mutagenesis of the petH Gene. Plasmid pWS1 contains a unique *Sph*I site located in the 3′ portion of the *petH* gene (see Figure 1; nucleotides 1829–1834 in Figure 2). A 1.32-kbp fragment encoding the *aphII* gene

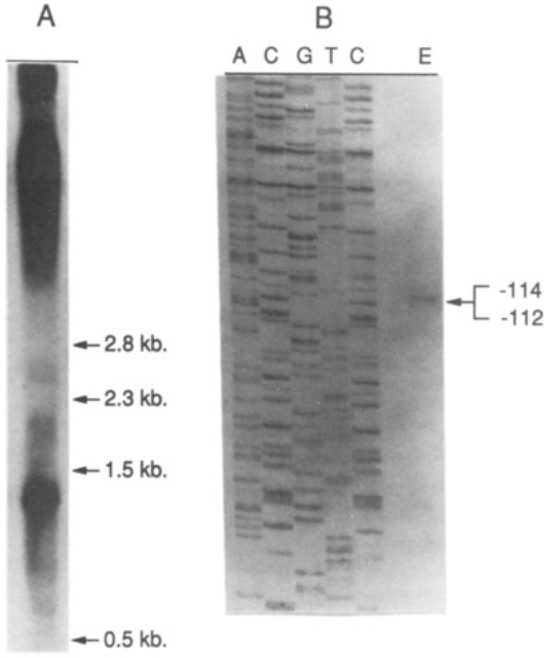

FIGURE 5: Northern blot hybridization (panel A) and primer extension analysis (panel B) of the *Synechococcus* sp. PCC 7002 *petH* transcript. (A) The Northern blot was probed with a radiolabeled 486-bp *Hin*CII–*Sph*I gene-internal fragment of the *petH* gene (see Figure 1). The arrows at the right indicate the position of ribosomal RNA molecules whose approximate lengths in kilobases (kb) are indicated. (B) Lane E shows the primer extension product from the *petH* transcript compared to a sequencing ladder to determine its length. The synthetic 20-mer 5′-GAAGCCATAGGAGATTGTGC-3′, corresponding to nucleotides 1032–1013 in Figure 2, was used. The length of the primer extension product was compared to two different sequencing ladders which indicate that the length of the extension product is between 404 and 406 nucleotides. Hence, the 5′ end point of the *petH* mRNA occurs 112–114 nucleotides upstream from the translation start of the gene at nucleotides 626–628 in Figure 2.

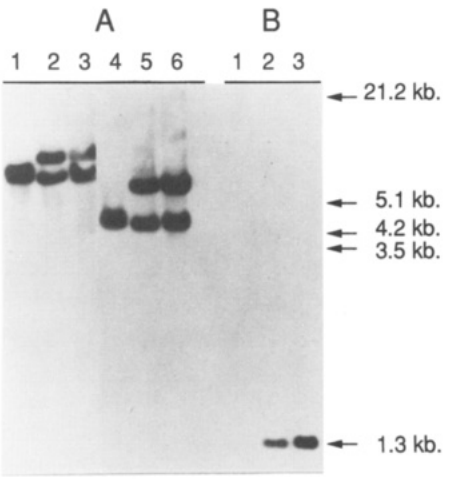

FIGURE 6: Fluorogram of a Southern blot hybridization experiment. Panel A: A radiolabeled 4.5-kbp *Hin*dIII–*Eco*RI insert from plasmid pWS1 was used as a probe for a Southern blot containing chromosomal DNAs digested with *Eco*RI (lanes 1–3) or with *Eco*RI and *Hin*dIII (lanes 4–6). Lanes 1 and 4 contained wild-type *Synechococcus* sp. PCC 7002 DNA whereas lanes 2 and 5 and 3 and 6 contained chromosomal DNAs from two independent transformants generated by the interposon construction shown in Figure 1 in which the *petH* gene is interrupted with the *aphII* gene at the *Sph*I site (see Figure 1). The transformants contain DNA fragments demonstrating that both unaltered and interrupted *petH* sequences occur in approximately equivalent amounts. Panel B represents *Xba*I digests of chromosomal DNAs from the wild-type strain (lane 1), and from two transformants (lanes 2 and 3). This blot was hybridized with a radiolabeled fragment containing the 1.32-kbp interposon cartridge encoding the *aphII* gene. Only the DNAs of the transformants exhibit hybridization to this probe as expected. The positions of size markers in kilobases are shown at the right.

of Tn5, which encodes aminoglycoside 3′-phosphotransferase II (an enzyme which confers resistance to the antibiotic kanamycin), was inserted into this site such that the *aphII* gene can be transcribed in the same direction as the *petH* gene (see Figure 1). The 5.85-kbp *Hin*dIII–*Eco*RI fragment from the resultant plasmid was excised, purified by agarose gel electrophoresis, and used to transform photoautotrophically grown *Synechococcus* sp. PCC 7002 cells. Kanamycin-resistant transformant colonies, which were more yellow-green in color than those of the wild-type strain, arose at high frequency; several transformants were selected and streaked repeatedly (at least four times) to allow segregation of the mutant and wild-type alleles. Chromosomal DNA was purified from several transformants, digested with various restriction endonucleases, and fractionated by agarose gel electrophoresis. The DNA fragments were transferred to a nitrocellulose filter by capillary transfer, and the resultant Southern blot was hybridized with two radiolabeled DNA probes: the first was the 4.5-kbp *Hin*dIII–*Eco*RI fragment insert of plasmid pWS1 (see Figure 1), and the second was the 1.32-kbp interposon cartridge encoding the *aphII* gene. As shown in Figure 6B, the DNAs of the transformants, but not that of the wild-type strain, contain a 1.32-kbp *Xba*I fragment (the *Xba*I sites arise from linker sequences flanking the interposon cartridge) that hybridizes to the *aphII* probe DNA as expected. In Figure 6A, lanes 1–3 contain genomic DNAs digested with *Eco*RI while lanes 4–6 contain genomic DNAs digested with *Eco*RI and *Hin*dIII. The 4.5-kbp *petH* probe fragment hybridizes to a unique 7.0-kbp *Eco*RI fragment (Figure 6A, lane 1) and to a unique 4.5-kbp *Hin*dIII–*Eco*RI fragment in the genomic digests of the wild-type strain. Approximately equivalent hybridization to *Eco*RI fragments of 7.0 and 8.4 kbp (Figure 6A, lanes 2 and 3) and to *Hin*dIII–*Eco*RI fragments of 4.5 and 5.85 kbp was observed for both transformants tested. The results obtained with the wild-type DNA indicate that the *petH* gene is a single-copy gene in *Synechococcus* sp. PCC 7002. The results obtained with the transformants indicate that stable diploids have been generated as previously observed in attempts to inactivate the *recA* gene of *Synechococcus* sp. PCC 7002 (Murphy et al., 1987, 1990). This implies that the *petH* gene product is required for viability of *Synechococcus* sp. PCC 7002 cells under photoautotrophic growth conditions.

Characterization of FNR Protein in Synechococcus sp. PCC 7002. The unexpectedly large size of *Synechococcus* sp. PCC 7002 FNR as predicted by the *petH* gene prompted us to perform experiments to verify that the protein indeed had a mass of 45 kDa. Immunoblotting experiments were performed with a rabbit antiserum directed against the FNR of spinach (generously provided by Drs. David Knaff, Texas Tech University, and Richard Malkin, University of California, Berkeley). The proteins present in whole-cell extracts were separated by SDS–PAGE, electrophoretically transferred to nitrocellulose filters, and probed with the anti-spinach FNR antibodies. Two strongly immunoreactive species, with apparent masses of approximately 45 and 43 kDa, were observed in whole-cell extracts (Figure 7A, lane 5). In many but not all experiments, a strongly immunoreactive species at approximately 100 kDa could also be observed in whole-cell extracts; this cross-reactive species at 100 kDa could represent an undissociated dimeric form of FNR or could represent a protein sharing some structural similarity to either the flavin-binding domain of FNR (e.g., nitrate or sulfite reductase; Karplus et al., 1991) or the ferredoxin-binding domain of FNR (e.g., nitrite reductase or glutamate synthase; Knaff & Hi-

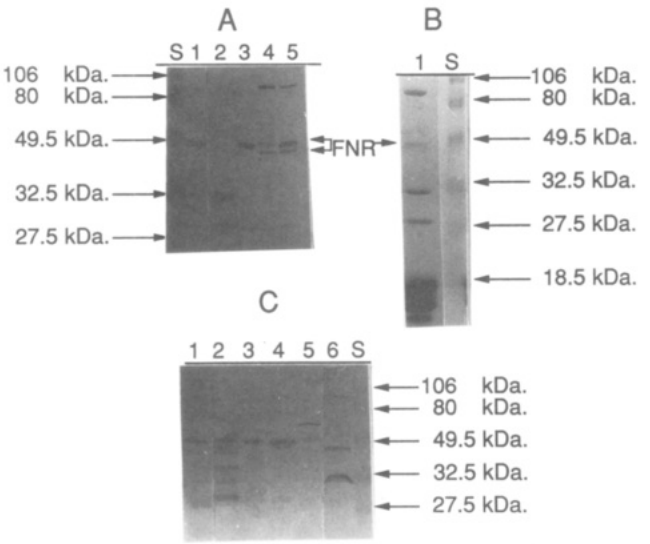

FIGURE 7: SDS–PAGE and immunoblot analysis of FNR from *Synechococcus* sp. PCC 7002 and other species of cyanobacteria. Proteins in cell extracts were separated by SDS–PAGE, electroblotted onto nitrocellulose filters, and probed with a spinach FNR antibody. Panel A represents an immunoblot of various preparations and extracts from *Synechococcus* sp. PCC 7002 strains. Lane 1 contains phycobilisomes from wild-type *Synechococcus* sp. PCC 7002; lane 2 contains peripheral rods isolated from phycobilisomes as described by Bryant et al. (1990); lane 3 contains phycobilisomes isolated from mutant strain PR6012 (de Lorimier et al., 1990b) which cannot produce the 33-kDa phycocyanin-associated rod-linker polypeptide CpcC; lane 4 contains a whole-cell extract from a mutant which does not accumulate any detectable phycobiliproteins or linker polypeptides (Bruce et al., 1989); and lane 5 contains a whole-cell extract from wild-type cells. The position of FNR is indicated by the arrows. The "S" lane shows prestained molecular mass markers whose sizes are indicated. Panel B shows Coomassie blue-stained polypeptides of *Synechococcus* sp. PCC 7002 phycobilisomes (lane 1) separated by SDS–PAGE; this sample is identical to that shown in the immunoblot in lane 1 of panel A. The "S" lane shows prestained molecular mass markers show sizes are indicated. Panel C shows an immunoblot of proteins in whole-cell extracts from various cyanobacterial species: *Nostoc* sp. PCC 8009 (lane 1); *Anabaena* sp. PCC 7120 (lane 2); *Synechocystis* sp. PCC 6714 (lane 3); *Synechococcus* sp. PCC 7002 (lane 4); *Synechocystis* sp. PCC 6803 (lane 5); and *Pseudanabaena* sp. PCC 7409 (lane 6). Immunoreactive proteins at 43–55 kDa can be observed in all extracts; additional immunoreactive species with masses between 27 and 35 kDa are probably proteolytic degradation products (see text).

rasawa, 1991). More weakly reactive polypeptides at 97, 33, and 27 kDa were also observed, and these three species correspond precisely with the apparent molecular masses of the *apcE*, *cpcC*, and *cpcG* gene products of *Synechococcus* sp. PCC 7002 (Bryant et al., 1990; Bryant, 1991). These three proteins, which have basic isoelectric points (p*I*s 7.85–10.03), have previously been shown to produce nonspecific interactions (probably via ionic interactions with the acidic immunoglobulins) with a commercially available anti-β-galactosidase antibody (Gasparich, 1989).

To confirm that these weak cross-reactions were indeed due to the phycobilisome linker polypeptides, several control experiments were performed. Figure 7A, lane 2, shows the strong, nonspecific cross-reaction obtained with the CpcC (33 kDa) and CpcG (27 kDa) phycocyanin-associated linker polypeptides of purified peripheral rods from the phycobilisomes of this cyanobacterium (Bryant et al., 1990). Figure 7A, lane 3, shows the results obtained with phycobilisomes from a mutant strain (PR 6012) of *Synechococcus* sp. PCC 7002 which does not produce the CpcC polypeptide (de Lorimier et al., 1990b), and as predicted, no 33-kDa species is recognized by the anti-FNR serum. Figure 7A, lane 4, shows results obtained with a whole-cell extract of a mutant strain of *Synechococcus* sp. PCC 7002 (Bruce et al., 1989) which does not accumulate any detectable phycobiliproteins or associated linker polypeptides, and no immunoreactive species are detected at 99, 33, or 27 kDa. The surprising result is that phycobilisomes from wild-type cells or from the strain PR6012 (lanes 1 and 3, respectively) react strongly and specifically with a polypeptide of 45 kDa which comigrates with the more slowly electrophoresing species observed in whole-cell extracts. As shown in Figure 7B for phycobilisomes of *Synechococcus* sp. PCC 7002 wild-type strain, this immunoreactive species corresponds to a low-abundance polypeptide of 45 kDa which can be observed in phycobilisomes by Coomassie blue staining. The N-terminal sequence of this polypeptide had been determined previously (V. L. Stirewalt and D. A. Bryant, unpublished results) as part of our characterization of the phycobilisomes of this cyanobacterium, and a reexamination of the data confirmed the polypeptide's identity as FNR. The sequence previously obtained matched that deduced from the gene perfectly and demonstrated that the N-terminal methionine is not posttranslationally removed from the polypeptide. It should be noted that dissociated phycobilisomes contained high levels of assayable FNR activity, even after prolonged storage at −20 °C.

Having confirmed that *Synechococcus* sp. PCC 7002 produces an FNR protein of 45 kDa, other phylogenetically diverse cyanobacterial strains were examined by the immunoblotting procedure. Whole-cell extracts in low-ionic-strength buffer were prepared from five additional cyanobacteria, and the results are presented in Figure 7C. In all species examined, immunoreactive species of 42–55 kDa could be observed. *Nostoc* sp. PCC 8009 (lane 1), *Anabaena* sp. PCC 7120 (lane 2), and *Synechocystis* sp. PCC 6714 (lane 3) exhibit strongly immunoreactive proteins at 45 kDa as does *Synechococcus* sp. PCC 7002 (lane 4). *Synechocystis* sp. PCC 6803 (lane 5) exhibited cross-reactive species at 55 and 43 kDa, and *Pseudanabaena* sp. PCC 7409 (lane 6) exhibited a cross-reactive species at 42 kDa. In *Nostoc* sp. PCC 8009 (lane 1), *Anabaena* sp. PCC 7120 (lane 2), and *Pseudanabaena* sp. PCC 7409 (lane 6), several additional cross-reactive species were observed in the 27–35-kDa range. Although some of these could be due to nonspecific cross-reactions to linker proteins as noted above, as the age of such extracts increased (even when stored at −80 °C), the amounts of these species increased and the 45-kDa species decreased (data not shown). This could also be observed with extracts of *Synechococcus* sp. PCC 7002 which had been stored for long periods of time (see lane 2, Figure 8, which is the same extract as shown in Figure 7A, lane 5, after approximately 1 month of storage). These results indicate that most, or perhaps all, cyanobacterial strains produce FNR proteins much larger than previously observed, but that these proteins are sensitive to endogenous proteolytic enzymes when extracts are prepared at low ionic strength.

Subcellular Localization and Posttranslational Modification Studies. The observation of two electrophoretically distinguishable forms of FNR in *Synechococcus* sp. PCC 7002 extracts prompted us to question whether these forms could somehow be related to the distribution of the protein within the cell and whether these forms reflect some posttranslational modification of the protein. To test these possibilities, whole-cell extracts prepared at low ionic strength were first fractionated into soluble-protein and membrane-protein fractions by streptomycin–sulfate precipitation of the membranes. As shown in Figure 8A, lanes 7 and 8, and in Figure 8B, lane 3, the supernatant from this precipitation, containing

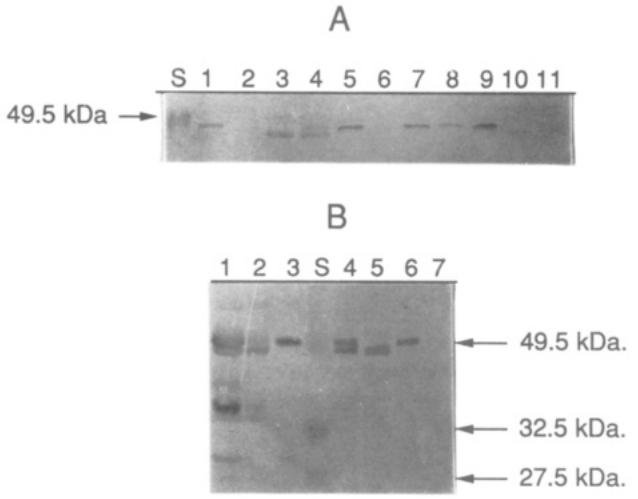

FIGURE 8: Immunoblot analysis of *Synechococcus* sp. PCC 7002 extracts treated with the detergent Triton X-114. Streptomycin sulfate was used to precipitate total cell membranes and thereby produce a membrane fraction and a soluble-protein fraction from a whole-cell extract. To achieve complete separation of hydrophobic proteins from hydrophilic proteins, phase partitioning with the detergent Triton X-114 was performed with whole-cell extract, with the streptomycin–sulfate supernatant fraction (soluble proteins), and with the streptomycin–sulfate pellet fraction (membrane-associated proteins). At 0 °C, Triton X-114 forms a homogeneous solution, but at room temperature this detergent phase-partitions. When centrifuged, the detergent micelles and hydrophobic proteins can be pelleted and the resulting supernatant contains the soluble proteins. Panel A shows FNR proteins from these various experiments. Lane 1, Triton X-114 supernatant after phase-partitioning of a streptomycin–sulfate pellet fraction; lanes 2 and 3, Triton X-114 pellet fractions after the second and first rounds, respectively, of phase-partitioning of a streptomycin–sulfate pellet fraction, respectively; lane 4, streptomycin–sulfate pellet fraction, untreated; lane 5, Triton X-114 supernatant after phase-partitioning of a streptomycin–sulfate supernatant fraction; lane 6, Triton X-114 pellet after phase-partitioning of a streptomycin–sulfate supernatant fraction; lane 7, streptomycin–sulfate supernatant fraction which was loaded directly; lane 8, streptomycin–sulfate supernatant precipitated with acetone and resuspended before loading; lane 9, Triton X-114 supernatant after phase-partitioning of a whole-cell extract; lane 10, Triton X-114 pellet after phase-partitioning of a whole-cell extract; and lane 11, the whole-cell extract with no treatment. Panel B shows results obtained after Triton X-114 phase-partitioning of streptomycin–sulfate fractions after treatment with hydroxylamine under conditions which would cleave ester-linked acyl groups from proteins. Lane 1, untreated whole-cell extract; lane 2, streptomycin–sulfate pellet from whole-cell extract; and lane 3, streptomycin–sulfate supernatant fraction from whole-cell extract. Lanes 4 and 5 represent Triton X-114 phase-partitioning of a streptomycin–sulfate pellet fraction after treatment with hydroxylamine; lane 4 contains proteins from the Triton X-114 supernatant phase (hydrophilic proteins), and lane 5 contains the proteins which continued to associate with the Triton X-114 pellet (hydrophobic proteins). Triton X-114 partitioning of the streptomycin–sulfate supernatant fraction after treatment with hydroxylamine is shown in lanes 6 and 7. Lane 6 contains the Triton X-114 supernatant fraction (hydrophilic proteins) and lane 7 contains the Triton X-114 pellet fraction (hydrophobic proteins) from this partitioning experiment. Lane "S" contains the prestained molecular mass markers, and their masses are indicated in kilodaltons to the right.

cytosolic proteins only, contains only the more slowly migrating, 45-kDa form of the FNR protein. Conversely, the form of FNR associated with the membrane fraction pelleted with streptomycin sulfate corresponded almost exclusively to the more rapidly migrating species with an apparent mass of 43 kDa (Figure 8A, lane 4, and Figure 8B, lane 2). In the older whole-cell extract used in the experiments shown in Figure 8B, it is also possible to detect substantial amounts of a degradation product with an apparent mass of approximately 34 kDa (Figure 8B, lanes 1, 2, and 4). Both the supernatant and pellet fractions contained substantial levels of assayable FNR activity; this result suggests that the cross-reactive species observed in the membrane fraction is indeed FNR.

The observed partitioning pattern of FNR between the two subcellular compartments led us to explore the properties of the two forms of the FNR protein by Triton X-114 phase-partitioning. Phase-partitioning of proteins with this detergent provides rapid and convenient method of separating hydrophobic, membrane-associated species from hydrophilic, water-soluble proteins (Bordier, 1981; Bricker & Sherman, 1982). When phase-partitioning was applied to a whole-cell extract, the hydrophilic, supernatant phase contained the more slowly migrating, 45-kDa form of FNR (Figure 8A, lane 9), while the precipitated detergent-micelle phase contained the faster-migrating, 43-kDa form of FNR (Figure 8A, lane 10). These results suggested that the more rapidly migrating form of the protein was more hydrophobic than the more slowly migrating form. Moreover, it might be expected that the more hydrophobic FNR protein would be localized in the membrane fraction.

To test this possibility, phase-partitioning was applied to the cytosolic and membrane fractions generated by the streptomycin–sulfate precipitation procedure described above. The more slowly migrating form of FNR was found in the cytosol partitions exclusively as a hydrophilic protein (Figure 8A, lane 5), and no FNR was found in the micelle-pellet fraction (Fig. 8A, lane 6). Phase-partitioning of the membrane fraction produced complementary results. The more rapidly migrating form of FNR found in the membranes partitioned as a hydrophobic protein into the micelle pellet (Figure 8A, lane 3). A second partitioning of the supernatant did not extract additional FNR into the detergent phase (Figure 8A, lane 2). The supernatant after this second partitioning did contain a small amount of the more slowly migrating FNR species of 45 kDa (Figure 8A, lane 1). It is likely that this represents a portion of the hydrophilic (cytosolic) form that was trapped inside membrane vesicles produced during cell breakage and manipulation.

The results described above suggest that the two electrophoretically separable forms of FNR differ significantly in their hydrophobicity and that the 43-kDa form behaves as a hydrophobic protein. An examination of the amino acid sequence made it seem unlikely that such a difference would be created by proteolytic degradation of the larger protein. One mechanism for altering the hydrophobicity of proteins is the posttranslational addition of fatty acyl groups (Stoffel et al., 1983; Towler et al., 1988; Schmidt, 1989; Hayashi & Wu, 1990; Magee, 1990; James & Olson, 1990; Kent & Fleming, 1990). Treatment of proteins carrying ester-linked acyl groups with hydroxylamine can be used to remove such modifications (James & Olson, 1989; Kent & Fleming, 1990). When the cytosolic proteins from the streptomycin–sulfate precipitation were treated with 0.5 M hydroxylamine at pH 10, the 45-kDa form of FNR was unaffected and continued to partition as a hydrophilic protein during Triton X-114 phase-partitioning (Figure 8B, lanes 6 and 7). On the other hand, hydroxylamine treatment of the membrane-protein fraction from the streptomycin–sulfate precipitation procedure led to the appearance of a substantial amount of FNR in the hydrophilic phase (Figure 8B, lane 4). Additionally, the hydrophilic phase contained a new species whose electrophoretic mobility resembled that of the membrane-associated form. Some FNR continued to partition as a hydrophobic protein in the detergent-micelle phase (Figure 8B, lane 5), indicating that, under the conditions employed, the reaction was not complete. These results support but do not prove the hypothesis that a portion

of the FNR protein of *Synechococcus* sp. PCC 7002 is modified by a posttranslational modification which could be fatty acylation.

Discussion

Ferredoxin–$NADP^+$ oxidoreductase has been the object of extensive enzymological characterization in cyanobacteria, but it appears from the results presented here that most of these studies have been carried out with a proteolytic degradation product of approximately 34 kDa which retains enzymatic activity. The larger form of the enzyme was detected immunologically in one previous study in *Anabaena variabilis* (Scherer et al., 1988), but the anomalously larger size of the protein detected in that work (reported to be 52 kDa) was suggested to be due to comigration of FNR on SDS–PAGE with a binding protein. The complete amino acid sequence of FNR from *S. platensis* has been reported from amino acid sequencing (Yao et al., 1984), and the amino-terminal sequence of the protein purified from *A. variabilis* has also been reported (Sancho et al., 1988). Interestingly, these sequences do not initiate at similar positions, consistent with the observations reported here that proteolytic degradation of FNR in whole-cell extracts of cyanobacteria produced at low ionic strength can be extensive. The previously reported *petH* nucleotide sequence from *Anabaena* sp. PCC 7119 does not extend far upstream from the reported N-terminus of the FNR protein (Fillat et al., 1990). The sequence does not contain any in-frame stop codons at the 5′ end, and no Shine–Dalgarno-like sequence appears 5′ to the suggested translational start codon. Hence, the sequence reported may not correspond to the complete *petH* gene. Resolution of this point will require additional nucleotide sequencing of the *Anabaena* sp. *petH* gene.

An examination of the deduced amino acid sequence of the *Synechococcus* sp. PCC 7002 FNR protein between the CpcD-like domain (amino acids 1–85) and the "FNR domain" (amino acids 110–402) provides some clues to the proteolytic sensitivity of the protein. These thirty amino acids (80–110) are almost exclusively (88%) small and/or hydrophilic residues. Secondary structure prediction algorithms suggest that these residues should form a highly flexible, hinge-like structure. The region is highly enriched in residues (P, E/D, S, T, Q/K, G, A) which are frequently the target sites for proteolytic cleavages of proteins (Rogers et al., 1986). Moreover, the X-ray structure of the spinach FNR protein indicates that the N-terminus of the protein points away from the main body of the protein in a manner which probably contributes to the extreme sensitivity of this protein to proteolytic damage as well (Karplus et al., 1991).

Although the role of the CpcD-like domain of FNR and the putative acylation is presently unknown, it is possible to suggest some plausible roles for these modifications. As noted above, there is an extensive literature suggesting that FNR is tightly associated with chloroplast thylakoids via one or more proteins. Although the phycobilisome may be viewed as an unusual site for attachment of another protein to the thylakoid surface, it could nonetheless do so effectively. Phycobilisomes are firmly seated on the stromal surfaces of the thylakoids and are believed to contact the photosystem II reaction centers closely (Bryant, 1987, 1991); the phycobilisome–photosystem II complexes are further organized into long, parallel rows on the thylakoids. Since there are 2- to 4-fold more photosystem I than photosystem II reaction centers in cyanobacteria (Bryant, 1987), one can imagine that the highly organized photosystem II–phycobilisome complexes must be largely surrounded by photosystem I reaction centers. A physical association of FNR with the phycobilisomes would thus localize the protein in close proximity to its ultimate source of reducing power, photosystem I. Additionally, the interaction with the large phycobilisome structure could effectively shield the protease-sensitive region of the FNR protein.

It has been known for many years that the majority of isolated cyanobacterial phycobilisomes contain small amounts of a protein of approximately 45 kDa (Tandeau de Marsac & Cohen-Bazire, 1977; Yamanaka et al., 1978; Anderson & Eiserling, 1986; Bryant et al., 1990). In *Synechococcus* sp. PCC 7002 some phycobilisome preparations contain barely detectable amounts of this component, although the component is usually present and detectable after SDS–PAGE analyses. The phycobilisomes of *Synechococcus* sp. PCC 6301 contain approximately one molecule of this component per phycobilisome, and this component coprecipitates with all other phycobilisome components when these structures are challenged with an antiserum produced against allophycocyanin (Yamanaka et al., 1978). This experiment demonstrates that the 45-kDa protein is in fact tightly associated with the phycobilisomes and not just a copurifying contaminant. The 45-kDa protein within the phycobilisomes of *Synechococcus* sp. PCC 7942 has been purified by preparative electrophoresis and has an amino-terminal sequence quite similar to that reported here for FNR (Jackie Collier and Dr. A. Grossman, personal communication). Moreover, antibodies raised against the 45-kDa protein of *Synechococcus* sp. PCC 7942 cross-react with the FNR protein of *Synechococcus* sp. PCC 7002 (even when the protein is produced in *E. coli*, see below). Given the nature of the amino-terminal CpcD-like domain, we propose that a substantial proportion of FNR in cyanobacteria is associated with the peripheral rods of phycobilisomes at their core-distal ends. Consistent with this suggestion, the 45-kDa polypeptide is no longer associated with phycobilisomes in mutants which do not produce peripheral rods (Elmorjani et al., 1986).

The role of the putative acylation of FNR in *Synechococcus* sp. PCC 7002 is less certain. Two possibilities can be suggested. Phycobilisomes are not generally found on cyanobacterial cytoplasmic membranes, which nonetheless are important sites of respiratory electron transport in cyanobacteria (Peschek, 1987; Scherer, 1990). If FNR plays an important role in respiratory processes as suggested by some workers (Scherer et al., 1988), then complete localization of the FNR on the thylakoid-associated phycobilisomes could slow electron-transport processes. One hypothesis would be that fatty acylation of some FNR might be a mechanism by which some FNR could be specifically targeted to the cytoplasmic membrane. Whether this suggestion is true will require additional studies to determine with which membrane system the hydrophobic form of FNR is associated. An alternative suggestion would be that this modification targets FNR to the membrane so that it may interact closely with some other electron-transport complex. It should be noted that this second modification has thus far only been demonstrated for *Synechococcus* sp. PCC 7002 and therefore may not generally occur in cyanobacteria.

Another possible role of the putative fatty acylation of FNR in *Synechococcus* sp. PCC 7002 could be to modify the activity of the enzyme. Preliminary results suggest that the specific activity of the membrane-associated FNR may be greater than that of the soluble form of the enzyme. At present it is not known whether the proportion of the two forms of the enzyme might change in response to environmental stresses. These possibilities, as well as the identification of the modifying

group, will require further investigation.

The studies described have generated a large number of new research questions concerning the structure and function of FNR in cyanobacteria. As an additional step toward understanding this important electron-transport enzyme, the *petH* gene of *Synechococcus* sp. PCC 7002 has been expressed in *E. coli* (W. M. Schluchter and D. A. Bryant, unpublished results). Purification of the enzymatically active protein for further studies and crystallization is in progress.

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