IRON SULFUR CLUSTER ASSEMBLY IN PHOTOSYSTEM I OF CYANOBACTERIA: THE ROLE OF SufR (SUF) AND Fdx (ISC)

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ABSTRACT

The biogenesis/assembly of Photosystem I is poorly understood. As a multi-subunit membrane pigment-protein complex, many factors affect PS I assembly. Among them, three [4Fe-4S] clusters are functionally and structurally essential. A change in a single cysteine ligand results in a diminution or complete loss of the PS I complex in cyanobacteria. Fe-S cluster assembly is a complicated bioprocess involving many protein factors. Thus far, three Fe-S cluster assembly pathways have been identified, namely NIF, ISC, and SUF. Cyanobacteria are distinguished from other organisms with regard to Fe-S assembly, since they contain four nifS-like homologs representing three possible pathways. slr0387 and sllo704 are two iscS-like homologs (ISC), slr0077 is a sufS-like homolog (SUF), and slr2143 does not belong to any Fe-S cluster assembly pathways known thus far. To investigate their roles in the Fe-S cluster assembly in PS I, two strategies were applied: 1) pseudorevertant screening of the C14S_{PsaC} mutant in Synechocystis sp. PCC 6803 to find regulators of Fe-S cluster assembly; and 2) molecular and genetic manipulation of homologous genes encoding components of known Fe-S cluster assembly pathways. For the first strategy, pseudorevertant screening revealed that sllo088 is involved in the assembly of PS I. An amino acid sequence alignment of homologs indicates that Sllo088 contains an HTH DNA binding domain and a unique cysteine motif CX_{12}CX_{13}CX_{14}C. In almost all the cyanobacterial genomes sequenced thus far, an sllo088-like gene is located immediately upstream of a suf gene cluster and is divergently transcribed from the suf genes. To investigate if sllo088 encodes a transcriptional regulator, the sllo088-like gene in
*Synechococcus* sp. PCC 7002 was insertionally inactivated. RT-PCR analysis showed that the *sufBCDS* genes are organized as an operon, and their transcription is co-coordinately up-regulated in the *sll0088*-like inactivation mutant. The *sll0088*-like gene is thus renamed *sufR* (*suf* repressor). SufR also controls other regulons, such as *slr0387* (*iscS* homolog), which is down-regulated in the *sufR* null mutant. The SufR protein was overexpressed in *E. coli* and purified. EPR and Mössbauer studies indicate that reconstituted SufR contains a [4Fe-4S] cluster. The ratio is one Fe-S cluster per dimer. Amino acid substitution studies suggest that the 1st, 2nd and 4th cysteine residues in the cysteine motif provide ligands to the Fe-S cluster. Replacement of the 3rd Cys with either serine or glycine resulted in negligible changes to the EPR spectrum. Thus, this cysteine may not participate in Fe-S cluster ligation. The reconstituted Fe-S cluster is oxygen-sensitive. Physiologically, the SufR protein may use a [4Fe-4S] cluster as a sensor to respond to oxidative stress and iron deficiency, since under both conditions the *sufBCDS* genes were derepressed. The SufR protein was also found to be distributed in two compartments as shown by an immunoblot assay. The majority of the SufR protein exists in the cytosol, but about one third associates with the thylakoid membranes, suggesting that SufR might be a multi-functional protein. The function of the membrane-bound SufR protein remains unknown.

For the second strategy, we focused on an *fdx* (ISC) homolog, *slr0148* in *Synechocystis* sp. PCC 6803. *slr0148* was shown to be the *fdx* homolog of the ISC Fe-S cluster assembly pathway based on amino acid sequence similarity, and based on the finding that the recombinant SLR0148 protein can coordinate a [2Fe-2S] cluster. To further demonstrate that Slr0148 is the Fdx component of the ISC machinery, the *slr0148* gene was
insertionally inactivated, and the growth phenotype of the slr0148 mutant was compared with that of the slr0387 and sll0704 inactivation mutants under standard, high light, low temperature, and iron limitation conditions. Their transcription under these growth conditions and oxidative stress conditions, together with that of the other two nifS-like genes, were also investigated by RT-PCR. The four nifS-like genes were differentially expressed under various conditions, clearly indicating that each individual NifS-like protein alone or in combination plays a leading role under certain stress conditions. slr0148 is found in a large gene cluster extending from slr0144 through slr0152 in Synechocystis sp. PCC 6803. RT-PCR data indicate that this gene cluster forms a large operon spanning from slr0144 through slr0151. The microarray findings that the genes involved in this operon are down-regulated in response to high light, cold, H$_2$O$_2$ treatment, and iron starvation was confirmed, although there are a few exceptions under certain conditions. The function of this gene cluster might be related to photosystem I assembly and/or turnover. Based on these studies, it is concluded that: 1) the SUF machinery plays a major and essential role in the Fe-S cluster assembly in cyanobacteria, and that the ISC machinery may be only a backup pathway; 2) SufR regulates PS I biogenesis and/or assembly as the transcriptional repressor of suf operon and transcriptional activator of iscS-like genes; 3) Slr0148 is the Fdx component of the ISC pathway in Synechocystis but may have a dual function related to the operon.
# TABLE OF CONTENTS

**LIST OF FIGURES** ........................................................................................................... ix

**LIST OF TABLES** ............................................................................................................. xiv

**ABBREVIATIONS AND DEFINITIONS** .......................................................................... xvi

**ACKNOWLEDGEMENTS** .................................................................................................. xviii

**Chapter 1 Introduction** ..................................................................................................... 1

Photosynthetic reaction centers ......................................................................................... 1

Components of PS I complex .............................................................................................. 3

PS I biogenesis/assembly .................................................................................................... 4

Fe-S cluster biogenesis ......................................................................................................... 11

Research goal ...................................................................................................................... 28

References .......................................................................................................................... 31

Figure Legends ................................................................................................................... 54

**Chapter 2 The sufR Gene (sll0088 in Synechocystis sp. PCC 6803) Functions as a Repressor of the sufBCDS Operon in Iron-Sulfur Cluster Biogenesis in Cyanobacteria** ..................................................................................................................... 63

Abstract ............................................................................................................................. 63

Introduction .......................................................................................................................... 64

Materials and Methods ...................................................................................................... 68
Chapter 5. *slr0148*, an ORF involved in an operon spanning from *slr0144* through *slr0151*, is the *fdx* Homolog of the ISC Fe-S Cluster Assembly Pathway in *Synechocystis* sp. PCC 6803
LIST OF FIGURES

**Figure 1.1** photosynthetic machinery of cyanobacteria ..............................55

**Figure 1.2** Fe-S cluster assembly pathways.................................................56

**Figure 1.3** NifU contains three domains ...................................................57

**Figure 2.1** Map of gene organization of sll0088 (sufR) and the sufBCDS operon ....112

**Figure 2.2** Conserved domains in SufR proteins from four cyanobacterial strains ....113

**Figure 2.3** The primer pairs used for the RT-PCR experiment and the co-transcription of suf genes .................................................................114

**Figure 2.4** Mutagenesis of the sll0088-like gene in Synechococcus sp. PCC 7002 ...115

**Figure 2.5** Growth curves of three sll0088-like inactivation mutant strains in Synechococcus sp. PCC 7002 .................................................................116

**Figure 2.6** Immunodetection of the SufR protein in the cells of the wild-type and two sufR null mutants of Synechococcus sp. PCC 7002 ...............................117
Figure 2.7 Transcription analyses of the suf operon in Synechococcus sp. PCC 7002...

Figure 2.8 Comparison of the mRNA levels of the nifS-like genes.........................119

Figure 2.9 RT-PCR analysis of the expression of the sufS and sufB genes in the wild-type and sufR mutant of Synechococcus sp. PCC 7002 .........................................................120

Figure 2.10 Growth curves of the wild-type and sufR mutant of Synechococcus sp. PCC 7002........................................................................................................121

Figure 2.11 Growth of the wild-type and sufR null mutant under stress conditions .....122

Figure 2.12 RT-PCR analysis of expression of the psaC gene in Synechococcus 7002
.....................................................................................................................................123

Figure 2.13 Immunoblot analysis of the PsaC protein in thylakoid membranes of Synechococcus sp PCC 7002 wild-type and sufR null mutant.........................124

Figure 2.14 Alignment of the Cys-containing C-terminal domains of the cyanobacterial SufR and homologous proteins from several other organisms .......................125

Figure 3.1 Localization of the SufR protein in cyanobacterial cells ......................145
Figure 3.2 Association of native SufR with thylakoid membranes of *Synechocystis* sp. PCC 6803 ..............................................................146

Figure 3.3 RT-PCR analysis of the expression of *sufR* under various conditions ......147

Figure 3.4 Immunoblot analysis of SufR in *Synechocystis* sp. PCC 6803 grown at different conditions .................................................................148

Figure 4.1 Comparison of the size of the original SufR-240 protein and the truncated SufR-217 protein .................................................................189

Figure 4.2 UV/visible absorption and EPR spectra of the recombinant SufR-240 protein .................................................................190

Figure 4.3 Mössbauer spectra of $^{57}$Fe-enriched wild-type SufR protein..............191

Figure 4.4 EPR spectra of reconstituted the mutant and wild-type SufR proteins ......192

Figure 4.5 Power and temperature dependence of EPR signals of the mutant and wild-type SufR proteins .................................................................193

Figure 4.6 Spin quantitation of the reconstituted wild-type SufR protein ..............194
Figure 4.7 Gel filtration analysis of the reconstituted mutant and wild-type SufR proteins .................................................................195

Figure 5.1 Amino acid sequence alignment of Slr0148 with Fdx homologs from other organisms.................................................................229

Figure 5.2 Gene organization of the gene cluster spanning slr0143 through slr0152 ..............................................................................230

Figure 5.3 Purified recombinant Slr0148 protein forms two bands on SDS-PAGE gel. ..............................................................................231

Figure 5.4 Insertional inactivation of slr0148 in Synechocystis sp. PCC 6803 ........232

Figure 5.5 EPR spectra of the Slr0148 recombinant protein .......................233

Figure 5.6 Growth curves of the slr0148 mutant and the wild-type Synechocystis sp. PCC 6803 ........................................................................234

Figure 5.7 Co-transcription of slr0143 through slr0151 detected by RT-PCR....................................................................................235
Figure 5.8 Transcription levels of slr0144 through slr0151 in *Synechocystis* sp. PCC 6803 cells growing under various conditions .........................................................236

Figure 5.9 Differential expression of four nifS-like homologous genes in *Synechocystis* sp. PCC 6803 growing under various growth conditions .................................237

Figure 5.10 Growth curves of the slr0148, slr0387, and sll0704 mutants of *Synechocystis* sp. PCC 6803 ...............................................................238
LIST OF TABLES

Table 1.1 PS I subunits and their mutants ..........................................................58

Table 1.2 Protein factors involved in PS I biogenesis .......................................59

Table 1.3 Site-directed mutations to $F_X$, $F_A$, and $F_B$ in Synechocystis 6803 ..........60

Table 1.4 Homologous genes encoding ISC components in Synechocystis 6803 ……61

Table 1.5 Homologous genes encoding ISC components in Synechocystis 6803 ……62

Table 2.1 Sequences of oligonucleotides used for RT-PCR analysis and cloning of the $sll0088$ gene.................................................................104

Table 2.2 $suf$ homologs in Arabidopsis thaliana.................................................106

Table 4.1 Sequences of oligonucleotides used for site-directed mutagenesis and truncation of the original SufR-240 protein ......................................................182

Table 4.2 List of the wild-type and mutant SufR proteins used in Chapter 4..........183
Table 4.3 The number of iron and sulfide per polypeptide of reconstituted SufR protein
………………………………………………………………………………………………184

Table 4.4 Calculated molecular weights corresponding to UV-absorption peaks of mutant
and the wild-type SufR proteins ………………………………………………….185

Table 5.1 Primers and their sequences used for RT-PCR analysis, mutagenesis and cloning
of slr0148 for protein overexpression …………………………………………..224
ABBREVIATIONS AND DEFINITIONS

$A_0$ primary electron acceptor of Photosystem I

$A_1$ secondary electron acceptor of Photosystem I

ATP adenosine triphosphate

CAPS 3-(cyclohexylamino)-1-propanesulfonic acid

Chl chlorophyll

CTAB cetyl trimethyl ammonium bromide

DBMIB 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone

DCMU 3-(3,4-dichlorophenyl)-1,1-dimethylurea

DTT dithiothreitol

EDTA ethylenediaminetetraacetic acid

EPR electron paramagnetic resonance

$F_A$ terminal electron acceptor of Photosystem I

$F_B$ terminal electron acceptor of Photosystem I

Ferene 5,5'(3-(2-pyridyl)-1,2,4-triazine-5,6 diyl)-bis-2-furansulfonic acid

Fe-S iron-sulfur

$F_X$ electron acceptor of Photosystem I preceding $F_A$ and $F_B$

HiPIP high potential iron-sulfur protein

$Km^R$ kanamycin resistance gene

LAHG light-activated heterotrophic growth

LHCl light-harvesting complex of Photosystem I
NADP ↗ nicotinamide adenine dinucleotide phosphate

OD ↗ optical density

ORF ↗ open reading frame

P680 ↗ primary electron donor of Photosystem I

P700 ↗ primary electron donor of Photosystem I

PCR ↗ polymerase chain reaction

PMSF ↗ phenylmethylsulfonyl fluoride

RT-PCR ↗ reverse transcription-polymerase chain reaction

PS I ↗ Photosystem I

PS II ↗ Photosystem II

PQ ↗ two-electron reduced $Q_B$

$Q_A$ ↗ secondary electron acceptor of Photosystem II

$Q_B$ ↗ terminal electron acceptor of Photosystem II

Sarkosyl ↗ sodium lauryl sarcosinate

SDS ↗ sodium dodecyl sulfate

PAGE ↗ polyacrylamide gel electrophoresis

$Sp^\text{R}$ ↗ spectomycin resistance gene

Tris ↗ tris(hydroxymethyl) aminoethane

Ycf ↗ hypothetical chloroplast open reading frames
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Chapter 1. Introduction

1.1 Photosynthesis and reaction center complexes

Photosynthesis is one of the most fundamental bioprocesses on Earth because it converts light energy into chemical energy and stores it in carbohydrates. The conversion of absorbed solar energy into chemical free energy in the early steps of photosynthesis involves light-induced charge separation followed by electron transport in the reaction center complexes. In photosynthetic organisms there are two distinct groups of reaction centers, type I and type II, which use [4Fe-4S] clusters and quinones as their terminal electron acceptors, respectively. Preceding these categorizing electron transfer components, both type I and type II reaction centers use a core set of six pigment molecules for light-induced charge separation, and a quinone as the secondary electron acceptor (Golbeck, 1993). Based on the similarities of the constitution and arrangement of the electron transfer cofactors and the secondary structure motif of the reaction center cores, it has been hypothesized that both types of reaction centers share a common evolutionary origin (Golbeck, 1993; Schubert et al., 1998).

Cyanobacteria and chloroplasts in plants and algae have both type I (Photosystem I, PS I) and type II (Photosystem II, PS II) reaction centers and thus can perform oxygenic photosynthesis. Both PS I and PS II are multimeric pigment-protein complexes embedded in the thylakoid membranes. They carry out unique redox chemistry mediated by the photosynthetic electron transport chains. PS II is composed of at least 17 polypeptide subunits and more than 40 cofactors (Ferreira et al., 2004; for review, see Barber, 2002; Hankamer et al., 1997). The reaction center proteins D1 and
D2 bind all of the redox-active cofactors, namely P680, the primary electron donor; pheophytin A, the primary electron acceptor; and Qₐ/Qₐ, the secondary and terminal electron acceptors. P680⁺, produced by the absorption of light, is a strong oxidant that is capable of splitting water and evolving oxygen, and is mediated by a tyrosine residue (Y₂) in the D1 protein. PS I contains 12 subunits and about 100 cofactors (for review, see Chitnis, 2001, and Golbeck, 1994; Jordan et al., 2001). The PsaA/PsaB heterodimeric core (PS I core) harbors most of the electron transfer cofactors, from the primary electron donor P700, a chlorophyll a/a_pair, to A₀, a monomeric chlorophyll a; A₁, a phylloquinone; and Fx, a [4Fe-4S] cluster. The low molecular weight PsaC protein binds Fₐ and Fₐ, two [4Fe-4S] clusters, as the terminal electron acceptors (Golbeck, 1993). Reduced Fₐ/Fₐ are strong reductants capable of reducing NADP⁺ to NADPH through the low molecular weight soluble [2Fe-2S] ferredoxin. PS II and PS I are linked together by a third thylakoid membrane protein complex, cytochrome b₆f (illustrated in Figure 1.1). The cytochrome b₆f monomer consists of 7 protein subunits. The redox cofactors are distributed in three subunits, the [2Fe-2S] Rieske protein, cytochrome bₐ, and cytochrome f (for review, see Hervas et al., 2003). There are three sites that control electron transfer in cytochrome b₆f: 1), the plastoquinol oxidase, or Qₐ site; 2), the plastoquinone reductase, or Q₁ site; and 3), the cytochrome f/plastocyanin oxidoreductase site. Electron transfer from PS II to cytochrome b₆f is mediated by the plastoquinone (PQ, 2-electron reduced Qₐ). Electron transfer between cytochrome b₆f and PS I is mediated by cytochrome c₆ or plastocyanin. Site-specific electron transport inhibitors, such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which binds to the Qₐ site in PS II (Trebst, 1980), and 2,5-
dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB), which binds to the $Q_o$ site of the cytochrome $b_6$ complex (Trebst, 1980), are often used to block electron transfer routes. During the past two decades, various biochemical, biophysical, crystallographic, and molecular genetic studies have greatly contributed to the elucidation of the structure and functions of these protein complexes and of their components (Manna et al., 1999). As a result, the structures of the three thylakoid membrane protein complexes have been solved to various degrees of resolution (Ben-sham et al., 2003; Ferreira et al., 2004; Jordan et al., 2001; Kurisu et al., 2003; Zouni et al., 2001).

1.2 Components of PS I complex

PS I functions as a plastocyanin-ferredoxin oxidoreductase. It accepts electrons from plastocyanin on the lumenal side of the thylakoid membrane and transfers them through a series of electron transport cofactors to ferredoxin docked on the stromal side. PS I contains 12 different polypeptide subunits in cyanobacteria (Golbeck, 1994; Jordan et al., 2001). Most are integral membrane proteins, with the exception of the PsaC, PsaD, and PsaE proteins, which are stromal subunits. Two transmembrane proteins, PsaA and PsaB, form the heterodimeric core of PS I, and harbor two branches of electron transfer chains as well as about 100 antenna chlorophylls and 22 carotenoids. There is also a pair of chlorophyll $a$ molecules (the accessory chlorophylls) between P700 and $A_0$, which may be involved in both forward electron transfer and energy transfer between chlorophyll molecules (Jordan et al., 2001). As stated earlier, the two terminal electron transfer acceptors, $F_A$ and $F_B$ are coordinated by the low molecular weight ferredoxin-like
protein PsaC (Golbeck, 1993). The remaining polypeptides, PsaD-F, PsaI-M, and PsaX, coordinate the remaining antenna chlorophyll a molecules and are ancillary to electron transfer in function. The three-dimensional structure of cyanobacterial PS I has been improved from 6 Å to 2.5 Å (Jordan et al., 2001; Krauss et al., 1993; Schubert et al., 1997). Recently, the crystal structure of the spinach PS I at 4.4 Å resolution was published (Ben-shem et al., 2003). The structures of the cyanobacterial and spinach PS I complexes have significant similarities in the arrangement of their subunits as well the many cofactors. Out of 96 chlorophyll a molecules in the cyanobacterial PS I complex, only three are missing in spinach. These minor but significant adjustments and ten additional chlorophyll a molecules in the spinach PS I complex are obviously adapted by spinach in a manner to facilitate docking of LHCI to PS I and energy transfer from LHCI to P700.

1.3 PS I assembly/biogenesis

Because of the detailed information on its structure and because mature biophysical techniques are available in its study, PS I serves as an excellent model system for the investigation of the biogenesis/assembly of multi-subunit membrane protein complexes. Several aspects must be considered in regard to the biogenesis/assembly of this pigment-protein complex: the coordinated expression of the PS I subunits; the formation of PS I complex from the individual subunits, including the insertion of cofactors into the PS I subunits before, during, or after PS I assembly; and the turnover of PS I complex in response to environment changes. It is believed that there must exist
proteins that are involved in the regulation of the biogenesis, assembly, and/or degradation of the PS I complex. In contrast to its well-documented structure and function, the biogenesis/assembly of PS I is poorly understood. Our knowledge of PS I biogenesis/assembly has been mainly dependent on inference from mutagenesis studies on the PS I subunits, from in vitro reconstitution experiments, and from studies on PS I assembly during light-induced chloroplast development (Chitnis, 2001).

1.3.1 PS I components and PS I assembly

The first step in PS I assembly is the formation of the PsaA/PsaB heterodimeric core (Smart et al., 1993). In *Chlamydomonas reinhardtii*, PsaA has been shown to be a CES (control by epistasy of synthesis) subunit whose translation is dependent on the presence of PsaB (Choquet et al., 2001). Both PsaA and PsaB are essential for the biogenesis/assembly of PS I. Their accumulation is a concerted process that controls the build-up of the other PS I subunits. The absence of either PsaA or PsaB in cyanobacteria (Shen et al., 1993, 1995; Smart et al., 1991, 1993) and *C. reinhardtii* (Redding et al., 1999; Webber et al., 1993) do not allow the formation of monomeric or homodimeric chlorophyll-protein complexes from PsaB or PsaA. The formation of the PsaA/PsaB heterodimeric core is followed by the insertion of the bridging [4Fe-4S] cluster F₅. Site-directed mutagenesis of the F₅-coordinating Cys565 (listed in Table 1.3) and the adjacent residue Asp566 in PsaB of *Synechocystis* sp. PCC 6803 resulted in a reduced PS I content (Smart et al., 1993; Vassiliev et al., 1995; Warren et al., 1993). Similar mutations introduced in *C. reinhardtii* led to the complete loss of PS I (Rodday et al., 1995; Webber
et al., 1993). Once the PS I core is assembled, the PsaC protein with bound F_A and F_B can associate on the heterodimeric core. In cyanobacteria, the PsaA/PsaB heterodimeric core can still be assembled in the absence of PsaC, while PsaD and PsaE cannot be assembled in the absence of PsaC (Mannan et al., 1991, 1994; Yu et al., 1995). In contrast, inactivation of the psaC gene in C. reinhardtii led to disappearance of the whole set of PS I subunits (Takahashi et al., 1991). PsaD was demonstrated to be required for the stable assembly of PsaC into the PS I core by in vitro reconstitution experiment using overexpressed PsaC and PsaD proteins with a PsaA/PsaB heterodimeric core (Li et al., 1991a). PsaD may also contribute to the assembly and/or accumulation of other small PS I subunits (Chitnis et al., 1989; Nechushtai and Nelson, 1981). The assembly of the small integral membrane proteins proceeds in parallel with the assembly of the peripheral proteins (Wollman et al., 1999). The deletion of the small subunits that do not bind prosthetic groups affects PS I biogenesis/assembly in different and limited degrees but it does not inhibit it. For example, inactivation of psaE, psaF, and psaL in Synechocystis sp. PCC 6803 had no or only a minor effect on PS I assembly (Chitnis et al., 1989, 1991, 1993). Inactivation of psaL only affects the trimerization of PS I rather than its function (Chitnis et al., 1993; Schluchter et al., 1996). PsaI helps PsaL function in PS I trimerization. A similar functional interaction was also observed between PsaJ and PsaF. While integration of PsaF into the thylakoid membrane is relatively independent of the presence of the PS I core (Shen et al., 1995), its content in a psaJ-less mutant of Synechocystis sp. PCC 6803 dropped to 20% that of the wild-type (Xu et al., 1994).
Genes encoding the PS I subunits and the phenotypes of their mutants are summarized in Table 1.1.

1.3.2 Cofactors and PS I biogenesis

Cofactors, such as chlorophyll $a$, Fe-S clusters, and quinones play an important role in PS I biogenesis/assembly. Chlorophyll $a$ as the photon capturer as well as light activated electron transporter, is absolutely required for the biogenesis of PS I. In a $chlL$-less mutant of the cyanobacterium *Plectonema boryanum*, in which chlorophyll biosynthesis was stopped at protochlorophyllide and thus became light-dependent, the amount and activity of PS I decreased during the etiolating process induced by darkness, although transcription levels of the PS I subunits remained unchanged (Kada et al., 2003). The phylloquinone-less mutants in *Synechocystis* sp. PCC 6803 recruit a plastoquinone into the $A_1$ site of PS I (Johnson et al., 2000). While the stability of the assembled PS I complexes was not affected, the levels decreased to 70% of that of the wild-type. No PS I complexes with an empty $A_1$ site seemed to exist, suggesting that a quinone in the binding site may be essential for the assembly of PS I. The presence of the [Fe-S] clusters in $F_X$, $F_A$, and $F_B$ are also required for the stable assembly of PS I. This issue will be dealt with separately in the following paragraphs.

1.3.3 Protein factors involved in PS I assembly

Factors that are not components of PS I but are involved specifically in PS I assembly have only been identified in the last several years (listed in Table 1.2). BtpA, a
thylakoid-bound peripheral protein in *Synechocystis* sp. PCC 6803, is a necessary regulatory factor for the stabilization of the PsaA and PsaB proteins, especially under low-temperature conditions (Zak et al., 1999, 2000). A Valine to Glycine (V51A) mutation in this protein significantly affected the accumulation of PsaA and PsaB, and thus resulted in 80% decrease in PS I activity (Bartsevich et al., 1997). However, another research group found that inactivation of *btpA* in *Synechocystis* sp. PCC 6803 led to the complete absence of PS I (Schwabe et al., 2003). *slr0228*, an FtsH-like ORF in *Synechocystis* sp. PCC 6803 caused a 60% reduction in the abundance of the functional PS I based on an insertional mutagenesis study (Mann et al., 2000). Recently, the same group identified another role for Slr0228 in D1 protein degradation during PS II repair under photoinhibition conditions (Silva et al., 2003). Inactivation of a ycf37 homolog in *Synechocystis* sp. PCC 6803 led to a lower PSI/PSII ratio and a higher phycocyanin/chlorophyll ratio (Wilde et al., 2001). Two other genes, ycf3 and ycf4 and their homologs, have also been identified to be required for the maximal yield of PS I. Disruption of ycf3 in *Synechocystis* sp. PCC 6803, *C. reinhardtii*, and *Nicotiana tabacum* L. caused a complete loss of PS I (Boudrea et al., 1997; Ruf et al., 1997; Schwabe et al., 2003). Ycf3 appears to be a chaperone that interacts directly with PsaA and PsaD and functions specifically in PS I assembly instead of its stability (Naver et al., 2001). Disruption of ycf4 led to a complete loss of PS I in *C. reinhardtii*, but only caused partial decrease of PS I content in *Synechocystis* sp. PCC 6803 (Boudrea et al., 1997; Schwabe et al., 2003; Wilde et al., 1995). A low molecular weight ferredoxin-like protein, RubA in *Synechococcus* sp. PCC 7002 (Shen et al., 2002a&b) and HCF101 in *Arabidopsis*
(Lezhneva et al., 2004; Stockel and Oelmuller, 2004) were proposed to disrupt the PS I assembly by affecting the Fe-S cluster biosynthesis based on genetic, biochemical, and biophysical analysis. Thus far all of the above-mentioned factors were proposed to function in the PS I assembly post-translationally. In comparison, PmgA, a protein that has been well established to function in PS I/PS II stoichiometry adjustment by mutagenesis studies (Hihara et al., 1998; Sonoike et al., 2001), was recently demonstrated to participate in the down-regulation of the transcription level of PS I genes upon shifting to high light conditions (Muramatsu et al., 2003). However, none of these factors thus far have been shown the functional mechanisms in the PS I biogenesis/assembly.

1.3.4 Fe-S cluster and PS I assembly

PS I uses three [4Fe-4S] clusters, namely $F_X$, $F_A$, and $F_B$ as the terminal electron acceptors. These three [4Fe-4S] clusters are functionally and structurally essential for PS I complex assembly. $F_X$ is an inter-polypeptide iron-sulfur cluster located between the PsaA and PsaB subunits. Each protein provides two cysteine ligands. Both $F_A$ and $F_B$ are bound by the low molecular weight PsaC protein, which contains two CXXCXXCXXXCP motifs. The first three cysteine residues in one motif and the fourth cysteine residue in the second motif provide ligands to a [4Fe-4S] cluster (Golbeck, 1999). In Synechocystis sp. PCC 6803, $F_A$ is ligated by cysteines 48, 51, 54, and 21; while $F_B$ is ligated by cysteines 11, 14, 17 and 58. Historically, a series of site-directed mutations were introduced into these cysteines in an attempt to resolve the electron transfer pathway from $F_X$ to $F_A/F_B$ (reviewed by Golbeck, 1999). These site-directed PsaC
mutants were overexpressed in *E. coli* and purified for *in vitro* reconstitution studies. [4Fe-4S] clusters can be formed by incubating sulfide, ferrous or ferric iron, and 2-mercaptoethanol and inserted into the apo-PsaC protein. The PsaC protein with reconstituted Fe-S clusters exhibited the same EPR properties as the native PsaC protein isolated from spinach thylakoids (Golbeck, 1999). Reconstituted PsaC was able to assemble onto the PsaA/PsaB heterodimeric core in the presence of the PsaD protein and form a fully functional PS I complex (Golbeck et al., 1988; Li et al., 1991b; Parrett et al., 1990; Zhao et al., 1990). These studies also found that the apo-PsaC protein cannot be reinserted into the PS I complex. The mutant PsaC proteins can also harbour [4Fe-4S] or [3Fe-4S] clusters at the modified F_A or F_B sites. Most of the mutant PsaC proteins can be rebound to the PsaA/PsaB heterodimeric core. However, functional reconstitution has not yet been achieved for mutants (Mehari et al., 1995; Jung et al., 1996). Since two [4Fe-4S] clusters were found in the PsaC protein in all the reconstituted PS I complexes, their presence may be a necessary precondition for binding to the PS I cores.

In contrast to these *in vitro* findings, *Synechocystis* sp. PCC 6803 strains with the altered PsaC, in which cysteine ligands at positions 14 or 51 to F_B and F_A respectively were replaced with an aspartate, serine, or alanine, were unable to grow photoautotrophically (Jung et al., 1997; Yu et al., 1997) (as listed in Table 1.3). Physiological and biochemical analysis indicated that the aspartate and serine PsaC mutants had dramatically lower steady-state levels of chlorophyll *a* and PS I per cell. Also, the alanine and double aspartate mutants contained no detectable levels of PsaC, PsaD, and PsaE. PS I complexes isolated from the aspartate or serine mutants showed
competent charge separation between P700 and $F_A/F_B$ and high rates of electron transfer from cytochrome $c_6$ to flavodoxin, indicating that a lower PS I content rather than any inherent problem with primary photochemistry is the underlying reason for the lack of photoautotrophic growth. More specifically, these subtle PsaC mutations may affect the proper formation of [4Fe-4S] clusters and/or their stability at the mutated site and may eventually affect PS I biogenesis. Thus, both in vitro and in vivo investigations clearly demonstrate the indispensable role of Fe-S clusters in PS I assembly. These studies led us to the question as to how these three [4Fe-4S] clusters are assembled in vivo.

1.4 Fe-S cluster biogenesis

In vitro Fe-S cluster assembly was first achieved in 1966 by incubating apo-proteins with ferrous/ferric ion and sulfide in the presence of a reductant under anaerobic conditions (Malkin et al., 1966). However, the concentrations of free iron and sulfide must be much lower in living cells than that used in the in vitro reconstitution because of their intrinsic toxicity. It has thus long been believed that the Fe-S cluster biogenesis/assembly in vivo is a complicated process involving protein factors. Although the first biological Fe-S cluster was identified about 40 years ago (Mortenson et al., 1962), significant progress in Fe-S cluster biogenesis has only been made in last several years. The breakthrough in this research area was the discovery of $nifS$ and $nifU$ (NIF machinery, Figure 1.2) that are essential for the Fe-S assembly in nitrogenase in Azotobacter vinelandii (Fu et al., 1994; Zheng et al., 1993). The NIF system is specific for the maturation of nitrogenase. Shortly thereafter, a second Fe-S cluster assembly
pathway, namely ISC encoded by the *iscRSUA-hscBA-fdx* gene cluster (Figure 1.2) was found to be involved in Fe-S cluster formation or repair of other Fe-S proteins in *A. vinelandii* (Zheng et al, 1998). A wide conservation of *iscSUA* in archaea, eubacteria, and eukaryotes suggests that the ISC machinery represents a general Fe-S cluster assembly pathway in nature. The absence of both the NIF and ISC pathways in some archaea, such as *Methanococcus janaschii* and *Thermoplasma acidophilum*, even though these organisms possess many genes encoding Fe-S proteins, led to the discovery of a third Fe-S cluster assembly pathway, named SUF (Takahashi et al., 2002).

### 1.4 Components of the NIF and ISC systems and their homologs

In addition to *nifU* and *nifS*, the *nifUS* gene cluster also contains *nifV* that encodes a homocitrate synthase, *cysE* that codes for a cysteine synthase (Evans et al., 1991), and an *iscA*-like homolog that is named *nifiscA* (Krebs et al., 2001). Because only NifS and NifU are directly involved in the biosynthesis of Fe-S clusters, and because they show high sequence similarity with IscS and IscU of the ISC pathway respectively, NIF and ISC are introduced here together.

#### 1.4.1.1 NifS/IscS

NifS/IscS are homodimeric, pyridoxal phosphate-dependent cysteine desulfurase enzymes that catalyze sulfur mobilization from L-cysteine to produce alanine and an enzyme-bound cysteine persulfide (Zheng et al., 1993, 1994; Flint, 1996). NifS/IscS-like proteins are the best-characterized components at both the molecular and structural
levels. Many organisms have more than one copy of nifS/iscS homologs. For example, E. coli contains three ORFs encoding proteins homologous to NifS/IscS, namely iscS, csdA, and csdB (sufS) (Mihara et al., 2002). IscS is a component of the ISC machinery. It shows 40% identity with A. vinelandii NifS, and exhibits similar L-cysteine desulfurase activity (Flint, 1996). By contrast, CsdA and SufS are only 24% identical to E. coli IscS. They represent a class distinct from that of NifS/IscS. CsdA shows broad substrate specificity for a number of cysteine analogs, such as L-cysteine sulfinic acid, L-selenocysteine, and L-cystine (Mihara et al., 1997). Its physiological function is unknown. SufS alone has significantly lower activity toward L-cysteine (Mihara et al., 1999). Its L-cysteine desulfurase activity can be elevated 8-10- and 30- fold in the presence of other proteins (Outten et al., 2003; Loiseau et al., 2003). Indeed, SufS and five other protein components represent a third Fe-S cluster pathway (Takahashi et al., 2002). All three IscS/NifS homologs in E. coli have been purified, and the crystal structures of IscS and SufS are available (Cupp-Vickery et al., 2003; Fujii et al., 2000; Lima et al., 2002). Based on their sequence similarities, NifS-like proteins are classified in two groups: group I containing NifS/IscS, and group II consisting of CsdA and SufS (Mihara et al., 1997). NifS/IscS proteins also participate in the biosynthesis of thiamine and thiouridine required for modification of the tRNAs in E. coli (Kambampati et al., 1999, 2000, 2003; Lauhon et al., 2000, 2002), Salmonella enterica serovar typhimurium (Leipuviene et al., 2004; Nilsson et al., 2002), and Saccharomyces cerevisiae (Nakai et al., 2004). Human IscS is also reported to be located in the nucleus and cytoplasm (Land et al., 1998).
1.4.1.2 NifU/IscU

NifU is a modular scaffold protein that contains three domains (Agar et al., 2000; Yuvaniyama et al., 2000) (see Figure 1.3). The N-terminal ISU domain has three conserved cysteines. Mutagenesis studies on NifU from A. vinelandii showed that the three cysteines are essential for the full function of NifU in the maturation of nitrogenase (Agar et al., 2000). IscU is homologous to this domain. In vitro reconstitution studies demonstrated that one reductively labile [2Fe-2S] cluster per dimer could be assembled in the ISU domain of NifU (Yuvaniyama et al., 2000). A. vinelandii IscU overexpressed in E. coli was purified and found to contain one [2Fe-2S] cluster per dimer. When incubated with ferrous iron and cysteine in the presence of catalytic amounts of the NifS protein, NifU can be converted sequentially to a form containing two [2Fe-2S] clusters per dimer and finally to a form containing one [4Fe-4S] cluster per dimer. Both [2Fe-2S] and [4Fe-4S] clusters were reductively labile (Agar et al., 2000). There are two IscU homologs in yeast, Isu1p and Isu2p. Deletion of either isu1 or isu2 did not lead to any growth effect on rich medium supplemented with glucose. The Δisu1/Δisu2 double mutant is inviable, indicating that Isu plays an essential role in the mitochondria (Garland et al., 1999). Human Isu was also expressed in E. coli and purified as a [2Fe-2S] protein. It was further demonstrated to be able to transfer a [2Fe-2S] cluster to apo-ferredoxin protein (Wu et al., 2002). Direct evidence that Isu is an Fe-S cluster scaffold protein came from an in vivo study in yeast (Muhlenhoff et al., 2003).

The central region of NifU contains four conserved cysteine residues. NifU overproduced in E. coli was isolated as a homodimeric protein containing one redox
active [2Fe-2S] cluster per subunit on this domain, which was referred to as the permanent [2Fe-2S] cluster (Agar et al., 2000). This stable [2Fe-2S] cluster is proposed to function similar to Fdx in the ISC Fe-S cluster assembly pathway, and may play an essential role in electron transfer during Fe-S cluster assembly. Mutations introduced to any of the four cysteine residues led to low diazotrophic growth of A. vinelandii (Agar et al., 2000).

The C-terminal domain contains two evolutionally conserved cysteines. It is usually referred to as the NFU domain. Replacement of the two cysteine residues by alanine did not affect the diazotrophic growth of A. vinelandii, indicating that they are not required for the full function of NifU (Agar et al., 2000). Nevertheless, it is still possible that this domain acts as an alternative, yet inefficient, scaffold for Fe-S cluster assembly. This speculation comes from the finding that the nifU inactivation mutant of A. vinelandii showed much poorer diazotrophic growth than the mutants containing amino acid mutations at one of the cysteine residues of either the N-terminal or central domain. Nfu-like proteins have been found in all organisms investigated thus far. Mutagenesis studies have established an important role for Nfu1p in Fe-S cluster biogenesis in yeast mitochondria (Lill et al., 2000). Ssl2667, a Nfu-like protein in Synechocystis sp. PCC 6803, which is the only homolog to NifU in this organism, was shown to coordinate a reductively labile [2Fe-2S] cluster and to transfer it to apo-ferredoxin (Nishio and Nakai, 2000). There are at least five Nfu-like proteins in Arabidopsis thaliana. Nfu2 was capable of binding a labile [2Fe-2S] cluster. Nfu1 and Nfu2 could even restore the wild-type
growth of the $\Delta isu1/\Delta nfu1$ yeast mutant in when they were targeted into yeast mitochondria (Leon et al., 2003).

**1.4.1.3 IscA**

IscA is proposed to be an alternative scaffold protein based on its ability to incorporate an Fe-S cluster and transfer it to apo-ferredoxin *in vitro* (Krebs et al., 2002; Ollagnier-de-Choudens et al., 2001; Wollenburg et al., 2003; Wu et al., 2002). This is consistent with its nonessential role (Jensen et al., 2000; Tokumoto et al., 2001). The idea that IscA acts as a nonessential alternative scaffold is supported by the absence of IscA homologs in the ISC machinery of *Encephalitozoon cuniculi*, a simple, fungus-related, intracellular parasite possessing the smallest eukaryotic genome known at present (Katinka et al., 2001; Seeber et al., 2002). Although a recent review mentioned that all of the ISC components are essential in *A. vinelandii* (Frazzon and Dean, 2003), only *iscS* and *hscA* were inactivated (Zheng et al., 1998). The deletion of another *iscA* ($^{Nif}$iscA) in the *nifUS* operon did not lead to a phenotype (Jacobson et al., 1989). There are two IscA homologs in yeast, Isa1p and Isa2p. Deletion of either or both resulted in a similar, non-lethal growth defect (Jensen et al., 2001). A crystal structure of *E. coli* IscA at 2.3 Å resolution was just published (Bilder et al., 2004). In contrast to the highly mobile secondary structural elements within the IscU protein, the structure of the IscA protein is very rigid, with the exception of a few amino acid residues within the C-terminal tail that contains two out of three invariant cysteines. IscA and IscU share no significant sequence similarities. Thus far, IscA is only demonstrated to interact with the Fdx protein.
The IscA-like proteins are still among the least-understood components of the Fe-S cluster assembly machinery.

1.4.1.4 HscA/HscB

The Hsc70- and Hsc20-like chaperone proteins were reported even before the ISC Fe-S cluster pathway was identified (Cupp-Vickery et al., 2001; Lelivelt et al., 1995; Vickery et al., 1997). However, their function in Fe-S cluster assembly was only recognized after the identification of the isc gene cluster (Zheng et al., 1998). Their participation in Fe-S cluster assembly was first demonstrated by insertional inactivation of the hscA gene of A. vinelandii (Zheng et al., 1998). The mutants with an insertion of aphII in hscA could not be fully segregated, indicating that hscA is essential in this diazotrophic organism. A similar conclusion was reached in E. coli (Nukamura et al., 1999; Takahashi and Nakamura, 1999; Tokumoto et al., 2001). The deletion of ssq1 or jac1, homologs to hscA and hscB respectively, disrupted the Fe-S cluster assembly in yeast mitochondria and led to a slow growth phenotype. The Δssq1/Δjac1 double mutant showed more compromised growth (Kim et al., 2001). By employing yeast mutants that facilitate the rapid depletion of various ISC components, Lill’s research group provided strong evidence that Ssq1p and Jac1p assist in the delivery of the Fe-S cluster instead of de novo Fe-S biosynthesis on the Isu1p scaffold protein (Muhlenhoff et al., 2003). Interactions between IscU/Isu1p and HscBA/Ssq1p/Jac1p have been well documented (Dutkiewicz et al., 2003; Hoff et al., 2000, 2003; Tokumoto et al., 2002).
1.4.1.5 Fdx

Fdx has been shown to be a [2Fe-2S] protein in *E. coli* (Takahashi and Nakamura, 1999), *A. vinelandii* (Zheng et al., 1998), and *Pseudomonas ovalis* (Ohmori et al., 2003). Mutagenesis studies showed that *fdx* is essential in the bacterium *A. vinelandii* and the yeast *S. cerevisiae* (Zheng et al., 1998; Lange et al., 2000). In *A. vinelandii*, *fdx* and *fpr* (which encodes a ferredoxin reductase) had similar expression patterns in response to oxidative stress (paraquat treatment) (Yannone et al., 1998). In yeast, depletion of Yah1p (a Fdx homolog) or Arh1p (which encodes a ferredoxin reductase) resulted in the same phenotypes (Li et al., 2001). These studies suggest the involvement of an Fdx reductase-Fdx system in an electron transfer step during Fe-S cluster biogenesis (Mihara et al., 2002). Fdx might also be required for the transient Fe-S cluster biosynthesis on the scaffold Isu1p protein, as the iron level bound to the Isu1p protein decreased when Yah1p was depleted (Muhlenhoff et al., 2003). The same study showed that the assembly of the [2Fe-2S] cluster onto Yah1p required Isu1p. However, a protein–protein interaction study conducted in *E. coli* did not detect an interaction between IscU and Fdx. Instead, Fdx interacted with the IscA and IscS proteins (Ollagnier-de-Choudens et al., 2001; Tokumoto et al., 2002).

In yeast and likely in all other eukaryotes, the mitochondrion is the place where Fe-S clusters are assembled for both mitochondrial and cytoplasmic Fe-S proteins (For reviews, see Lill et al., 1999, 2000; Muhlenhoff and Lill, 2000). Because of this, yeast mitochondria have additional components responsible for transporting Fe-S clusters (Kispal et al., 1997, 1999; Lange et al., 2001), such as Atm1p and Erv1p. In addition, more than one copy of some of the ISC components exist in yeast mitochondrion. For
example, there exist two IscA homologs (Jensen et al., 2000; Kaut et al., 2000; Pelzer et al., 2000), two IscU homologs, and one Nfu homolog with similarity to the C-terminus of NifU (Schilke et al., 1999). The yeast mitochondrion also contains at least two more components that do not have counterparts in the bacterial ISC system. The terminology of these genes and proteins is different between bacteria and yeast.

1.4.2 Mechanism of Fe-S cluster biosynthesis in ISC pathway

*De novo* Fe-S cluster assembly is believed to proceed in two basic steps: formation of a transient [2Fe-2S] cluster on scaffold proteins such as NifU/IscU and/or IscA, followed by the delivery of the cluster to target apo-proteins. The sulfur is provided by IscS/NifS. Sulfur transfer from IscS to IscU is believed to be the first step in Fe-S cluster biosynthesis (Smith et al., 2001). Sulfur can be directly transferred from IscS to IscU without involvement of any intermediates. An interaction between IscS and IscU was shown by surface plasmon resonance studies and isothermal titration calorimetry measurements (Urbina et al., 2001). The C-terminus of IscS is important for IscU binding. It is proposed that sulfur transfer is initiated by the attack of Cys63 in IscU on the $\square$ atom of Cys328 in IscS that is bound to sulfane sulfur derived from L-cysteine. A disulfide bridge between the Cys328 of IscS (*E. coli*) and the Cys63 of IscU (*E. coli*) is formed after sulfur transfer (Kato et al., 2002). Ferrous iron is believed to be the source of iron for Fe-S cluster biosynthesis (Krebs et al., 2001). Its oxidization can yield an electron for sulfide reduction. This redox coupling ensures that no toxic sulfide is generated when Fe$^{2+}$ availability is limited. A calculation on the number of electrons
involved in Fe-S cluster assembly indicated that ferrous iron itself is not enough to provide all of the electrons needed (Krebs et al., 2001). More electrons may be provided by the ferredoxin-ferredoxin-NADPH reductase redox system (Mihara et al., 2002). [2Fe-2S] clusters appear to be the building unit for more complicated Fe-S clusters, such as [4Fe-4S] clusters. The formation of [4Fe-4S] clusters from [2Fe-2S] clusters might be mediated by reductive coupling. The chemistry involved in this process is still speculative. The exact mechanism of the delivery of transient Fe-S cluster from the scaffold proteins to target apo-proteins is not clear. The HscB and HscA chaperone proteins are proposed to be involved in this process.

### 1.4.3 SUF Fe-S cluster pathway

As stated earlier, some archaea lack all of the homologous components of the NIF and the ISC pathways even though these organisms possess many genes encoding Fe-S proteins, suggesting the existence of other Fe-S cluster assembly pathways (Zheng et al., 1998). Furthermore, the apparently essential role of the ISC pathway in *A. vinelandii* (Zheng et al., 1998) vs. the non-lethality albeit severe growth defect in *E. coli* upon deletion of the entire *isc* gene cluster (Takimoto et al., 2002) also implies that *E. coli* contains an alternative Fe-S cluster assembly pathway. A suppressor screening on the *E. coli* mutant strain YT1014 led to the identification of the *sufABCDSE* operon encoding protein components of the SUF machinery (Takahashi et al., 2002) (see Figure 1.2). A mild overexpression of the *suf* operon restored the growth phenotype and the activity of Fe-S proteins in the strain YT1014, indicating a functional redundancy between the SUF
and ISC pathways. Disruption of the suf operon alone did not cause significant growth defects. However, a synthetic lethality occurred when both the suf and isc operons were inactivated.

Before the SUF machinery was found to be the third Fe-S cluster assembly pathway, several studies independently had linked functions of the Suf proteins to Fe metabolism, Fe-S cluster assembly, and resistance to oxidative stress. A DNA microarray analysis indicated that the transcription level of all six suf genes was elevated in response to oxidative stress (hydrogen peroxide treatment) (Zheng et al., 2001). Up-regulation of the sufBCDS genes was also observed in Mycobacterium tuberculosis after treatment with cumene hydroperoxide (Wilson et al., 2002). Inactivation of sufD or sufS in E. coli resulted in the inability to use ferroxamine B as an iron source, a phenotype similar to that of the fhuA mutant. SufD and SufS were then proposed to be responsible for the [2Fe-2S] cluster assembly in the FhuF protein (Patzer et al., 1999). The sufABCDSE operon also exists in the plant pathogen Erwinia chrysanthemi. Inactivation of the entire locus or each of the suf genes individually resulted in an increase of the intracellular free iron concentration and more sensitivity to oxidative stress. Transcription of sufB was induced under iron deficiency conditions and was repressed by Fur in the presence of iron (Nachin et al., 2001). Several groups failed to obtain fully segregated mutants when trying to inactivate sufB, sufC, or sufS in Synechocystis sp. PCC 6803 (Law et al., 2001; Shen, Golbeck, and Bryant, personal communication; Tirupati and Bollinger, personal communication), indicating that the suf genes are essential in cyanobacteria. A mutation in A. thaliana involving the atABC1 (a sufB homolog) gene led to the accumulation of
protoporphyrin IX (Moller et al., 2001). This phenotype was later reinterpreted in terms of iron stress induced in this mutant rather than disruption of light signaling coupled to a defect in protoporphyrin IX transport (Wilson et al., 2002). All of these data, albeit preliminary, suggest that the SUF pathway plays an important role in oxidative stress and/or Fe limitation conditions.

More recent studies on the SUF pathway involve the characterization of the individual Suf proteins. SufA is a homolog of IscA. Both IscA and SufA are classified as HesB-like proteins. They are almost indistinguishable in amino acid sequence and secondary structure. Thus, SufA may similarly function as a scaffold protein in the SUF Fe-S cluster pathway (Ellis et al., 2001). This is supported by two recent studies using SufA proteins from E. chrysanthemi (Ollagnier-de Choudens et al., 2003) and Synechocystis sp. PCC 6803 respectively (Wollenberg et al., 2003). Both are able to assemble a [2Fe-2S] cluster and transfer it to a [2Fe-2S] apo-ferredoxin and a [4Fe-4S] apo-protein, such as biotin synthase. SufB and SufD are paralogs that share approximately 44% sequence similarity. Numerous organisms contain at least one of them. Both SufB and SufD are predicted to be membrane components of a family of ABC transporters. However, secondary structure predictions suggest they are not integral membrane proteins (Ellis 2001 et al.; Wilson et al., 2002). This analysis does not exclude the possibility that they are peripheral proteins. Indeed, it was reported that SufB and SufC are associated with membranes in E. coli based on an immunolocalization study (Rangachari et al., 2002). In comparison, another study reported that only SufB is associated with the inner membrane of chloroplasts in A. thaliana. However, a third
group found that the SufB, SufD, and SufC proteins from *E. chrysanthemi* were definitely located in the cytosol (Nachin et al., 2003).

SufC shows clear-cut similarities to HisP, the ATP-binding subunit of the bacterial histidine permease in both sequence and predicted secondary structure (Wilson et al., 2002). The ATPase activity of SufC has been demonstrated by two research groups (Rangachari et al., 2002; Nachin et al., 2003). These studies also detected an interaction between SufB and SufC and the formation of a SufBCD protein complex.

SufE is also a component of the SUF pathway, but is often found missing in some organisms. In genomes lacking *sufE*, the position of *sufE* is often occupied by a *nifU*-like gene (Yuvaniyama et al., 2000). Bacterial SufE and NifU proteins have a predicted secondary structure similarity along their entire amino acid sequences. There exists a *sufE*-like homolog, namely *ygdK* in *E. coli*, which is located immediately downstream of *csdA*. The function of *csdA-ygdK* remains unknown. A similar gene organization was also found in *P. aeruginosa* (PA3667/8), *Vibrio cholerae* (VC2309/10), and *Haemophilus influenzae* (HI1295/3) (Ellis et al., 2001). These findings imply that an interaction between SufE and SufS resembles the interaction between IscU and IscS, and that there are some functional similarities between SufE and IscU even though SufE has only one conserved cysteine residue. This was confirmed by two recent findings: SufE from *E. coli* stimulated the L-cysteine desulfurase activity of SufS by 8-fold alone, and up to 32-fold in the presence of the SufBCD proteins (Outten et al., 2003); The binding of SufE to SufS from *E. chrysanthemi* resulted in a 50-fold increase of cysteine desulfurase activity (Loiseau et al., 2003). In addition, the SufBCD proteins from *E. coli* were co-
purified by anion exchange and gel filtration chromatography. This very stable protein complex exhibited ATPase activity. Homodimeric SufE from *E. coli* was further defined as a sulfur acceptor (Outten et al., 2003). The sulfur produced by L-cysteine desulphuration was likely transferred from SufS to SufE via a sulfane-sulfur bridge. Substitution of the conserved Cys51 residue in SufE with serine completely abolished its ability to stimulate SufS activity, indicating that this cysteine residue plays a crucial role in sulfur transfer (Outten et al., 2003). The ability of SufE to bind sulfur and the direct sulfur transfer from the SufS persulfide to SufE were further confirmed by mass spectroscopy (Ollagnier-de-Choudens et al., 2003).

The distribution of the *suf* genes is limited to major groups of archaea and bacteria, as well as plastid-containing organisms (Ellis et al., 2001). Very few organisms contain all the *suf* genes. Only *sufB* and *sufC* occur invariably together in the bacterial genomes. Although a few pathogenic bacteria appear to lack the entire *suf* operon, *sufB* is still maintained in the plastid genome of the apicomplexan parasite *Plasmodium falciparum* (Wilson et al, 1996). Only two genes (including *sufB*) out of 60 in this 35 kb DNA sequence encode proteins rather than the machinery of protein synthesis (Wilson et al., 2002). A clear pattern for the distribution of the *suf* genes cannot be presented at the present time.

**1.4.4 Fe-S cluster assembly pathways in cyanobacteria**

Three *nifS*-like ORFs can be identified in the genome of *Synechocystis* sp. PCC 6803, *slr0387*, *sll0704*, and *slr0077*, by BLAST search. These three genes have been
cloned into *E. coli* and their gene products have been overexpressed and purified (Tirupati and Bollinger, personal communication). Both the Slr0387 and the Sll0704 proteins exhibited efficient L-cysteine desulfurase activity and thus are homologs to the group I NifS/IscS-like proteins. Slr0077 belongs to the group II SufS-like proteins. Its cysteine desulfurase activity can be elevated 8-10 fold by the addition of the SufE-like Slr1419 protein (Tirupati and Bollinger, personal communication). A fourth NifS-like protein, L-cyst(e)ine desulfurylase (C-DES), which was identified by chromatographic purification from *Synechocystis* sp. PCC 6714 cell lysates, represents a group distinct from both group I and II (Leibrecht et al., 1997). C-DES has only 27% amino acid sequence similarity with the NifS proteins from *E. coli* and *A. vinelandii*. C-DES is a monomeric protein that catalyzes the production of pyruvate and sulfide from cysteine instead of alanine and sulfur (Lang et al., 1999). It is capable of directing [2Fe-2S] cluster assembly in ferredoxin *in vitro* (Lang and Kessler, 1999; Leibrecht and Kessler, 1997). Its crystal structure is available (Kaiser et al., 2003). An ORF in *Synechocystis* sp. PCC 6803, *slr2143*, encodes a protein corresponding to this unique enzyme. Thus, three cysteine desulfurases and one cyst(e)ine desulphurylase, representing three likely Fe-S cluster assembly pathways, i.e., ISC (*slr0387* and *sll0704*), SUF (*slr0077*), and an unknown pathway (*slr2143*), co-exist in the cyanobacterium *Synechocystis* sp. PCC 6803. This makes cyanobacteria distinguishable from other organisms in the field of Fe-S cluster biogenesis/assembly.
1.4.4.1 Cyanobacterial ISC components

Homologs for all of the ISC components with the exception of IscU have been found in the genome of Synechocystis sp. PCC 6803 (listed in Table 1.4). The only NifU-like homolog in Synechocystis sp. PCC 6803, ssl2667, corresponds only to the C-terminal third of A. vinelandii NifU protein. The recombinant Ssl2667 protein was capable of assembling a labile [2Fe-2S] cluster and transferring it to apo-ferredoxin (Nishio and Nakai, 2001), indicating that it is competent as a scaffold protein. Because there are only two conserved cysteine residues in a C-X-X-C motif in this 76 amino-acid protein, the [2Fe-2S] cluster is most likely ligated in a homodimer. In contrast to the absence of a phenotype when either slr0387 or sll0704 is inactivated in Synechocystis sp. PCC 6803 (Tirupati and Bollinger, personal communication), ssl2667 is essential and cannot be completely inactivated (Nakai et al., 2002). There are two iscA-like homologs in Synechocystis sp. PCC 6803, slr1417 and slr1565, which were assigned cyanobacterial sufA and iscA, respectively, based on their amino acid sequence similarities to SufA and IscA from E. coli and A. vinelandii (Bryant and Golbeck Labs). Slr1417 and Slr1565 share 39% sequence identity to each other and 38% identity to A. vinelandii IscA. Slr1565 can form a protein complex with a novel prokaryotic HEAT-repeat-containing protein, IaiH (encoded by slr1098) in cyanobacterial cells (Morimoto et al., 2002). The recombinant Slr1565 protein was able to bind a reductively-labile [2Fe-2S] cluster (Morimoto et al., 2002). The efficient formation of the Slr1565/IaiH protein complex required the presence of the [2Fe-2S] cluster in Slr1565. The IaiH protein can stabilize the [2Fe-2S] cluster in Slr1565 (Morimoto et al., 2003). The physiological function of the
Slr1565/IaiH protein complex is unknown, although it may sense the redox state in cyanobacterial cells. Thus far, no studies have been reported for HscBA homologs in cyanobacteria. *slr0148*, an ORF in *Synechocystis* sp. PCC 6803, encodes a protein sharing 39% amino acid sequence identity with Fdx from *E. coli*. It is found in a gene cluster spanning *slr0144* through *slr0152*. Recent microarray analyses indicated that the transcription level of all the genes in this cluster was down-regulated in response to cold, high light, Fe deficiency, or hydrogen peroxide treatments (Hihara et al., 2001; Suzuki et al., 2001; Singh et al. 2004). As can be discerned from their gene numbers, genes encoding the ISC protein homologs in cyanobacteria are scattered throughout their genomes and are not organized in operons.

### 1.4.4.2 Cyanobacterial SUF components

The indispensability of the SufS homolog in *Synechococcus* sp. PCC 7002 indicates that the SUF machinery is an essential Fe-S cluster assembly pathway in cyanobacteria (the *suf* gene homologs are listed in Table 1.5). With few exceptions, the *sufBCDS* homologs form a conserved gene cluster in cyanobacteria (Figure 2.1). The *sufA* and *sufE* homologs are located elsewhere in the cyanobacterial genomes. The recombinant SufA protein from *Synechocystis* sp. PCC 6803 can assemble a transient [2Fe-2S] cluster and transfer it to a [2Fe-2S] ferredoxin and a [4Fe-4S] protein (Wollenberg et al., 2003). The [2Fe-2S] cluster in the cyanobacterial SufA protein is more labile than that in the cyanobacterial IscA protein (Morimoto et al., 2002). The cyanobacterial SufA protein contains three cysteine residues that are conserved among all of the IscA-like, SufA-like, HesB-like, and IscN-like proteins.
(Dombrecht et al., 2002). Two of the Cys residues were shown to provide ligands to the [2Fe-2S] cluster by a site-directed mutagenesis study (Wollenberg et al., 2003). Substitution of the other two cysteine residues did not affect Fe-S cluster assembly in the SufA protein, indicating that the active form of SufA might be a [2Fe-2S] homodimer (Wollenberg et al., 2003). The formation of a SufA homodimer was observed from the recombinant SufA protein and in *Synechocystis* sp. PCC 6803 cell lysates (Morimoto et al., 2002, 2003). The cyanobacterial SufE protein was demonstrated to be able to stimulate cysteine desulfurase activity of SufS approximately 10-fold (Tirupati and Bollinger, unpublished data). Thus far, no data are available on the properties of the cyanobacterial SufBCD proteins.

1.5 Research goal

The Fe-S clusters of PS I complexes in cyanobacteria contain the vast majority of functional iron in these bacteria. The goal of my thesis research was to study the regulation of Fe-S cluster assembly in PS I complex. Limited information on Fe-S cluster assembly in cyanobacteria showed that cyanobacteria represent one of the most complicated organisms with regard to Fe-S cluster biosynthesis. Although many genes encoding components of the ISC and the SUF pathways have been found in cyanobacteria based on BLAST searches, our knowledge of the cyanobacterial Fe-S cluster assembly process is still rudimentary. Even the components of the ISC and the SUF pathways remain to be identified because of the existence of multiple homologs (such as IscS and IscA), the lack of a homolog (such as IscU), or the low sequence similarities (such as Fdx). A study on the regulation of the Fe-S cluster assembly in PS I would include identifying which Fe-S cluster assembly machinery
is involved in delivering Fe-S clusters to $F_X$, $F_A$, and $F_B$ of PS I, and elucidating how the Fe-S cluster biosynthesis pathway is regulated in cyanobacteria. An ideal candidate for this purpose would be a regulator of one general Fe-S cluster pathway through which the Fe-S cluster assembly in PS I is regulated. An ORF, sll0088 in *Synechocystis* sp. PCC 6803, which was discovered to be involved in PS I assembly by pseudorevertant screening, is such a regulator. One of my research goals was to elucidate how the SlI0088 protein functions in Fe-S cluster assembly and thus in PS I biogenesis/assembly in cyanobacteria. Another project is focused on *slr0148*, an ORF in *Synechocystis* sp. PCC 6803 encoding a protein homologous to Fdx of the ISC Fe-S cluster assembly pathway. The study of *slr0148* is part of a project aimed at addressing the role of the ISC machinery in Fe-S cluster assembly and in PS I biogenesis/assembly of cyanobacteria.

Cyanobacteria are believed to be the origin of chloroplasts. Cyanobacteria and chloroplasts contain very similar photosynthetic units in structure, composition, and function. For these reasons, cyanobacteria have long been a model system for photosynthesis research. The freshwater cyanobacterium *Synechocystis* sp. PCC 6803 and the marine cyanobacterium *Synechococcus* sp. PCC 7002 were used in my thesis research. *Synechocystis* sp. PCC 6803 is the first cyanobacterium whose genome DNA has been completely sequenced (Kaneko et al., 1996). It is naturally competent for transformation, and has an active homologous recombination mechanism (Williams et al., 1988). Furthermore, it can grow photoheterotrophically in the presence of glucose. *Synechococcus* sp. PCC 7002 possesses all of the advantages of *Synechocystis* sp. PCC 6803 as a model organism for oxygenic photosynthesis study, with the exception that it uses glycerol instead
of glucose for photoheterotrophic growth. It contains a smaller genome, and grows much faster than *Synechocystis* sp. PCC 6803 under optimal growth conditions. The genome of *Synechococcus* sp. PCC 7002 is available (Genbank accession number AY375041), and the *sll0088* homolog in this strain has been annotated. The availability of the genome sequences of cyanobacteria and higher plants, such as *A. thaliana* and *Oryza sativa*, allows one to make comparisons of Fe-S cluster assembly between cyanobacteria and chloroplasts and to gain further insight into this important bioprocess.
REFERENCES


Barber, J. 2002. Photosystem II: a multisubunit membrane protein that oxidizes water

*Curr Opin Struct Biol* 12, 523-530


Kambampati, R., and C. T. Lauhon. 1999. IscS is a sulfurtransferase for the *in vitro* biosynthesis of 4-thiouridine in *Escherichia coli* tRNA. *Biochemistry* 38(50):16561-8


Lang, T., and D. Kessler. 1999. Evidence for cysteine persulfide as reaction product of L-Cyst(e)ine C-S-lyase (C-DES) from Synechocystis. Analyses using cystine analogues and recombinant C-DES. J Biol Chem 274(1):189-95


Rodday, S. M., A. N. Webber, S. E. Bingham, and J. Biggins. 1995. Evidence that the FX domain in photosystem I interacts with the subunit PsaC: site-directed changes in PsaB destabilize the subunit interaction in *Chlamydomonas reinhardtii.* *Biochemistry* 34(19):6328-34


gene expression in the cyanobacterium Synechocystis sp. PCC 6803. Physiologia Plantarum 120: 27–35


insertional inactivation of the psaC gene encoding the iron sulfur protein destabilizes photosystem I. *EMBO J* 10(8):2033-40


FIGURE LEGENDS

**Figure 1.1** Photosynthetic machinery of cyanobacteria (adapted from Jones, 2001)

**Figure 1.2** Genes involved in Fe-S cluster assembly pathways. Yeast contains only the ISC pathway. The terminology is different between yeast and bacteria. The corresponding homologs to ISC components in yeast are: Nfs1 (IscS), Isu1/Isu2 (IscU), Nfu1 (homologous to C-terminal domain of NifU), Isa1/Isa2 (IscA), Ssq1(HscA), Jac1(HscB), and Yah1 (Fdx). Yeast also has several additional components that do not exist in bacteria: Yfh1 (frataxin), Atm1, Erv1, Bat1/Bat2. The last four are involved in Fe-S cluster export from the mitochondria to the cytoplasm.

**Figure 1.3** NifU contains three domains (adapted from Mihara et al., 2002).
Figure 1.1

(Adapted from Jones, 2001)
Figure 1.2

1), nif

\[
\text{NifiscA} \quad \text{nifU} \quad \text{nifS} \quad \text{nifV} \quad \text{cysEl}
\]

2), isc

\[
\text{iscR} \quad \text{iscS} \quad \text{iscU} \quad \text{iscA} \quad \text{hscB} \quad \text{hscA} \quad \text{fdx}
\]

3), suf

\[
\text{sufA} \quad \text{sufB} \quad \text{sufC} \quad \text{sufD} \quad \text{sufS} \quad \text{sufE}
\]

\[
\text{csdA} \quad \text{sufE-like}
\]
Figure 1.3

(Adapted from Mihara et al., 2002)
Table 1.1 PS I subunits and their mutants (adapted from Chitnis et al., 2001)

<table>
<thead>
<tr>
<th>Subunits</th>
<th>ORFs in <em>Synechocystis</em></th>
<th>Mass (kDa)</th>
<th>Cyanobacterial mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>PsaA</td>
<td>Slr1834</td>
<td>83.0</td>
<td>No PSI in <em>Synechocystis</em> mutants</td>
</tr>
<tr>
<td>PsaB</td>
<td>Slr1835</td>
<td>82.4</td>
<td>No PSI in <em>Synechocystis</em> mutants</td>
</tr>
<tr>
<td>PsaC</td>
<td>Ssl0563</td>
<td>8.9</td>
<td><em>Synechocystis</em> and <em>Anabaena</em> mutants contain PSI core, but lack terminal electron donors and PsaD and PsaE proteins</td>
</tr>
<tr>
<td>PsaD</td>
<td>Slr0737</td>
<td>15.6</td>
<td>Ferredoxin or flavodoxin reduction decreased or absent in <em>Synechocystis</em> mutants</td>
</tr>
<tr>
<td>PsaE</td>
<td>Ssr2831</td>
<td>8.0</td>
<td>Ferredoxin or flavodoxin reduction decreased in <em>Synechocystis</em> mutants</td>
</tr>
<tr>
<td>PsaF</td>
<td>Sll0819</td>
<td>15.7</td>
<td>No effect on photosynthesis in <em>Synechocystis</em>, <em>Synechococcus</em>, or <em>Synechococcus elongatus</em> mutants</td>
</tr>
<tr>
<td>PsaI</td>
<td>Smr0004</td>
<td>4.3</td>
<td>PsaL assembly and trimer formation altered in the <em>Synechocystis</em> and <em>Synechococcus</em> mutants</td>
</tr>
<tr>
<td>PsaJ</td>
<td>Sml0008</td>
<td>4.4</td>
<td>PsaF assembly and organization affected in the mutants</td>
</tr>
<tr>
<td>PsaK/K2</td>
<td>Ssr0390/sll0629</td>
<td>8.5/13.7</td>
<td>No effect on PSI activity when PsaK or PsaK2 or both genes inactivated in <em>Synechocystis</em></td>
</tr>
<tr>
<td>PsaL</td>
<td>Slr1655</td>
<td>16.6</td>
<td>No PSI trimers in the mutants of <em>Synechocystis</em> and <em>Synechococcus</em></td>
</tr>
<tr>
<td>PsaM</td>
<td>Smr0005</td>
<td>3.4</td>
<td>Less trimer formation in the <em>Synechocystis</em> mutants</td>
</tr>
<tr>
<td>PsaX</td>
<td>Identified by X-ray Crystallography</td>
<td>N/A[]</td>
<td>N/A[]</td>
</tr>
</tbody>
</table>

[] N/A means not available
<table>
<thead>
<tr>
<th>Proteins</th>
<th>Function(s)</th>
<th>Evidence(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BtpA</td>
<td>Stabilization of PSI</td>
<td>Rapid degradation of PSI in the BtpA-less mutants of <em>Synechocystis</em> when grown at low temperature</td>
</tr>
<tr>
<td>FtsH</td>
<td>Not understood</td>
<td>Targeted mutants in a <em>ftsH</em> homologue (<em>slr0228</em>) of <em>Synechocystis</em> contain 60% less PSI</td>
</tr>
<tr>
<td>Ycf3</td>
<td>PSI assembly; may function as a member of a multiprotein complex</td>
<td>Complete loss of PSI in <em>ycf3</em>-less mutants of <em>C. reinhardtii</em>, tobacco, and <em>Synechocystis</em></td>
</tr>
<tr>
<td>Ycf4</td>
<td>PSI assembly</td>
<td>Mutants of <em>C. reinhardtii</em> completely lack PS I, whereas cyanobacterial mutants have reduced PS I levels</td>
</tr>
<tr>
<td>RubA</td>
<td>Fe-S cluster assembly</td>
<td>Cyanobacterial <em>rubA</em> mutants contain PS I without F_X, F_A, F_B, and peripheral proteins</td>
</tr>
<tr>
<td>PmgA</td>
<td>PSI/PSII ratio adjustment</td>
<td>Less PS I complexes and less chlorophyll in the <em>pmgA</em> mutant of <em>Synechocystis</em></td>
</tr>
<tr>
<td>Sll0088</td>
<td>PSI assembly, possibly in Fe-S cluster assembly</td>
<td>Secondary mutations in this ORF restores wild-type growth of primary C14D_pscC mutants</td>
</tr>
</tbody>
</table>
Table 1.3 Site-directed mutations to cysteine residues that provide ligands to $F_X$, $F_A$, and $F_B$ in *Synechocystis* sp. PCC 6803

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_X$ mutant:</td>
<td></td>
</tr>
<tr>
<td>$F_A/F_B$ mutant:</td>
<td></td>
</tr>
<tr>
<td>C14X (X=D,S,A)</td>
<td>Can not grow photoautotrophically; Low PS I quantity; Less or no detectable PsaC, PsaD, PsaE protein.</td>
</tr>
<tr>
<td>C51X (X=D,S,A)</td>
<td></td>
</tr>
<tr>
<td>C14D/C51D</td>
<td></td>
</tr>
</tbody>
</table>
### Table 1.4 Homologous genes encoding ISC components in *Synechocystis* sp. PCC 6803

<table>
<thead>
<tr>
<th>Genes</th>
<th>Cyanobacterial homologs</th>
<th>Proposed functions of products</th>
<th>Mutagenesis study</th>
</tr>
</thead>
</table>
| *nifS* | *slr0387, sll0704,*  
**str0077 (sufS)** | Cysteine desulfurase  
Cysteine desulfurase | Inactivated  
Essential |
| *nifU* | *ssl2667 (synifU)* | [Fe-S] scaffold | Essential |
| *iscA* | *slr1417, slr1565* | Alternative scaffold | Inactivated. Double |
| *fdx* | *slr0148* | Ferredoxin assembly | mutant |
| *hscB* | *sll1666, sll0897* | DnaJ, Hsp homolog | Inactivated |
| *hscA* | *sll0058, sll0170, sll1932* | DnaK, Hsp homolog | N/A[]  
N/A[] |

[]N/A means not available
Table 1.5 Homologous genes encoding ISC components in *Synechocystis* sp. PCC 6803

<table>
<thead>
<tr>
<th>Genes</th>
<th>Cyanobacterial homologs</th>
<th>Functions of products</th>
<th>Mutagenesis study</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>sufS</em></td>
<td><em>slr0077</em></td>
<td>Cysteine desulfurase</td>
<td>essential</td>
</tr>
<tr>
<td><em>sufA</em></td>
<td><em>slr1417</em></td>
<td>scaffold?</td>
<td>inactivated</td>
</tr>
<tr>
<td><em>sufB</em></td>
<td><em>slr0074</em></td>
<td>ABC transporter component</td>
<td>essential</td>
</tr>
<tr>
<td><em>sufC</em></td>
<td><em>slr0075</em></td>
<td>ATPase of ABC transporter</td>
<td>essential</td>
</tr>
<tr>
<td><em>sufD</em></td>
<td><em>slr0076</em></td>
<td>sufB homolog</td>
<td>N/A</td>
</tr>
<tr>
<td><em>sufE</em></td>
<td><em>slr1419</em></td>
<td>sulfur transfer</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\[N/A\] means not available
Chapter 2. The sufR Gene (sll0088 in Synechocystis sp. PCC 6803) Functions as a Repressor of the sufBCDS Operon in Iron-Sulfur Cluster Biogenesis in Cyanobacteria

ABSTRACT

The suf operon is composed of four genes (sufB, sufC, sufD and sufS) and is highly conserved in the genomes of cyanobacteria. Open reading frame sl10088 in Synechocystis sp. PCC 6803 is located near the 5’ end of the suf operon, but is divergently transcribed from it. The same gene organization was also found in several other cyanobacterial strains. The C14S_PsaC mutation in Photosystem I of Synechocystis sp. PCC 6803 abolished photoautotrophic growth. A secondary mutation in two independent suppressor strains of C14S_PsaC mapped to sl10088. The protein encoded by sl10088 has two significant features: 1) a DNA-binding domain near the N-terminus; and 2) four highly conserved cysteine residues near the C-terminus. The protein has high sequence similarity to transcription regulatory proteins with a conserved DNA binding domain and can be classified in the DeoR family of helix-loop-helix proteins. The protein falls into a further subclass that contains a C-X\textsubscript{12}-C-X\textsubscript{13}-C-X\textsubscript{14}-C motif near the C-terminus, which may represent a metal-binding site. Compared to the wild-type, the expression level of the sufBCDS genes was elevated when cells were grown under conditions of oxidative and iron stress, and were even more elevated in a null mutant of Synechococcus sp. PCC 7002 in which the sl10088 homolog was insertionally inactivated. In agreement with the proposed role of the sufBCDS genes in iron metabolism, the null mutant exhibited a significantly faster growth rate than the wild-type under iron-limiting conditions. It is proposed that the protein encoded by sl10088 is a transcriptional repressor of the suf operon, and the gene is named sufR.
INTRODUCTION

It is well established that the three [4Fe-4S] clusters, \( F_X \), \( F_A \), \( F_B \), are functionally and structurally essential for PS I function. Although there are potentially three Fe-S cluster assembly pathways represented by four \( nifS \)-like homologs in cyanobacteria, it is not clear which is responsible for the Fe-S cluster assembly onto proteins of the PS I complex. Further complications concerning the exact mechanism of Fe-S cluster assembly in PS I have arisen from recent studies indicating that Fe-S cluster assembly in PS I is assisted by cofactors that are not components of any Fe-S cluster assembly machineries uncovered thus far. For example, the RubA protein is proposed to be specific for the assembly of \( F_X \) Fe-S clusters in PS I. A \( rubA \) interruption mutant in \textit{Synechococcus} sp. PCC 7002 cannot grow photoautotrophically (Shen et al., 2003). Biophysical analysis showed that in the \( rubA \) mutant \( F_X \), \( F_A \), and \( F_B \), the three [4Fe-4S] clusters in PS I were missing in contrast to the intact Rieske Fe-S protein and the non-heme iron in PS II complex. Correspondingly, the PsaC, PsaD, and PsaE proteins cannot be detected in this mutant. In \textit{A. thaliana}, the HCF101 protein was recently shown to be specific for [4Fe-4S] cluster assembly. As a result, the \textit{hcf101} mutant cannot accumulate PS I. In contrast, the levels of the [2Fe-2S] ferredoxin protein are not affected (Lezhneva et al., 2004). To determine which Fe-S assembly pathway is involved in PS I, we can begin with the \( nifS \)-like homologs using mutagenesis followed by monitoring the PS I assembly, or we can begin with the site-directed PsaC mutants in \textit{Synechocystis} sp. PCC 6803. For the first strategy, three \( nifS \)-like and one \( fdx \)-like homologs have been subject to mutagenesis. For the second strategy, which is the topic of this chapter,
Pseudorevertant screening is applied to PsaC mutants such as C51D$_{\text{PsaC}}$ and C14S$_{\text{PsaC}}$. Pseudorevertant screening has been successfully used for studies of the biogenesis of many membrane complexes, such as the cytochrome $bc_1$ complex of *Saccharomyces cerevisiae* (Brasseur et al., 2001), the rhodopsin I-transducer complex (Jung et al., 1998), and the folding studies of the yeast cytochrome $b$ (di Rago et al., 1995). The C51D$_{\text{PsaC}}$ and C14S$_{\text{PsaC}}$ mutants are sensitive to high light intensities in the range from 20 to 60 $\mu$E m$^{-2}$ s$^{-1}$ and cannot grow photoautotrophically or photomixotrophically. The mutants had two additional phenotypes: the amount of PS I, but not PS II, is lower than the wild-type on a per cell basis, and the mutants are able to grow photomixtrophically at a light intensity of 20 to 60 $\mu$E m$^{-2}$ s$^{-1}$ only in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of PS II. Thus, failure to grow photoautotrophically is apparently due to the electron toxicity caused by the lowered ratio of PS I to PS II in the mutant cells rather than an inefficiency in forward electron transfer in any individual PS I complexes. The mutants are maintained under LAHG conditions (Smart et al., 1991). To isolate potential pseudorevertants, the mutants were restreaked and placed under photoautotrophic growth conditions for up to forty days. Colonies that appeared spontaneously under these selective conditions were selected and subjected to physiological and genetic studies. Three classes of pseudorevertants could be categorized (See Figure below, adapted from Yu et al., 2003): 1) mutations that occurred on a PS I subunit other than PsaC; 2) mutations that occurred within PS II; and 3) mutations that occurred in genes encoding cofactors that are not components of either PS I and PS II.
Two of the intragenic suppressor strains, C14S<sub>PsaC</sub>-R18 and C14S<sub>PsaC</sub>-R62, which were capable of photoautotrophic growth at normal light intensities, were selected for further study. These two suppressor strains retained the primary mutation, which was verified through amplification of the <i>psaC</i> gene fragment by PCR and sequencing. The suppressor mutations were mapped to a specific gene, <i>sll0088</i>, by phenotypic complementation and identified by DNA sequencing (Yu et al., 2003). C14S-R62 has an arginine to proline mutation (R161ÆP) and C14S-R18 has an insertion of three amino acids, glycine-tyrosine-phenylalanine, near the C-terminus between 230 and 231 amino acids. Apparently, a mutant form of <i>Sll0088</i> allows for assembly of the subtly-altered C14S-PsaC subunits into PS I core, and restored growth due to wild-type levels of PS I. The pseudorevertants R-62 and R-18 were found to be able to restore photoautotrophic growth of C51D, indicating that <i>Sll0088</i> can recognize more than one site (Yu and McIntosh, unpublished). However, for unknown reasons, attempts to inactivate the
The slt0088 gene in *Synechocystis* sp. PCC 6803 failed under all photoautotrophic, mixotrophic, and photoheterotrophic growth conditions (Yu and McIntosh, personal communication).

In this study, we show that the protein encoded by slt0088 functions as a transcriptional repressor that regulates the expression of the *sufBCDS* operon. Thus, it is similar in function to *iscR*, which codes for an iron-sulfur protein that functions as a transcriptional repressor of the *isc* operon in *E. coli* (Schwartz et al., 2001).
MATERIALS AND METHODS

Growth of the wild-type and mutant cells under optimal conditions.

*Synechococcus* sp. PCC 7002 is a unicellular or filamentous marine cyanobacterium isolated by Chase van Baalen in 1962. It grows photoautotrophically and can also undergo photoheterotrophic growth in the presence of glycerol. The laboratory wild-type *Synechococcus* sp. PCC 7002 was originally obtained from the Pasteur Culture Collection, Unité de Physiologie Microbiéenne, Institut Pasteur, Paris, France. *Synechococcus* sp. PCC 7002 was grown under photoautotrophic conditions in A medium supplemented with 1 mg NaNO$_3$ ml$^{-1}$ (denoted A+ (Stevens et al., 1980)). Solid medium for maintaining the mutant strains of *Synechococcus* sp. PCC 7002 was supplemented with 0.3% (w/v) sodium thiosulfate and 100 µg kanamycin ml$^{-1}$. *Synechococcus* sp. PCC 7002 cells were grown under photoheterotrophic conditions in A+ liquid medium containing 5 mM glycerol. *Synechocystis* sp. PCC 6803 cells were grown under photoautotrophic conditions in BG11 medium as described in (Shen et al., 1994). All liquid cultures were bubbled with air supplemented with 1% (v/v) CO$_2$. Fluorescent bulbs provided continuous illumination with white light. The light intensity was varied by addition or removal of fluorescent bulbs and/or covering plates/aquarium with sheets of paper. The light intensity was monitored using a model QSL-100 quantum scalar irradiance meter (Biospherical Instruments, Inc., San Diego, CA). The temperature was maintained by a water bath at 32°C for *Synechocystis* sp. PCC 6803 and 38°C for *Synechococcus* sp. PCC 7002. Growth of the wild-type and mutant strains was monitored by measuring the optical density at 730 nm with a Cary-14
spectrophotometer modified for computerized data acquisition by On-Line Instruments, Inc. (Bogart, GA).

**Growth of the wild-type and mutant cells under stress conditions.**

For growth studies under iron stress conditions, the wild-type and mutant cells of *Synechococcus* sp. PCC 7002 were grown to mid-exponential phase, collected by centrifugation at 4,000 g for 10 min, and resuspended to OD$_{730} = 0.1$ in fresh medium containing 50 µM 2,2’-dipyridyl or 0.25 µM streptonigrin. For growth studies under iron starvation conditions, FeCl$_3$ was eliminated from the A$^+$ medium. For growth studies under oxidative stress conditions, the wild-type and mutant cells of *Synechococcus* sp. PCC 7002 were grown to mid-exponential phase under standard growth condition with the addition of 5 mM glycerol in the medium. The cell cultures were diluted with fresh medium to OD$_{730} = 0.1$ and grown to OD$_{730} = 0.5$. A total of 0.5 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, an inhibitor of PS II), 10 µM 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB, an inhibitor of cytochrome $b_{6}f$), or 150 µM hydrogen peroxide (H$_2$O$_2$) was then added into the medium. The cells were grown for 10 to 12 h, during which time they undergo no more than one division, and collected for total RNA isolation.

**Construction of the plasmids for mutagenesis**

The *sll0088* homolog gene and flanking regions were amplified from chromosomal DNA of *Synechococcus* sp. PCC 7002 by PCR using primers 7002s88F and 7002s88R (Table I). The resulting 1788 bp fragment was digested with *PstI* and *KpnI* and cloned into
plasmid pUC19 cleaved with the same enzymes, creating the plasmid pUCs88. To create
insertional mutant, a 1321-bp *aphII* gene derived from plasmid pRL161 by digestion with
*HincII* was inserted into the unique *StuI* restriction site within the *sll0088*-like gene
sequence on the plasmid pUCs88, yielding the plasmid pUCKs88. The insertion was
verified by cleavage with *PstI*.

For deletion mutagenesis of the *sll0088*-like gene in *Synechococcus* sp. PCC 7002,
the pUCs88 plasmid was first partially digested with *SspI*. A 3475 bp fragment containing
the pUC19 plasmid DNA sequence and 20 bp 5’ DNA sequence of *sll0088* was picked up
and ligated with the 1100 bp streptomycin resistant gene derived from the plasmid pSAR2
cleaved with *HincII*, yielding the plasmid pUCSs88. The insertion was checked by cleavage
with *EcoRI* and *EcoRV*.

*Transformation of cyanobacteria and Selection*

*Synechococcus* sp PCC 7002 is naturally transformable. It can uptake free DNA and
incorporate it into its chromosomal DNA by homologous recombination. To transform
*Synechococcus* sp PCC 7002, 1 ml of the wild-type culture with OD$_{730}$ = 1-1.5 was
incubated with 10 μg linearized plasmid DNA for 4 hours under normal growth condition
with very slow bubbling and spread on A$^+$ plate. After growth under reduced light
conditions for 2 days, 3 ml melted 0.8% agar containing appropriate antibiotics was overlaid
on the plate. The plate was then transferred to appropriate light conditions for selection.
Resistant colonies were re-streaked to obtain single colonies with at least five serial transfers
to obtain full segregation of the mutation. Complete segregation was verified by PCR and Southern blot hybridization analysis.

**DNA isolation and Southern blot hybridization**

*Synechococcus* sp. PCC 7002 cells (10-20 ml of exponentially growing culture) were collected by centrifugation at 5000 g and resuspended in 400 l TES buffer (5 mM Tris/HCl pH 8.5, 50 mM NaCl, 5 mM EDTA pH 8.0) containing 10 mg/ml lysozyme in a 1.5 ml eppendorf tubes. After incubating at 37°C for 15 min, the cells were lysed by adding 50 l 10% (w/v) sarkosyl and 600 l phenol. This mixture was agitated by vortexing or shaking for 10 min. The organic and aqueous phases were separated by centrifugation at maximum speed in a standard tabletop centrifuge for 5 min. The aqueous layer was transferred into a new tube and incubated with 5 l 10 mg/ml RNase at 37°C for 15 min to remove RNA. Then 100 l 5M NaCl, 100 l CTAB-NaCl solution (700 mM NaCl, 10% (w/v) CTAB), and 600 l chloroform were subsequently added to remove polysaccharides. After centrifugation for 5 min, the upper aqueous layer was transferred into a new tube. Chromosomal DNA was precipitated by the addition of an equal volume of isopropanol to the aqueous phase.

For Southern blot hybridization, cyanobacterial chromosomal DNA digested with appropriate restriction enzymes was separated by electrophoresis on 1% (w/v) agarose gel in TBE buffer. The gel was photographed alongside a fluorescent ruler under UV light using the Biophotonics (Ann Arbor, MI) Gel Print 2000i video imaging system. The DNA was denatured by soaking the gel in denaturation solution (0.5M NaOH, 1.5M NaCl) for 30 min
and subsequently neutralized in neutralizing solution (0.5M Tris/HCl pH 8.0, 1.5M NaCl) for another 30 min. The DNA was transferred onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by capillary action in 10X SSC (0.15M sodium citrate pH 7.0, 1.5M NaCl) overnight. The membrane blot was dried in the air for 30 min, crosslinked for 1 min under UV light, and baked for 1 hour under vacuum at 80°C. The blot was then transferred into a tube with 30-40 ml hybridization buffer. The hybridization buffer contains: 5% SDS (w/v), 0.5M NaPO₄, and 1% (w/v) bovine serum albumin (fraction V from Sigma, St. Louis). Hybridization was typically carried out at 65°C over night (12-16 hours). After hybridization, the blot was washed with 6X NET buffer (0.18M Tris pH 8.0, 6 mM EDTA, 0.9M NaCl) at hybridization temperature for at least 4 times or until there were minimal radioactive counts in the wash eluent.

**RNA isolation and reverse transcriptase PCR analysis**

For RNA preparation, cells of the wild-type and mutant strains of *Synechococcus* sp. PCC 7002 were harvested during exponential growth. Total RNA was isolated using the High Pure™ RNA isolation kit according to the protocol provided by the manufacturer (Roche Diagnostics, Indianapolis, IN). Contaminating DNA was eliminated by incubating the sample with DNase I for 1 hr at room temperature during the preparation procedure. The absence of DNA contamination in RNA samples was confirmed by RT-PCR analysis in the presence of RNase. RNA concentrations were determined by absorption spectroscopy.

For Northern blot hybridization analysis, 10 μg total RNA from the wild-type and mutant strains was loaded onto gel per lane, and the samples were electrophoresed in 1.3%
(w/v) denaturing agarose-formaldehyde gels. The RNA was transferred onto nylon membranes by capillary transfer and fixed by UV illumination for 1 min and baked for 1 h at 80 °C under vacuum. The sufR gene-specific probe was generated by PCR using *Synechococcus* sp. PCC 7002 genomic DNA as template. The PCR product was purified by electrophoresis on an agarose gel, and the DNA was extracted and purified by using the QIAGEN Gel Extract kit (Valencia, CA). The probe was labeled by random priming as described in Shen et al. (1995). Hybridization with 32P-labeled DNA was performed overnight at 42°C. After washing, the membranes were incubated for fluorography at −80°C.

Reverse transcriptase-polymerase chain reaction (RT-PCR) experiments were performed by using the QIAGEN OneStep RT-PCR kit (Valencia, CA). The primers were designed to amplify specifically the sufBCDS genes of *Synechococcus* sp. PCC 7002 (Table I). Total RNA (2.4 ng) was used as template for the wild-type and mutant strains. The RT-PCR reactions were programmed with a 30-min reverse-transcription reaction at 50°C, a 15-min initial heating step at 95°C, and thirty-two 3-step cycles (1 min at 94°C, 1 min at 59°C and 1.5 min at 72°C).

*Expression and purification of the protein encoded by sll0088*

The *sll0088* gene from *Synechocystis* sp. PCC 6803 (Kaneko et al., 1996) was amplified by PCR. NdeI and HindIII restriction enzyme sites were incorporated via the primers Esll0088F and Esll0088R during PCR as listed in Table 1. The amplified *sll0088* gene fragment was digested with NdeI and HindIII and cloned into the expression vector pET24a (Novagen, Madison, WI), resulting in the pET24a/sll0088 expression plasmid. The
resulting construct was sequenced to ensure the correctness of the PCR amplification. The plasmid pET24a/sll0088 was transformed into *E. coli* strain BL21 (DE3). Overproduction of the Sll0088 protein in *E. coli* cells was induced by the addition of 0.5 mM IPTG. The cells were harvested and washed once in TS buffer (10 mM Tris-HCl, pH 8.0, and 50 mM NaCl). The cell pellets were resuspended in TS buffer with 1 mM PMSF and 0.1 mg/ml DNase I (Sigma, St. Louis, MO) and passed twice through a chilled French pressure cell. The overexpressed protein was present in inclusion bodies, which were collected by low-speed centrifugation at 7000 g for 20 min. The pellet was washed twice with TS buffer and solubilized in 7 M urea in TS buffer by the addition of 10 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 12,000 g to remove the insoluble material, the supernatant was applied to a Sepharose G-100 column that was pre-equilibrated with TS buffer containing 5 mM DTT.

*Production of antibodies*

To obtain polyclonal antibodies, the recombinant Sll0088 protein was partially purified by Sepharose G-100 chromatography and was subjected to preparative polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). An extra lane was loaded identically and used for Coomassie Blue staining to locate the position of the protein band. The protein-containing region of the gel was excised, and the protein was eluted using a Bio-Rad Electroelutor (Bio-Rad, Hercules, CA). Polyclonal antibodies against the SufR protein were generated in rabbits at The Centralized Biological Laboratory at The Pennsylvania State University.
Protein quantitation

Protein concentrations were measured by using the Coomassie Plus protein assay with bovine serum albumin as the standard (Pierce, Rockford, IL).

Protein electrophoresis and immunoblotting

Methods used for SDS-PAGE and immunoblotting were identical to those described previously (Shen et al., 1995). Samples, including uninduced cells, induced cells, inclusion bodies and purified proteins, were solubilized in loading buffer and boiled for 5 min before loading on an SDS PAGE gel. A 12% (w/v) acrylamide gel was used to resolve proteins, which were stained with Coomassie Blue. To prepare cyanobacterial extracts, cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl buffer, pH 8.0, and broken by sonication. For immunoblots, proteins separated by electrophoresis were transferred electrophoretically onto nitrocellulose membranes (Schleicher & Schuell, Keane, NH) by using the Semi-Dry system (Bio-Rad, Hercules, CA). For immunoreaction with rabbit polyclonal antibodies against SufR, the membranes were firstly placed in 5% (w/v) nonfat milk in TBS buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl) for 1 hour on a shaking platform at room temperature. The membranes were then incubated in a 1% nonfat milk/TBS solution with the SufR protein antibody diluted 1:3000 to 1:5000 for at least 2 hours at room temperature. After incubation with the primary antibody, the membranes were washed with TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween-20) for three times (5 min each). The membranes were then incubated with the secondary antibody (goat, anti-rabbit IgG-alkaline phosphatase conjugate, Sigma, A-8025, St. Louis,
MO) diluted 1:3000 in 1% (w/v) nonfat milk/TBS for 1 hour. The membranes were rinsed briefly with TBST buffer for three times (5 min each). The color of the blots was developed in 50 ml of alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) containing 330 μl of nitroblue tetrazolium (50 mg/ml in 70% (v/v) dimethyl formamide) and 165 μl of bromochlorindolyl phosphate (50 mg/ml in ddH₂O). After color development was complete, the blots were rinsed in distilled water and stored in the dark.
RESULTS

The sll0088 gene is adjacent to the suf operon in Synechocystis sp. PCC 6803

The majority of functional studies described in this paper were carried out in Synechococcus sp. PCC 7002 because unlike Synechocystis sp. PCC 6803, it was possible to generate a fully segregated, null mutation in the sll0088 gene of this cyanobacterium. Because the genome sequence is not yet available for Synechococcus sp. PCC 7002, it was necessary to determine the gene organization surrounding the sll0088 homolog. A DNA fragment containing the sll0088 homolog and its upstream flanking region were cloned and sequenced from the genome of Synechococcus sp. PCC 7002 (Genbank accession number AY375041). DNA sequence analysis of the sll0088 homolog and the open reading frames in the region upstream from this gene indicate that the gene organization is identical to that in Synechocystis sp. PCC 6803 (Kaneko et al., 1995; 1996) and Anabaena sp. PCC 7120 (Kaneko et al., 2001). As shown in Figure 2.1, sequence comparisons show that four conserved genes in cyanobacterial genomes, sufB, sufC, sufD and sufS, have homologs in the suf operon of Escherichia coli (Blattner et al., 1997; Patzer et al., 1999). The genes in the suf operon have been shown to function in iron-sulfur cluster biogenesis under conditions of oxidative stress (Takahashi et al., 2002; Nachin et al., 2001). In cyanobacteria, the genes homologous to sll0088 are located near the 5’ end of the presumed suf operons, but are divergently transcribed from the sufBCDS genes. The sufA and sufE genes also exist in cyanobacteria (slr1417 and slr1419, respectively, in Synechocystis sp. PCC 6803), but they are not contiguous with the sufBCDS genes as they are in E. coli and certain other bacteria.
As shown in Figure 2.2, the amino acid sequences of the proteins coded by the sll0088 homologs are highly conserved in *Synechococcus* sp. PCC 7002, *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120, and *Synechococcus* WH8102. A BLAST search shows that highly similar proteins also exist in the photosynthetic prokaryotes *Trichodesmium erythraeum* IMS101, *Nostoc punctiforme*, *Thermosynechococcus elongatus*, and *Prochlorococcus marinus* str. MIT 9313. Three features can be identified in these proteins using PROSITE program analysis: 1) near the N-terminus a basic region preceded by the elements of a putative protein kinase C phosphorylation site (Ser-Thr-Lys); 2) a DNA-binding domain that contains a basic region and a putative helix-loop-helix motif, with a high sequence similarity to DNA-binding proteins from other organisms; and 3) near the C-terminus four highly conserved cysteine residues in a C-(X)_{12}-C-(X)_{13}-C-(X)_{14}-C motif with a possible function in metal binding. Comparative analyses show that the sll0088 homologs have high sequence similarity to transcription regulatory proteins that bind DNA by a conserved helix-loop-helix motif near the N-terminus. The C-terminus, with the similarly spaced cysteine residues, may confer functional specificity to this class of regulatory proteins.

*The sufBCDS genes compose an operon in cyanobacteria*

The *sufABCDSE* genes in *E. coli* (Takahashi et al., 2002) and *E. chrysanthemi* (Nachin et al., 2001) are organized into operons. In cyanobacteria, the adjacent *sufBCDS* genes suggest that they also compose an operon (see Figure 2.1). To determine whether *sufB, sufC, sufD, and sufS* are co-transcribed in *Synechococcus* sp. PCC 7002, the 5’-primers
for *sufB*, *sufC*, and *sufD* were added to an RT-PCR reaction together with the 3′-primer for *sufC*, *sufD*, and *sufS*, respectively, to form primer pairs (Figure 2.3A). As shown in Figure 2.3B, three fragments were amplified with sizes corresponding to *sufBC* (2.2 kbp), *sufCD* (2.1 kbp), and *sufDS* (2.6 kbp). The co-transcription of the *sufB* and *sufC*, *sufC* and *sufD*, and *sufD* and *sufS* genes clearly indicates that in cyanobacteria the four genes also compose an operon.

Insertional inactivation of *sll0088*-like in *Synechococcus* sp. PCC 7002

A 1.8 kbp *KpnI*-EcoRV DNA fragment containing the *sll0088*-like gene was cloned from the genome of *Synechococcus* sp. PCC 7002. A 1.3-kbp *aphII* gene conferring kanamycin resistance was inserted at the unique *StuI* site in the *sll0088*-like gene as shown in Figure 2.4A. This construct was used to transform *Synechococcus* sp. PCC 7002 wild-type cells. To verify the complete segregation of the *sll0088*-like interruption in the transformed strains, genomic DNA of the wild-type and *sll0088*-like transformants was fragmentated by *EcoRI/PstI* digestions, and the blot was probed using a 1.8-kb PCR fragment from the wild-type (Figure 2.4B, upper panel). The probe hybridized to a 2.6-kb DNA fragment in the wild type as predicted. Two hybridizing fragments were detected in the mutant *sll0088*-like::*KmI*(II) and *sll0088*-like::*KmII*(II) strains as a result of introducing the *PstI* site in the kanamycin-resistance cartridge gene. PCR analysis also verified that no DNA fragment with the same size of the wild-type at 1.8-kbp could be identified in genomic DNAs of the *sll0088*-like inactivation mutants (Figure 2.4B, lower panel). Only a 3.1-kbp fragment was amplified from the *sll0088*-like inactivation mutant, which is evidence for the
insertion of the 1.3-kbp kanamycin resistance cartridge. These results demonstrate complete segregation of the sl0088-like::Km'(II) in the Synechococcus sp. PCC 7002 transformants.

The sl0088-like gene was also deleted by substitution of its coding region for spectomycin resistantance gene (Figure 2.4A) as described in the Materials and Methods. The resulting plasmid pUCSs88 was used to transform the sl0088-like::Km'(II) mutant rather than the wild-type strain to facilitate segregation. The complete segregation of the sl0088-like deletion mutants was verified by PCR analysis using the primer pairs as shown in Figure 2.4C. The primers were designed to amplify a DNA fragment corresponding to the size of the spectomycin resistantance gene in the sl0088-like deletion mutants.

*Increased sufBCDS mRNA levels in a null mutant of the sl0088 homolog*

The proximity of the sl0088 homologs to the sufBCDS operon in several cyanobacteria and the identification of the product of the sl0088 gene from Synechocystis sp. PCC 6803 as a possible transcription regulatory protein suggested that Sll0088 might play a role in the regulation of the expression of the sufBCDS operon. To test this idea, we compared the mRNA levels for the sufBCDS genes in the wild-type and a null strain of the sl0088 homolog in Synechococcus sp. PCC 7002. As we have shown above, sl0088 null mutants were created by inserting the aphII gene into the Stul restriction site of the sl0088-like gene in Synechococcus sp. PCC 7002 either parallel (//) or antiparallel (\//) to the transcription direction of the sl0088 homolog (Figure 2.4A). Both mutants were completely segregated as verified by PCR and Southern blot analyses. Their growth rates under low (50 \( \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \)) and normal (250 \( \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \)) light intensities were very similar (Figure 2.5),
suggestions that the insertion direction of *aphII* gene has no effect on the mutant phenotype. The complete absence of the Sll0088 homolog in the null strains was confirmed by immunoblot analysis (Figure 2.6). Given these results, all further studies reported here in which the wild-type and the null mutant were compared used only the strain in which the *aphII* gene was inserted into the *sll0088* homolog parallel to its transcription orientation. We chose this orientation because in principle, a parallel insertion is likely to have less effect on the other genes downstream of the *sll0088* homolog than an antiparallel insertion.

The transcript abundance for the *sufS* gene, probed by northern blotting analysis, was found to be higher in the null strain than in the wild-type (Figure 2.7A). The intensities of the hybridization signals for the *sufS* gene were quantified using the NIH Image Program and the expression level was 3 to 4 times higher in the null strain. RT-PCR analysis was further performed to probe more sensitively the transcript abundances for the *sufB, sufC, sufD* and *sufS* genes in cells of the wild-type and null strains. As shown in Figure 2.7B, mRNA levels for the *sufB, sufC, sufD*, and *sufS* genes are consistently higher in the *sll0088*-like null strain. In particular, the absence of the Sll0088-like leads to an increase in the transcript levels of the *suf* operon. These results therefore implicate the *sll0088*-like gene product in the regulation of the *suf* operon expression in *Synechococcus* sp. PCC 7002. It is proposed that the *sll0088*-like be named *sufR* and that the protein coded by this gene be named SufR.
Does SufR regulate expression of other nifS-like homologs in cyanobacteria?

In addition to *slr0077*, there are three additional *nifS*-like homologs in cyanobacteria. To investigate if these *nifS*-like genes are also subject to regulation by SufR, RT-PCR analysis was performed to measure transcription levels in the wild-type and the *sufR* null mutant of *Synechococcus* sp. PCC 7002. These results are presented in Figure 2.8. In contrast to the increased expression level of *sll0077*-like (*sufS*) in the *sufR* null mutant, the mRNA level of *slr0387*-like is significantly lower in the *sufR* null mutant than in the wild-type. The expression of the *sll0704*-like gene is also slightly lower in the *sufR* null mutant. By contrast, no obvious change can be observed for the transcription of *slr2143*-like. These data suggest that all four *nifS*-like homologs are differentially expressed in the *sufR* null mutant, and that SufR may directly or indirectly regulate both the SUF and ISC pathways.

Transcript levels of *sufB* and *sufS* are elevated in the *sufR* null strain under stress conditions

Proteins encoded by the *suf* operon in *E. coli* and *Erwinia chrysanthemi* are thought to be involved in the assembly of iron-sulfur clusters, particularly under conditions of oxidative stress (Nachin et al., 2001; Takahashi et al., 2002; Zheng et al., 2001). To probe the expression of the *suf* genes in cyanobacteria under stress conditions, the wild-type and the *sufR* null strain were grown in normal media and stressed with DCMU (an inhibitor of PS II), DBMIB (an inhibitor of cytochrome *b*$_{6}$*f*), H$_{2}$O$_{2}$, and Fe deprivation for varying lengths of time before cells were harvested and processed for RNA isolation. The transcription levels of the *sufB* and *sufS* genes under these conditions were compared with those under normal growth conditions. As shown in Figure 2.9, the expression level of *sufB*
and suffS is elevated in the presence of DCMU, DBMIB, H2O2, and in the absence of iron in the medium. In the sufR null strain (see Figure 2.9), the expression level of sufB and suffS is elevated and constant regardless of the stress imposed. Thus, the expression levels of the sufB and suffS genes in the sufR null mutant are constant and higher under all conditions compared to the wild-type. This would be expected if the sufBCDS operon were no longer negatively controlled by SufR. These observations therefore suggest that the iron-sulfur cluster assembly proteins encoded by the sufBCDS operon have increased functional importance under conditions of oxidative stress in cyanobacteria.

**Growth rates of the sufR null strain are higher under stress conditions.**

The increased mRNA levels of the sufBCDS operon in the sufR null strain would be expected to have physiological consequences if the proteins coded by these genes normally function in response to stress. As shown in Figure 2.10A, the growth rate of the sufR null strain at normal (250 μmol·m⁻²·s⁻¹) light intensities was lower than for the wild-type, with doubling time of 6.5 and 4 hours respectively, suggesting that the over-expression of the Suf proteins is mildly inhibitory in cells grown under near-optimal nutrient and illumination conditions. To probe the relationship between sufR and iron metabolism, cells were transferred to fresh growth medium containing 2,2'-dipyridyl, an iron chelator, and streptonigrin, an iron-activated antibiotic, during mid-exponential phase and the growth rates were measured (see Materials and Methods for details). In the presence of 2,2'-dipyridyl (Figure 2.10B), the 15 h doubling time of the wild-type decreased to 10.5 h in the sufR null mutant. This might be expected if the iron-sulfur cluster biosynthetic enzymes in
the *sufR* mutant were up-regulated and were thereby able to scavenge low levels of iron more efficiently than the wild-type. To test this idea, the growth rates of the wild-type and *sufR* null strain of *Synechococcus* sp. PCC 7002 were compared when iron was eliminated from the growth medium. When the cells were transferred to fresh medium without added FeCl$_3$ during mid-exponential phase, the *sufR* null strain showed a significantly faster growth rate than the wild-type (Figure 2.10C). When the cells were transferred to fresh growth medium in the presence of streptonigrin (Fig. 10D, open symbols), growth of the wild-type and the mutant were inhibited for three and seven days respectively. The higher sensitivity of the mutant than the wild-type could indicate that iron accumulated in the mutant cells, thereby providing the cells with a higher intracellular iron content than the wild-type. The inhibiting effect of streptonigrin could be alleviated by iron starvation; the *sufR* mutant resumed growth after the second day, and the wild-type after the third day (Fig. 10D, filled symbols). Thus, the *sufR* mutant survived the presence of streptonigrin under iron starvation conditions better than under normal growth medium. In contrast, growth of the wild-type showed no significant differences under these conditions, probably because iron starvation exerted contradictory effects: on the one hand, it inhibited the wild-type growth, and on the other hand, it alleviated the toxicity of streptonigrin.

Figure 2.11 shows the appearance of the wild-type and the *sufR* null mutant cultures grown under standard, streptonigrin-treated, 2,2'-dipyridyl-treated, and Fe-starvation conditions. Their relative growth rates are reflected in the intensity of the green color of these cultures. These studies clearly show that the increased transcript levels for the *suf* genes in the *sufR* null mutant allows cyanobacterial cells to grow more efficiently in a low-
iron environment. This result is in clear contrast to the inhibitory effect exerted by increased $suf$ gene expression level on the growth of $sufR$ null mutants under standard growth conditions. The inhibitory effect of the high expression of the $suf$ genes is more obvious under iron-replete conditions, as shown by the streptonigrin-containing cultures. As shown previously in Figure 2.9, the expression level of $suf$ genes in the $sufR$ null mutant always exceeds that of the wild-type in response to oxidative stresses. High expression levels of the Suf proteins are also toxic in *E. coli* (Zheng et al., 2001). These studies show that increased mRNA levels for the $suf$ genes in the $sufR$ null mutant lead to the predicted physiological response of enhanced survivability under iron-limiting conditions.

*Expression of the psaC gene in sufR null mutants of Synechococcus sp. PCC 7002*

Our initial physiological characterization indicates that the $sufR$ null mutant in *Synechococcus* sp. PCC 7002 can grow photoautotrophically. No obvious difference could be observed in assembly/stability of the PS I complexes (data not shown). To determine if $sufR$ is involved in the regulation of $psaC$ expression, the transcript level of $psaC$ in the wild-type and $sufR$ null mutant were measured. As shown in Figure 2.12A, no difference was found in the expression of the $psaC$ gene in *Synechococcus* sp. PCC 7002 in the presence or in the absence of the $sufR$. Also, the expression level of the $psaC$ gene is not subject to redox regulation when cells are grown under different oxidative stress conditions, as shown in Figure 2.12B. To examine further whether the $sufR$ gene is involved in regulation of the translation of the PsaC protein, its assembly in thylakoid membranes was compared between the *Synechococcus* sp. PCC 7002 wild-type and the $sufR$ null mutant. As
shown in Figure 2.13, no obvious difference can be seen in the amount of PsaC assembled in thylakoids of the wild-type and mutant (similar to *Synechocystis* PCC 6803, Yu et al., 2003). These results indicate that the *sufR* gene in *Synechococcus* sp. PCC 7002 is not directly involved in regulation of expression of the *psaC* gene at the transcriptional or translational levels.
DISCUSSION

The *sufR* gene is usually located adjacent to the *sufBCDS* operon in cyanobacteria.

The *sll0088* (*sufR*) gene is immediately upstream from the *sufBCDS* operon in *Synechocystis* sp. PCC 6803, and homologs occupy a similar position in other cyanobacteria, including *Synechococcus* sp. PCC 7002, *Anabaena* sp. PCC 7120, *Trichodesmium erythraeum* IMS 101, and *Prochlorococcus marinus* MIT9313. In *Synechococcus* sp. WH8102, the ferredoxin-thioredoxin reductase catalytic chain, a subunit of heterodimeric ferredoxin-thioredoxin reductase (*ftrC*, which corresponds to ORF *sll0554* in *Synechocystis* sp. PCC 6803), is located between *sufR* and the *sufBCDS* operon. There is only a 7 bp-long spacer between *ftrC* and the *sufBCDS* operon, suggesting that the *ftrC* homolog is co-transcribed with the downstream *sufBCDS* genes. Ferredoxin-thioredoxin reductase is a [4Fe-4S] protein through which thioredoxin undergoes redox regulation of protein function and signaling via thiol redox control. Thioredoxin has additional functions in defense against oxidative stress and is required by a number of transcription factors for DNA binding (Arner et al., 2000). The organization of the *sufR, ftrC* and *sufBCDS* genes in *Synechococcus* sp. WH8102 implies that SufR may also be involved in the regulation of the ferredoxin-thioredoxin system. A more pronounced exception to the gene organization exists in *Thermosynechococcus elongatus*, in which *sufB* is located immediately upstream of the *sll0088* homolog, and even though the *sufC* and *sufD* genes are adjacent to each other, they are located elsewhere in the genome, as is the *sufS* gene. This difference raises the possibility that SufR might act to control a regulon rather than simply the *sufBCDS* operon. An indication that this might be the case is that the mRNA abundance for the bacterioferritin
gene, *sufA*, and *sufE* of *Synechococcus* sp. PCC 7002 also increases in the *sufR* mutant strain (data not shown). Moreover, the decreased transcription level of *slr0387*-like and *sll0704*-like in *sufR* null mutant SufR clearly indicates that SufR might also act as a transcription activator.

*Homologs of the sufR gene may exist in non-photosynthetic pathogenic bacteria*

Non-photosynthetic bacteria, particularly pathogens and organisms that live under extreme conditions, also contain genes with strong similarity to *sufR*. Bacterial pathogens have developed efficient iron acquisition systems to counteract the defensive sequestration of iron by their hosts (Dussurget et al., 1998). These organisms must deal with oxidative stress exerted by either the host defense response or the environment. Although all of the four cysteines are well conserved in *sufR* homologs of cyanobacteria, only three of them (the 1st, 2nd, and 4th) are conserved in *sufR* homologs of non-photosynthetic bacteria. An exception is *Vibrio cholerae*, which contains two cysteines in addition to the four conserved cysteine residues that are found in cyanobacterial SufR. The third position in the bacterial *sufR* homologs is often occupied by Ala (*Bacillus anthracis, Thermoplasma acidophilum*), Thr (*Mycobacterium leprae, Streptomyces coelicolor*), or Val (*Yersinia pestis*), just to name a few possibilities (See Figure 2.14). This group of homologs also includes *Chloroflexus aurantiacus*, a thermophilic photosynthetic green non-sulfur bacterium in which the SufR homolog contains only three cysteine residues. It should be noted that certain non-photosynthetic bacteria have SufR homologs with additional cysteine residues near the C-terminus. As will be shown in chapter 4, the cysteine motif in SufR protein does provides
ligands to a [4Fe-4S cluster]. The iron-sulfur cluster coordinated by the common cysteine motif near the C-terminus of these proteins may sense oxidative stress and thus control the expression of their target genes. Because there are only three conserved cysteine residues in the SufR homologs of non-photosynthetic organisms, a non-cysteine residue may be required as the fourth ligand to the iron-sulfur cluster. Indeed, IscR, a transcriptional repressor of the iscSUA operon, contains only three conserved cysteines, but EPR studies show that it binds a stable [2Fe-2S] cluster (Schwartz et al., 2001). Among the bacteria that contain sufR homologs, it is only in Mycobacterium tuberculosis strain H37RV and Mycobacterium leprae that four suf genes and one iscU-like gene (sufBB’CS-iscU) are located adjacent to the sufR homolog. In T. acidophilum only three suf genes (sufCBD) are adjacent to the sufR homolog, and in the other organisms that bear a sufR homolog, sufR and the other suf genes are separated by significant distances on the genome. This strengthens the notion suggested above that SufR may act to control a regulon rather than only the sufBCDS operon. E. coli and E. chrysanthemi also contain a suf operon that is composed of all six suf genes (sufABCDSE), but no sufR homolog could be discerned.

The SufR protein is a member of the DeoR family of helix-loop-helix proteins

An amino acid alignment and BLAST search against the GenBank database shows that the proteins coded by the sufR homologs contain three putative motifs: a protein kinase C phosphorylation site near the N-terminus, a helix-loop-helix DNA binding domain, and a motif of four cysteine residues separated by 12, 13, and 14 amino acids near the C-terminus. The DNA-binding motif is highly conserved among sufR homologs of cyanobacteria and
can be classified in the DeoR family of helix-loop-helix proteins. These are typically highly diverse proteins, which includes DeoR (Mortensen et al., 1989), LacR (Rosey et al., 1992), and FucR (Lu et al., 1989). SufR appears to be a member of yet another DeoR subclass that contains a highly conserved C-(X)_{12}-C-(X)_{13}-C-(X)_{14}-C motif near the C-terminus.

The SufBCDS proteins function in iron-sulfur cluster biogenesis in cyanobacteria

As have been summarized in Chapter 1 (Introduction), proteins encoded by the sufABCDSE genes have been proposed to be involved in the biogenesis and assembly of iron-sulfur clusters in bacteria (Patzer et al., 1999; Nachin et al., 2001, Takahashi et al., 2002). There is evidence showing that SUF is the only possible Fe-S cluster pathway in the chloroplast of Arabidopsis thaliana. First, A. thaliana contains homologs to all six suf genes; at least one copy of each suf gene encodes a protein with a transit peptide sequence targeted to chloroplast (Table 2.2). In addition, Homologs of sufB and sufC, named ycf24 and ycf16 respectively, have also been located in the chloroplasts of the red alga Porphyra purpurea (Sugiura et al., 1995) and the cyanelle Cyanophora paradoxa (Stirewalt et al., 1995, GB/EMBL/DDBJ cross-reference U30821). SufC is also encoded in the plastid genome of the cryptomonad Guillardia theta (Douglas et al., 1998). Second, there are two nifS-like homologs in A. thaliana, one of them, named AtNFS2 (Leon et al, 2002) or AtCpNIFS (Pilon-Smits et al., 2002), is targeted to the chloroplast. The recombinant and expressed AtNFS2 protein showed cysteine desulfurase activity (Pilon-Smits et al., 2002). The other homolog is less similar to nifS, and is predicted to be targeted to the mitochondria (Lee McIntosh, unpublished data). Both mitochondria and chloroplasts contain high
concentrations of iron-sulfur clusters due largely to the presence of NADH dehydrogenase and fumarase in the mitochondria and PS I in the chloroplast. Third, the assembly of Fe-S clusters in plant ferredoxin has long been shown to occur in isolated chloroplasts. Fourth, the *sufB* mutant in *A. thaliana* accumulates protoporphyrin IX in cells, which is caused by disruption of iron metabolism (Moller et al., 2001; Wilson et al., 2002). Based on these findings, it is reasonable to propose that the *suf* operon in cyanobacteria is also involved in iron-sulfur cluster assembly for the photosynthetic complexes. Hence, the SufB, SufC, SufD and SufS proteins probably work together to assemble iron-sulfur clusters in cyanobacteria. Thus, it is proposed that SufR plays an indirect role in controlling PS I biogenesis by regulating *suf* gene expression for the control and synthesis of iron-sulfur clusters.

*The C14S*<sub>PsaC</sub> *suppressor mutations are functionally equivalent to the sufR null mutants*

The relationship between *sll0088* (renamed here *sufR*) and PS I biogenesis/assembly was established by the earlier finding that a secondary mutation to *sufR* in the C14S<sub>PsaC</sub> mutant of *Synechocystis* sp. PCC 6803 restored photoautotrophic growth (Yu et al., 2003). The phenotype in the C14S<sub>PsaC</sub>-R62 and C14S<sub>PsaC</sub>-R18 suppressor mutants exhibits an increased number of PS I complexes per cell (Yu et al., 2003). Based on the result that no differences in both *psaC* gene expression and PsaC protein level are observed between the wild-type and the *sufR* null mutant in *Synechococcus* PCC 7002 (Figure 2.12 and 2.13), it can be ruled out that SufR acts directly on the *psaC* gene or its protein product. Thus, it is proposed that the substitution of Pro for Arg in C14S<sub>PsaC</sub>-R62 and the GYF insertion between Cys230 and Gly231 in C14S<sub>PsaC</sub>-R18 disrupt the structure, and hence the function,
of $sufR$ in *Synechocystis* sp. PCC 6803, and that as a consequence, the $C14_{PsaC}$-R62 and $C14S_{PsaC}$-R18 suppressor mutants are phenotypically identical to the $sufR$ null mutants in that both result in the up-regulation of the $sufBCDS$ genes that encode proteins involved in the synthesis of iron-sulfur clusters. PS I is one of the most abundant iron-sulfur protein complex in the cyanobacterial cell. The primary $C14S_{PsaC}$ mutation results in the occurrence of a mixed-ligand (3 Cys• 1 Ser) [4Fe-4S] cluster in the $F_B$ site. It is proposed that these PS I complexes are targeted as faulty, resulting in the turnover of the entire PS I complex. The level of PS I in the $C14S_{PsaC}$ mutant would then represent a balance between the rate of synthesis and the rate of turnover. If the rate-limiting step in the biosynthesis of PS I is indeed the formation of iron-sulfur clusters, then the over-expression of enzymes involved in iron-sulfur cluster biosynthesis would explain why the $C14S_{PsaC}$-R62 and $C14S_{PsaC}$-R18 mutants are able to increase the steady-state population of PS I complex per cell in the secondary mutants. The nearly normal ratio of PS I to PS II would support photoautotrophic growth, which was the criterion by which the secondary suppressor mutants were initially selected. Thus, the mixed-ligand iron-sulfur cluster in the $F_B$ site of the $C14S$ mutant is fully functional *in vivo*, as was implied by the ability of isolated PS I complexes to support high rates of electron transfer from cytochrome $c_6$ to flavodoxin *in vitro* (Yu et al., 1997; Yu et al., 2003).
REFERENCES


Table 2.1  Sequences of oligonucleotides used for RT-PCR analysis and cloning of the *sll0088* gene

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>sufRF</td>
<td>5’- ACCCTTTTCTGACACCCGCGCGCAAAAC</td>
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<tr>
<td>sufRR</td>
<td>5’- ATCTTTGCTTTGGATGATGAGCCG</td>
</tr>
<tr>
<td>sufBF</td>
<td>5’- AGTGCAGACCCTCAAAAACCCTTGTCA</td>
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<tr>
<td>sufBR</td>
<td>5’- CAACAGTGCTTCCGATTTGAGGTG</td>
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<tr>
<td>sufCF</td>
<td>5’- GAGTGAAGTTATCTTTAGCGATTA</td>
</tr>
<tr>
<td>sufCR</td>
<td>5’- ACAACCCCCCGAGCCAGTTTCTT</td>
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<tr>
<td>sufDF</td>
<td>5’- TCTAGCAGGATTCAATGACACGGCGGACGCCGAT</td>
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<td>sufDR</td>
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**Table 2.1** Sequences of oligonucleotides used for RT-PCR analysis and cloning of the *sll0088* gene (continued)

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<tr>
<td>Esll0088R</td>
<td>5’-CGTCAATTGATTTAAAAAGCTTTCTGCGGCCCG</td>
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Underlined letters indicate nucleotides of the restriction site sequence.
Table 2.2 Suf homologs in *Arabidopsis*

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<th>Identity(%)</th>
<th>Positive(%)</th>
<th>Predicted location</th>
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</tr>
<tr>
<td>SufD</td>
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</tr>
<tr>
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<td></td>
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<td></td>
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<td>48</td>
<td>cytoplasm</td>
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<tr>
<td>SufE</td>
<td>At4g26500</td>
<td>50</td>
<td>69</td>
<td>chloroplast</td>
</tr>
</tbody>
</table>
**FIGURE LEGENDS**

**Figure 2.1** Map of gene organization of *sll0088* (*sufR*) and the *sufBCDS* operon of three cyanobacteria and a comparison with the *sufABCDSE* operon from *Escherichia coli*.

**Figure 2.2** Conserved domains in SufR proteins from four cyanobacterial strains: 1) *Synechococcus* sp. PCC 7002; 2) *Synechocystis* sp. PCC 6803; 3) *Anabaena* sp. PCC 7120; and 4) *Synechococcus* sp. WH8102. Their amino acid sequences were aligned using the Clustal-W algorithm in the MacVector DNA analysis program (Accelrys, Madison, WI). The basic region, the helix-loop-helix domain, and the four conserved cysteines in the C-terminal region are indicated above the alignment. The diamond shape near the N-terminus indicates the protein kinase C phosphorylation site.

**Figure 2.3** The primer pairs used for the RT-PCR experiment (A) and the co-transcription of *suf* genes detected by RT-PCR (B). The primers *sufBF* and *sufCR* were used to amplify the *sufBC* fragment; the primers *sufCF* and *sufDR* were used to amplify the *sufCD* fragment; and the primers *sufDF* and *sufSR* were used to amplify the *sufDS* fragment. Products of the expected sizes for *sufB*, *sufC*, *sufC* and *sufD*, and *sufD* and *sufS*, establish that the four genes are co-transcribed.

**Figure 2.4** Mutagenesis of the *sll0088*-like gene in *Synechococcus* sp. PCC 7002. A), Restriction maps of DNA fragments from the wild-type and the *sll0088* inactivation and
deletion mutants. B), upper panel, Southern blot hybridization of the genomic DNAs of the wild-type and the \textit{sll0088} mutant strains. Genomic DNAs were digested by \textit{EcoRI} and \textit{PstI}. The hybridization probe was prepared using a 1.8-kb PCR fragment containing the \textit{sll0088} gene; lower panel, PCR analysis of segregation of the \textit{sll0088} insertional inactivation. C) PCR analysis of segregation of the \textit{sll0088} deletion mutants using primer pairs as shown at the bottom of the agarose gel picture. Arrows mark positions of the primers.

**Figure 2.5** Growth curves of three \textit{sll0088}-like inactivation mutant strains in \textit{Synechococcus} sp. PCC 7002. A), growth under low light condition (50 \text{E}.m^{-2}.s^{-1}); B), growth under normal light condition (250 \text{E}.m^{-2}.s^{-1}). Red square and blue diamond represent two \textit{sll0088}-like::\textit{Km}^R (//) mutants and green triangle represents a \textit{sll0088}-like::\textit{Km}^R (II) mutant.

**Figure 2.6** Immunodetection of the SufR protein in cells of the wild-type and two \textit{sufR} null mutants of \textit{Synechococcus} sp. PCC 7002 by use of antibodies against the recombinant SufR protein of \textit{Synechocystis} sp. PCC 6803. Proteins corresponding to 10 \text{g} chlorophyll from the whole cell lysates were loaded onto each lane. Strains \textit{sufR}(/) and \textit{sufR} (//) are mutants in which the inserted \textit{aphII} gene has the same direction of transcription (//) or the opposite direction of transcription (//) from the \textit{sufR} gene. Only the \textit{sufR}(/) strain was used for subsequent work; it is referred to in the text as the ‘\textit{sufR} null’ mutant.

**Figure 2.7** Transcription analyses of the \textit{suf} operon in \textit{Synechococcus} sp. PCC 7002 wild-type and \textit{sufR} null mutant. A) Northern blot analysis with hybridization probe for the
Synechococcus sp. PCC 7002 sufS gene. Arrow on the left points to the position of sufS. B) RT-PCR analysis of the suf gene transcription levels in Synechococcus sp. PCC 7002 wild-type and sufR null strains. Primers specific for the sufB, sufC, sufD, and sufS genes were used for the RT-PCR reactions as listed in Table I. The gene for 16S RNA was used as a control to assure that the RNAs of the wild-type and sufR null strains were added to the RT-PCR reactions at equal concentrations, and that both were equally competent for the RT-PCR reaction.

**Figure 2.8** Comparison of the mRNA levels of slr0704-like, slr0387-like, slr2143-like, and slr0707-like (sufS) in the wild-type and sufR null mutant strains of *Synechococcus* sp. PCC 7002 by RT-PCR analysis. 16S rRNA serves as the control. The slr0077-like gene is up-regulated in the sufR mutant. The slr0387-like gene is down-regulated in the sufR mutant. slr0704-like is also slightly down-regulated in the sufR mutant. The expression of slr2143 remains unchanged.

**Figure 2.9** RT-PCR analysis of the expression of the sufS and sufB genes in the wild-type and sufR strains of *Synechococcus* sp. PCC 7002. Total RNA was isolated from cells grown under: 1), standard growth conditions; 2), A+ medium after addition of 5 mM glycerol and 0.5 µM DCMU; 3), A+ medium after addition of 5 mM glycerol and 10 µM DBMIB; 4), A+ medium after addition of 150 µM H₂O₂; and 5), A+ medium without addition of FeCl₃ (grown for 2 days). The gene for 16S RNA was used as a control to assure that the RNAs of
the wild-type and $sufR$ strains were added to the RT-PCR reactions at equal concentrations, and that both were equally competent for the RT-PCR reaction.

**Figure 2.10** Growth curves of the wild-type ( ) and $sufR$ null (□) strains of *Synechococcus* sp. PCC 7002 under: A) standard conditions (250 μEm$^{-2}$s$^{-1}$, 38°C, A$^+$ medium); B) standard conditions in the presence of 2,2’-dipyridyl; C) standard conditions in A$^+$ medium without addition of FeCl$_3$; D) standard conditions in the presence of streptonigrin for the wild-type with (open symbols) or without (filled symbols) addition of FeCl$_3$ and the $sufR$ null strain with (open symbols) or without (filled symbols) addition of FeCl$_3$. These experiments were repeated three times; all showed similar growth profiles. Data from only one experiment are shown.

**Figure 2.11** Growth of the wild-type and $sufR$ null mutant under various stress conditions. The cultures were grown under standard, streptonigrin-treated, 2,2’-dipyridyl-treated, and Fe-starvation conditions and photographed on the second day, seventh day, third day, and third day of growth respectively. The figures depict a 2.5 cm high cross-section of a 2 cm x 18 cm culture tube. The orange-brown color of the Fe-starved cells is due to the loss of phycobiliproteins.

**Figure 2.12** RT-PCR analysis on expression of the *psaC* gene in *Synechococcus* sp. PCC 7002. Total RNA was isolated from cells of *Synechococcus* sp. PCC 7002 wild-type and $sufR$ null mutant (Figure A), and from cells of *Synechococcus* sp. PCC 7002 wild-type grown in A$^+$ media with the addition of 1) 0.5 μM DCMU; 2) 10 μM DBMIB; and 3) 150
$\text{M H}_2\text{O}_2$ (Figure B). 1.2 ng RNA was used as template for RT-PCR reaction using the One-Step RT-PCR kit from QIAGEN.

Figure 2.13 Immunoblot analysis of the PsaC protein in the thylakoid membranes of *Synechococcus* sp. PCC 7002 wild-type and *sufR* null mutant. Thylakoid membranes were isolated from cells grown at normal growth conditions. 6 µg Chl was loaded for each sample per lane. Proteins were resolved in SDS-PAGE, blotted onto nitrocellulose membrane, and probed with antiserum against the PsaC protein from *Synechococcus* sp. PCC 7002.

Figure 2.14 Alignment of the Cys-containing C-terminal domains of the cyanobacterial SufR and homologous proteins from several other organisms. The conserved sequences of the C-terminal part were from: 1) cyanobacterial consensus sequence that was drawn from sequence analysis of SufR from *Synechococcus* sp. PCC 7002 and published sequences of the SufR-like proteins in other cyanobacteria in the database; 2) *Vibrio cholerae* (NCBI Microbial Genomes Annotation Project); 3) *Chloroflexus aurantiacus* (NCBI Microbial Genomes Annotation Project); 4) the DeoR protein of *Bacillus cereus* 14579 (Ivanova et al., 2003); 5) *Shewanella oneidensis* MR-1 (Heidelberg et al., 2002); 6), *Mycobacterium tuberculosis* (Cole et al., 1998); 7) *Corynebacterium glutamicum* ATCC 13032 (NCBI Microbial Genomes Annotation Project); 8) *Thermoplasma volcanium* (Kawashima et al., 2000); 9) *Yersinia Pestis* (Parkhill et al., 2001); and 10) *Streptomyces coelicolor* (Bentley et al., 2002).
Figure 2.1

1 *Synechococcus* sp. PCC 7002

MutM  SufR  SufB-like  SufC-like  SufD-like  SufS-like  hypothetical

2 *Synechocystis* sp. PCC 6803

CheA  SufR  SufB-like  SufC-like  SufD-like  SufS-like  hypothetical

3 *Anabaena* sp. PCC 7120

hypothetical  SufR  SufB-like  SufC-like  SufD-like  SufS-like

4 *E. coli*

SufA  SufB  SufC  SufD  SufS  SufE

1000 kbp
Figure 2.2

---

basic region

---

helix 1

---

loop

---

helix 2

---

MTLSDTAETRSTKHIDILEYLLKHXQVKAVQLAKSLQISQPAGVRLKDLLEEELGIERHRAKQEG-LGRPNYFYGLSR 76
MTLSSS----HSTKDILCYYLKEGQSAYAAAEGLISPSQMAMKHKDLDEGELGIEHGQRQRG-MGRPQFLYQLSR 72
MATTQQ----ASTDQILEYLLKHDQATALKDLNVLDSQPQAIHRHKLLEELVVSNTMQAGMGRPQHVYQLSR 73
MTSPVQ----ASTDDALLSLLGERDADAALNLALSGARQVRLKSLAEAGLAESPSVSG-PGRPSNWRHLT 72

*: * **: : * **: :* :* **:*::**.* :*: *:* **:*:: **:..:** * **: :** :* **:*::**.* :*: *:* **: :**

---

loop

---

helix 2

---

AGRDRFPN----RYNDFAVSFLDTLAETAGEQVEVRQKFWQKKAHSYRDRLGDGSLPERLARLWQHKEEGYMAE 149
QGREQFPQ----RYGEFALSFLISDVLTVGHEQLAVLKKQWQRKAEYRQQIGQGPLSKRHKLVELRRQEGYMAE 145
QGRLHKNVSRDHGDFAVSLLDLTAETVGHQQFTIQKQRQWERKAEYRDRVQGKSLQERAVNLWQRLKNEFGMAE 150
QGRQTFPD----GSQRFALGLNLSRASPLTEBRTLVQMQAEKDAYSYDRIEGLQQREQLASLRDREGYTL 145

*: * **: :* **: :** :* :* **:*::**.* :*: *:* **:*:: **:..:** * **: :**

---

VIPAT---DHGHDGFLTEHHCAIAADVSAEFPVPGNELEMFEELVPDCTVTTRTQUINEHHCQGQYIQAKD 218
IHPLS----VEQAEKFIHCAIAADVASEPyTVCHELEMFAAILPDCAIERTHWNDEGTCYLIQSKPN 216
YHPVSSESTQKFIRFHCIAINSAEFPSVPGNELEMFAAELPDCPVERTHWNLTRLGEGTCYQACHPTSHI 227
CSPEE----DGVSRLQEVHCVQIARAEFPAVCDQVELLRVTPDCQVERVHWRLEGBHACFRTIPQLN 213

*: * **: :* **: :** :* :* **:*::**.* :*: *:* **:*:: **:..:** * **: :**
Figure 2.3

A

\[ \text{suf}B \quad \text{suf}C \quad \text{suf}D \quad \text{suf}S \]

B

\[ \text{bp} \quad \text{suf}BC \quad \text{suf}CD \quad \text{suf}DS \]

4000

3000

2500

2000

1500

1000
Figure 2.4

A

B

C

wt  sll0088-like

1  2  3  4

2.5kb  2.0kb  1.4kb  1.0kb  2.8kb  1.8kb

1500  1000  800

mutM  sll0088-like  sufB
Figure 2.5

A

OD at 730 nm

Time (hours)

0 20 40 60 80 100 120 140

sll0088-like-1

sll0088-like-2

sll0088-like(II)

B

OD at 730 nm

Time (hours)

0 5 10 15 20 25 30 35 40

sll0088-like-1

sll0088-like-2

sll0088-like(II)
Figure 2.6

WT  \textit{sufR}^{-}(\text{II})  \textit{sufR}^{-}(\text{II})

\begin{center}
\begin{tabular}{c}
\text{SufR} \rightarrow \\
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{c}
\text{29.9 kDa}
\end{tabular}
\end{center}
Figure 2.7

A  

WT  sufR(//)  sufR(a//)

[Gene expression image showing sufS and an arrow indicating 1.26 kb]

B

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[Genomic region diagram with bp scale: 2000, 1500, 1000, 800, 600, 400]
Figure 2.8

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[Image of gel electrophoresis showing bands for each condition]
Figure 2.9

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Figure 2.10
Table 2.11

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Figure 2.11
Figure 2.12

A

WT  sufR

psaC

B

1  2  3

psaC
Figure 2.13

WT  sufR

PsaC ➤
Figure 2.14

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Chapter 3. SufR Is Distributed in the Thylakoid Membranes and Cytosol in Cyanobacteria

ABSTRACT

A series of immunoblotting analyses were performed using polyclonal antibodies against the recombinant SufR protein of *Synechocystis* sp. PCC 6803. These data indicate that SufR is partitioned between the cytosol and the thylakoid membranes. The majority of the SufR protein (~65%) was in the soluble cytosol. About one third was associated with the thylakoid membranes. The nature of the association of SufR with the thylakoids was studied by washing membranes with high salt, detergent (Triton X-100), and reducing agents (dithiothreitol-DTT). The SufR protein was removed from the thylakoids with 0.4% Triton X-100 treatment, indicating that SufR is a peripheral protein and its association with the thylakoids is partly mediated by hydrophobic interactions. The removal of the SufR protein from thylakoids with 10 mM DTT suggests that this association is also mediated by the formation of a mixed disulfide bond with a thylakoid-associated protein. This two-compartment distribution phenomenon suggests a dual function for the SufR protein. Under several stress conditions, both the transcription level of the *sufR* gene and the translation level of the SufR protein were up-regulated, but no obvious changes were observed for the membrane-associated SufR protein. These data suggest that association and dissociation with the thylakoid membrane is not the mechanism by which SufR adjusts its functions in response to environment variations. While the SufR protein in the cytosol should function as a transcriptional regulator of the *suf* operon, the function of SufR bound to the thylakoids remains unknown.
INTRODUCTION

It is shown in Chapter 2 that SufR is a transcriptional repressor of the *suf* operon in *Synechococcus* sp. PCC 7002. Since transcription of mRNA occurs in the cytoplasm in bacteria, the transcriptional regulatory protein SufR should also exist in the cytosol. Certain gene regulators, such as RB60 (Trebitsh et al., 2000; 2001) and PutA (Surber et al., 1999), are bound to the membrane and thus have other functions. It is argued in chapter 2 that SufR participates in the PS I biogenesis/assembly through the regulation of the *suf* operon, but the possibility that SufR might be directly involved in PS I assembly is not excluded. If this is the case, SufR should also be associated with thylakoid membranes, since PS I is a membrane-bound protein complex. With the expectation that information on the localization of the SufR protein in cyanobacterial cells will help us better understand its function(s), polyclonal antibodies against purified recombinant SufR protein were produced, and a series of immunoblotting analyses were conducted in *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002. We found that the SufR protein was distributed between the cytosol and the thylakoid membranes in cyanobacteria, and its association with the thylakoid membranes was mediated by hydrophobic interactions between SufR and the membranes, and a disulfide bond between SufR and an unknown membrane-associated protein. Since SufR regulates the transcription of the *suf* operon, which functions under oxidative stress conditions, the expression of *sufR* was examined under several stress conditions. Similar to the *sufBCDS* genes, expression of *sufR* is also up-regulated under stress conditions.
MATERIALS AND METHODS

Membrane Isolation from Cyanobacteria

*Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 were grown as described in chapter 2. Thylakoid membranes from *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 were isolated as described in Shen et al. (2002). Cells in late-exponential growth stage were harvested and washed once in 50 mM Tris/HCl buffer pH 8.0. The cell pellets were resuspended in 50 mM Tris/HCl buffer pH 8.0 at about 1 gram cell pellet per 10 ml buffer and lysed by passing twice through a French pressure cell at 2,000 lb/in² (psi) at 4°C. Unbroken cells and cell debris were removed by centrifugation at 4,000 g for 10 min. Membranes were collected by centrifugation at 50,000 g for 1 hour. The membranes were resuspended in 50 mM HEPES/NaOH pH 8.0. Chlorophyll concentration was determined at 663 nm after extraction into 100% methanol (Lichtenthaler et al., 1987) using visible spectrophotometry with published extinction coefficients. This membrane preparation contains both thylakoid and plasma membranes.

Separation of plasma membrane and thylakoid membrane

Thylakoid and plasma membranes were separated as described in (Shen et al., 2002) by using an aqueous polymer two-phase partitioning method as described by Norling et al. (1998). Cells from 1 liter culture were resuspended in a 20 mM potassium phosphate buffer pH 7.8, to a final volume of 5 ml. Glass beads (diameter 0.2 mm) were added to the cell suspension, so that only 0.3-0.4 ml of solution was visible above the beads. The sample was vortexed 3 times at the highest speed for 2 min, with 1-min intervals, on ice, and then
centrifuged for 1 min. The cell lysate was collected and centrifuged at 4000 g for 10 min to pellet unbroken cells and cell debris. The supernatant was further centrifuged for one hour at 50,000 g. The resulting membrane pellet was resuspended and homogenized in 4 ml of 5 mM potassium phosphate buffer pH 7.8 containing 0.25 M sucrose. The two-phase aqueous polymer systems were prepared from 40% (w/w) polyethylene glycol 3350 (SIGMA) and 20% (w/w) Dextran T500 (Amersham Pharmacia) according to Norling et al. (1998). 3.75 g of the membrane sample at a concentration of 0.4 mg Chl/ml was applied to 6.25 g of the polymer mixture (5.8% Dextran T500 and 5.8% polyethylene glycol 3350 in 5 mM potassium phosphate pH 7.8 containing 0.25 M sucrose) and mixed by gently inverting the tubes 35 times at 4°C. A repartition system with the same final concentrations and a second repartition system with a final concentration of 6.2% (w/w) were also prepared. Phase separation was facilitated by centrifugation at 1000 g for 4 min. The upper yellow-green phase was enriched with plasma membranes and the lower dark green phase with thylakoid membranes. They were collected separately and were further purified by applying to the repartitioning system: the lower thylakoid phase was applied four times to the 5.8% upper repartition phase; the upper plasma membrane phase was applied two times to the 5.8% lower repartition phase and three more times to the 6.2% repartition phase.

Membrane treatment

To analyze the nature of the association between the native SufR protein and the membranes, the latter were resuspended in 50 mM Tris/HCl buffer pH 8.0 containing the following reagents: 1M NaCl; 10 mM DTT; 10 mM EDTA; and 0.05%, 01%, and 0.4%
Triton X-100, and incubated on ice for 30 min. After treatment, the samples were centrifuged for 5 min at full speed in a standard tabletop centrifuge. The supernatants were collected and transferred into fresh tubes. The membrane pellets were resuspended in the same volume of 50 mM Tris/HCl buffer pH 8.0. Both the supernatants and the resuspended pellets were loaded onto SDS-PAGE gels.

Methods for antibody production, immunoblotting analysis, RNA isolation, and RT-PCR reaction were as described in Chapter 2.
RESULTS

Identification and localization of the SufR protein by immunoblot analyses

To study if SufR protein homologs exist in other photosynthetic organisms, whole cells or chloroplasts from the cyanobacteria Synechocystis sp. PCC 6803, Synechococcus sp. PCC 6301, Synechococcus sp. PCC 7002, the green alga Chlamydomonas reinhardtii, and the higher plant Spinacia oleracea L. were subjected to immunoblotting assay using polyclonal antibodies raised against the SufR protein of Synechocystis sp. PCC 6803. Cross-reactions could be detected in all the tested organisms (data not shown), indicating that SufR protein is found in other photosynthetic organisms.

To identify the subcellular localization of the native SufR protein in the cyanobacterial cells, the Synechocystis sp. PCC 6803 cells were lysed, and the membranes were separated from the supernatant by centrifugation. Presence of the SufR protein in these two fractions was detected by immunoblot analysis (Figure 3.1A). The majority of the SufR protein (~65%) was in the soluble cytosol fraction of the cell lysates, and about one third was associated with the membranes.

To verify which membrane system (thylakoid or plasma membranes) the SufR protein is associated with, the plasma membranes and the thylakoid membranes were isolated from the Synechocystis sp. PCC 6803 cells by an aqueous polymer two-phase partitioning method. Both membrane samples were applied onto a 12% SDS-PAGE gel on an equal-protein basis. As shown in Figure 3.1B, the membrane-associated SufR protein was detected only from the thylakoid membrane sample and none was found in the plasma
membranes. This result clearly indicates that the membrane-bound SufR protein in cyanobacteria is associated with the thylakoid membranes.

*Nature of interaction of the SufR protein with thylakoid membranes*

The nature of the association between the native SufR protein and the membranes was investigated by washing the membranes with high salt, different concentrations of detergent Triton X-100, EDTA, and dithiothreitol (DTT). The membranes were then isolated by centrifugation, and the resistance of the membrane-bound SufR protein to each of these treatments was determined by immunoblot assay, which compares the amounts of membrane-associated and released soluble SufR protein. The results are presented in Figure 3.2. In panel A, each lane was loaded with the membranes after treatment. The SufR protein released from the membranes was loaded onto a second gel (Figure 3.2B). As shown in lane 2, the SufR protein could not be removed from the thylakoids by high salt treatment (1M NaCl), suggesting that SufR is tightly associated with the membranes. SufR was also resistant to 10 mM EDTA treatment (lane 3), indicating that the association between the SufR protein and the membranes is not mediated by membrane-bound polyribosomes. This result is confirmed by another immunoblot assay showing that RNase could not release the SufR protein from the membranes (data not shown). By contrast, more SufR protein was released from the membranes with increasing concentrations of the detergent Triton X-100 (lane 5, 6, and 7). More than 80% of the membrane-associated SufR protein was released to the wash buffer by 0.4% Triton X-100 (in lane 7). This result demonstrates that SufR is a peripheral protein, and its association with the thylakoids is partly mediated by hydrophobic
interactions. The membrane-associated SufR protein was obviously sensitive to chemical reduction. As indicated in lane 4, some SufR protein was removed from the membranes with inclusion of 10 mM DTT in wash buffer, suggesting that the membrane association of SufR is also mediated by the formation of a mixed disulfide bond with a membrane-associated protein. This issue was further examined by treating the membranes with DTT at various concentrations (Figure 3.2C). Although DTT at higher concentrations could remove more SufR protein from the membranes than could DTT at low concentrations, 100 mM DTT treatment did not significantly enhance the release of more SufR protein from the membranes than 20 mM DTT (Figure 3.2C), confirming that the membrane association of SufR is mediated by at least two combined forces.

Transcription of the sufR gene under stress conditions

It has been shown that transcription of the sufBCDS genes in Synechococcus sp. PCC 7002 is up-regulated under various stress conditions. Since SufR is located in both the cytosol and the thylakoid membranes, and since it is known to be a repressor of the suf operon, it is interesting to know how the sufR gene itself is expressed in cells grown under stress conditions. Figure 3.3A presents the RT-PCR data on the transcription levels of sufR in the Synechococcus sp. PCC 7002 cells grown under various stress conditions. Surprisingly, the sufR gene exhibited an expression profile similar to the sufBCDS genes. Transcription of the sufR gene was up-regulated under all stress conditions (lane 2-5) compared with standard growth conditions (lane 1). The transcription of the sufR gene was further investigated in Synechocystis sp. PCC 6803 grown under another series of stress conditions.
conditions, such as high light (lane 2), low temperature (lane 3), hydrogen peroxide (lane 4), and iron limitation (lane 5). As indicated by Figure 3.3B, the mRNA levels of the sufR gene under these stress conditions were also elevated albeit to different degrees.

*Expression of SufR protein under stress conditions*

The *Synechocystis* sp. PCC 6803 wild-type cells were grown at different light intensities and treated with DCMU (an inhibitor of PS II activity through binding at PS II Q$_b$ site) or DBMIB (an inhibitor to cytochrome b$_6$f complex). The level of the SufR protein in the whole cell lysates and the membrane fractions was investigated by immunoblot assay. The results are presented in Figure 3.4. Compared to cells grown under low light conditions (lane 1), the expression level of the SufR protein increased in cells growing in the presence of DCMU (lane 3) or DBMIB (lane 2) (Figure 3.4, upper panel). The protein level of SufR also increased slightly in cells grown under high light conditions (lane 4). However, the SufR protein associated with the membranes did not show significant changes under all of these conditions (Figure 3.4, lower panel), indicating that the cytosolic SufR protein contributed to the increase in the overall protein level.
DISCUSSION

The SufR protein is partitioned between the cytosol and the thylakoid membranes in *Synechocystis* sp. PCC 6803. This two-compartment distribution phenomenon is also found for other gene regulators, such as the light-regulatory protein RB60 from *C. reinhardtii*, which is a major protein component of the *psbA* 5’-PC complex that binds to the *psbA* mRNA 5’-UTR (Trebitsh et al., 2000; 2001). RB60 might be a bifunctional protein because it also exhibits homology to the PDI (protein disulfide isomerase) proteins.

Another example for a bipartitioning protein is PutA. *putA* and *putP* compose a short proline utilization operon (*put*) in *E. coli* and *Salmonella typhimurium* and allow these bacteria to use proline as a sole source of carbon, nitrogen and energy (Maloy et al., 1987). PutP is a membrane protein that functions as the proline permease. PutA functions as a dehydrogenase that catalyzes the oxidation of proline to glutarate when it is associated with the membrane (Menzel et al., 1981; Brown et al., 1992). In the absence of proline, the cytosolic PutA protein can bind to the DNA and act as an autogenous transcriptional repressor of the *put* operon. Depending on the availability of proline, PutA shuttles between the membrane and the cytoplasm to adjust its function. In addition, the functional transition of the PutA protein may be further modulated by phosphorylation/dephosphorylation. It was reported that the PutA protein from the bacterium *Salmonella typhimurium* became auto-phosphorylated on several threonine, serine, and tyrosine residues. The dephosphorylated PutA protein had a higher DNA binding affinity than the phosphorylated protein and thus may prevent toxic overexpression of the PutA protein in the absence of available membrane sites (Ostrovsky et al., 1995).
The SufR and PutA proteins show interesting similarities in several aspects. Both proteins are partitioned between the membrane and the cytosol and function as transcription repressors. The SufR protein contains one putative protein kinase C phosphorylation site at the N-terminus. Although the role of this phosphorylation site in SufR remains to be addressed, some weak but clear bands with size slightly larger than the major SufR protein band were detected in our immunoblotting studies (see Figure 3.1 and Figure 3.4). These bands may represent a small amount of phosphorylated SufR proteins. In addition, the putative phosphorylated SufR protein seems to exist mainly in the cytoplasm (comparing membrane and supernatant in Figure 3.1 and Figure 3.4). These similarities between SufR and PutA strongly suggest that SufR is also a bifunctional protein, with transcriptional repression in the cytoplasm and an unknown function on the thylakoid membranes. However, mechanisms of their functional adjustment in response to environmental changes may be very different. Unlike PutA that can shuttle between the cytoplasm and the membrane depending on the availability of proline, the membrane-bound SufR protein level did not show significant changes under various stress conditions. The association between SufR and the thylakoid membrane may be mediated by protein-protein interaction in addition to the protein-lipid hydrophobic interaction that is apparently the sole determinant for the membrane association of PutA (Surber et al., 1999). Therefore, a transition between the cytoplasm and the thylakoid membranes may not be a strategy used by SufR in response to stress conditions. In other words, functional adjustment, under different conditions, may only be operable on the cytosolic SufR protein. This can be accomplished by potential phosphorylation/dephosphorylation of the protein. However, as will be shown in the next
chapter, a major strategy for sensing stress conditions appears to be mediated by the presence or absence of Fe-S cluster on the SufR protein.

An increased expression at both transcriptional and translational levels under different stress conditions suggests that sufR may be subjected to autogenous regulation. That is, transcription of sufR is directly controlled by its product, the SufR protein. The rationale for the autogenous regulation of SufR is that under stress conditions, expression of sufR increases and the SufR protein competes for Fe-S clusters with other Fe-S proteins. The resulting increase of holo-SufR protein gradually shuts off the expression of itself and members of its regulons. Many transcription factors are known to regulate their own expression. An analogous system to SufR in this respect is the IscR protein that is a transcription repressor of the iscRUSA-hscBA-fdx gene cluster in E. coli (Schwartz et al., 2001). Both IscR and SufR are Fe-S proteins, and they regulate expression of components of two Fe-S assembly pathways, i.e. ISC and SUF respectively. Other examples include lacI repressor protein (Ptashne et al., 1992; Maloy et al., 1993), cAMP receptor protein (Aibia et al., 1983; Okamoto et al., 1986), Arg repressor (Tian et al., 1993), Ilv repressor (Wek et al., 1988), Trp repressor (Kelley et al., 1982), Met repressor (Maxon et al., 1989), Pur repressor (Meng et al., 1990; Rolfes et al., 1990), Cyt repressor (Gerlach et al., 1990), replication termination protein (Natarajan et al., 1991) in E. coli, Eut activator (Roof et al., 1992) in Salmonella typhimurium, and PagR repressor in Bacillus anthracis (Hoffmaster and Koehler, 1999) et al.. In addition, many enzymes that catalyze essential metabolic reactions in bacteria also regulate their own expression, such as the above-mentioned PutA protein; threonyl-tRNA synthetase (ThrS) that represses it own translation by binding to its mRNA.
(Romby et al., 1990); and biotin-protein ligase (BirA) whose expression is repressed by BirA-biotinoyl-AMP repressor complex that binds to the birA operator (Barker et al., 1981; Prakash et al., 1979). At present, there is no direct evidence supporting the hypothesis that SufR is an autogenous regulatory protein. Further study is therefore needed to address the regulation of SufR expression. The elucidation of the function of the membrane-associated SufR protein would also help to understand this issue.
REFERENCES


Ostrovsky, P. C., and S. Maloy. 1995. Protein phosphorylation on serine, threonine, and


gene encoding a membrane-associated rubredoxin in the cyanobacterium


FIGURE LEGENDS

Figure 3.1 Localization of the SufR protein in cyanobacterial cells. A) Immunoblotting analysis of soluble and membrane proteins of *Synechocystis* sp. PCC 6803 against the SufR protein antibody. For comparison, different amounts of proteins from the cytosol and the membranes were loaded. B) Localization of the SufR protein between thylakoid membranes and plasma membranes by immunoblot assay. For comparison, the same amount of protein (20 µg) was loaded on each lane. The molecular mass of protein markers (kDa) (Kaleidoscope Polypeptide Standards from BioRad) is indicated on the left.

Figure 3.2 Association of the native SufR protein with the thylakoid membranes of *Synechocystis* sp. PCC 6803. A) thylakoid membrane pellets after washing; B) supernatants after washing. Sample loading sequence in panel A and B: 1) control; 2) 1 M NaCl; 3) 10 mM EDTA; 4) 10 mM DTT; 5) 0.05% Triton X100; 6) 0.1% Triton X100; 7) 0.4% Triton X100; 8) 0.05% Triton X-100 + 1M NaCl. C) Treatment of the thylakoid membranes with DTT of different concentrations. 1) 20 mM; 2) 50 mM; 3) 100mM. Arrows point to the position of SufR protein band.

Figure 3.3 RT-PCR analysis of the expression of the *sufR* gene under various conditions. A) mRNA levels of the *sll0088* gene (*sufR*) in *Synechocystis* sp. PCC 6803 cells grown under: 1) standard growth conditions; 2) high light conditions (250 µE m⁻² s⁻¹); 3) low temperature (22°C); 4) standard growth conditions with addition of 5 mM H₂O₂; 5) iron starvation conditions. B) mRNA levels of the *sufR* gene in *Synechococcus* sp. PCC 7002 cells grown
under: 1) standard growth conditions; 2) standard growth conditions with addition of 10 µM DBMIB; 3) standard growth conditions with addition of 0.5 µM DCMU; 4) standard growth conditions with addition of 150 µM H₂O₂; 5) iron starvation conditions.

**Figure 3.4** Immunoblot analysis of the SufR protein in *Synechocystis* sp. PCC 6803 whole cells grown under different conditions and in membranes isolated from these cells. 20 µg protein was loaded on each lane. The loading sequences are: 1) cells grown under standard light conditions (50 µE m⁻² s⁻¹); 2) under standard growth conditions with addition of 10 µM DBMIB; 3) under standard growth conditions with addition of 0.5 µM DCMU; 4) under high light conditions (250 µE m⁻² s⁻¹);
Figure 3.1

A

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<tr>
<td>10µg</td>
<td>5µg</td>
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</table>

B

thylakoid  plasma
Figure 3.2

A

29.9 kDa

B

29.9 kDa

C

1 2 3

membrane

1 2 3

supernatant
Figure 3.3

A

\[
\begin{array}{cccccc}
\text{sufR} & 1 & 2 & 3 & 4 & 5 \\
\end{array}
\]

16S rRNA

Synechococcus sp. PCC 7002

B

\[
\begin{array}{cccccc}
\text{sufR} & 1 & 2 & 3 & 4 & 5 \\
\end{array}
\]

\[
\begin{array}{cccccc}
\text{rnpB} & 1 & 2 & 3 & 4 & 5 \\
\end{array}
\]

Synechocystis sp. PCC 6803
Figure 3.4

1        2          3        4

whole cell

membrane
Chapter 4. SufR Is a [4Fe-4S] Cluster Protein Ligated by Cysteine Residues in a Unique CX$_{12}$CX$_{13}$CX$_{14}$C Motif

ABSTRACT

SufR functions as a transcriptional repressor of the suf genes in the cyanobacterium *Synechococcus* sp. PCC 7002. It contains four cysteine residues in a unique CX$_{12}$CX$_{13}$CX$_{14}$C motif that is well-conserved within cyanobacteria. Three of the four cysteines are also conserved in SufR homologs from other organisms. The functional role of SufR under oxidative stress conditions and the arrangement of the four cysteine residues imply that SufR may be an Fe-S protein that uses the cluster as a sensor of stress conditions. EPR spectroscopy showed that reconstituted recombinant SufR protein was able to assemble a stable [4Fe-4S] cluster. Mössbauer spectroscopy confirmed that reconstituted SufR is a [4Fe-4S]$^{2+}$ protein in its oxidized form. In subsequent studies, each of the four cysteine residues was substituted for Ser. C191(3rd Cys) and C206 (4th Cys) were also mutated to Gly. Two double mutants, C191G/C206G and C177S (2nd Cys)/C206S were also engineered. All the Ser mutants contained an Fe-S cluster with different spectral features and g-tensors from that of the wild-type, but with the spin relaxation properties of a [4Fe-4S] cluster. The two Gly mutants also contained an Fe-S cluster but the g-tensors were more similar to the wild-type than the Ser mutants. The C191G/C206G double mutant contains an Fe-S cluster with EPR properties similar with that of the C206G single mutant. In contrast, no EPR signal could be detected in the reconstituted C177S/C206S mutant. Interestingly, all of the mutants except C177S/C206S showed a minor population of a [3Fe-4S] cluster. These results strongly suggest: 1) that SufR harbors an Fe-S cluster in the native state, and 2) that
it is present as a [4Fe-4S] cluster. We have found that the reconstituted SufR protein has a mass of approximately 50,000 kDa by gel filtration chromatography performed under anaerobic conditions. Since the predicted mass of SufR is 25,020 kDa when the Fe-S cluster is present, the SufR protein appears to be a dimer in the native state. The dimeric form was also observed on a non-denaturing protein gel. It is proposed that SufR represents a new class of transcriptional repressor that contains a [4Fe-4S] cluster coordinated by cysteine ligands in a unique cysteine motif, and that it plays an indirect role in controlling PS I biogenesis/assembly by regulating the $sufBCDS$ gene expression for the control and synthesis of Fe-S clusters.
INTRODUCTION

Significant progresses in Fe-S cluster assembly studies have been made in recent years. Three major Fe-S cluster assembly machineries have been identified, namely NIF, ISC, and SUF. Components involved in these pathways have also been studied in various organisms and their functions in the poorly-understood Fe-S cluster biosynthetic process are under active investigation. Because of the intrinsic relationship between iron and oxidative stress, free iron levels in living cells are extremely low and thus have to be finely regulated. Accordingly, the Fe-S cluster assembly machinery must also be well-controlled. Interestingly, regulators involved in the regulation of the Fe-S cluster assembly machineries have been found for the three bacterial Fe-S cluster pathways. The NIF machinery is specific for the maturation of the Fe proteins and the MoFe proteins in nitrogenase. However, nif genes do not specifically refer to those encoding components of the NIF machinery. There are more than twenty nif genes that are organized in several operons. In the free-living diazotrophs, such as Klebsiella pneumoniae and Azotobacter vinelandii, products of a nifLA operon regulate transcription of all other nif genes, including nifU and nifS. NifA activates transcription of the nif genes by the alternative form of RNA-polymerase, σ54-holoenzyme. The negative regulator, NifL modulates the activity of the transcriptional activator NifA in response to the presence of combined nitrogen and molecular oxygen. NifL binds an FAD (flavin adenine dinucleotide) at its N-terminal domain as a redox-sensitive cofactor (Schmitz et al., 2002).

Transcription of the iscRSUA-hscBA-fdx gene cluster in E. coli is regulated by the [2Fe-2S] IscR protein. Inactivation of iscR led to an increase in the expression of the isc
operon, suggesting that IscR functions as a transcriptional repressor. The [2Fe-2S] cluster appears to be important for IscR because auto-regulatory repression of the iscR expression is significantly reduced in strains containing null mutations of the Fe-S cluster assembly genes iscS or hscA. These findings led to the formulation of a feedback regulation model that suggests that IscR may be part of an auto-regulatory mechanism that senses the Fe-S cluster assembly status of cells (Schwartz et al., 2001).

The sufABCDSE operon in *E. coli* is up-regulated by OxyR (Lee et al., 2004; Outten et al., 2004; Zheng et al., 2001), IHF (integration host factor) (Lee et al., 2004; Outten et al., 2004), and an unknown transcription factor (Lee et al., 2004) in response to oxidative stress. OxyR is the global regulator of cellular response to H₂O₂ stress. IHF acts as the regulator of several stress response genes that are important for the cellular adaptations upon entering the stationary phase (Altuvia et al., 1994). The *cis*-acting elements for these three oxidant-responsive transcription regulators have been identified (Lee et al., 2004; Outten et al., 2004). Transcriptional induction of the *suf* operon under iron limiting condition is mediated by Fur, the global transcription regulator for iron metabolism in *E. coli* (Outten et al., 2004) and in *Erwinia chrysanthemi* (Nachin et al., 2001). A DNA microarray study confirms that the *suf* operon is part of the Fur regulon in *E. coli* (McHugh et al., 2003). Recently, it was found that the cyanobacterial *sufBCDS* operon was negatively regulated by the SufR protein. A comparison of the transcription levels of the *suf* genes in the wild-type and the *sufR* null mutant of *Synechococcus* sp. PCC 7002 growing under various stress conditions clearly demonstrated that the *suf* genes were regulated by SufR under oxidative stress conditions. The question is: how does the SufR protein sense the redox states in cells? As indicated by
the amino acid alignment (Figure 2.1), four cysteines at the C-terminus are arranged in such a manner that two neighboring cysteines are separated by 12, 13, and 14 amino acid residues respectively. Such an arrangement, together with its transcription repression function under oxidative stress and the presence of a DNA binding motif in the protein, suggest that SufR is a metal-binding protein and may use an Fe-S cluster as the sensor. Fe-S clusters are widely used by biological systems for sensing and regulatory purposes. In addition to IscR, several other proteins also use Fe-S clusters to adjust their functions. Two of them, SoxR and FNR, are also transcriptional regulators. The DNA binding activity of SoxR is controlled by the oxidization state of its \([2\text{Fe-2S}]^{1+/2+}\) cluster. FNR activates expression of over 100 genes in response to oxygen limitation. Its ability to act as a global regulator depends on the integrity of its \([4\text{Fe-4S}]\) cluster. In this chapter, it is shown that the recombinant SufR protein is able to assemble a \([4\text{Fe-4S}]\) cluster. Amino acid substitution studies indicate that replacement of the third cysteine residue (Cys191) results in little change in EPR signal, suggesting that it may not participate in ligation of the Fe-S cluster in the SufR protein. This is consistent with the finding that Cys191 is not conserved in SufR homologs from organisms other than cyanobacteria (Figure 2.14). The physiological role of the \([4\text{Fe-4S}]\) SufR protein is discussed.
MATERIALS AND METHODS

Site-directed mutagenesis

The annotated DNA sequence of the open reading frame sll0088 (hereafter renamed sufR-240, and SufR-240 for the protein) in Cyanobase (http://www.kazusa.or.jp/cyanobase/) contains two closely-spaced ATG start codons near the beginning of the ORF. Based on a comparison with the SufR sequences from other cyanobacteria (Figure 2.1, Chapter 2), it is likely that the authentic start codon is the second ATG. Therefore, the SufR protein of Synochocystis sp. PCC 6803 is likely to be 217 amino acids in length instead of 240. To obtain the authentic SufR protein (hereafter named SufR-217), an NdeI restriction site was introduced to the second ATG codon by PCR using the pET24a/sll0088 plasmid (Chapter 2) as the template and S88EcufF and S88EcufR (Table 4.1) as the primers. A 72-bp DNA sequence at the 5’ end of the annotated sufR-240 gene was deleted by incubating the PCR product with restriction enzyme NdeI. The truncated plasmid (renamed pET24a/sufR) was transformed into BL21(DE3) for production of the true SufR protein. pET24a/sufR was thereafter used as the template for subsequent amino acid substitution manipulations. All PCR reactions were performed by using Hotstart pfu DNA polymerase under experimental conditions recommended by the manufacturer (Stratagene, La Jolla, CA). Primers for site-directed mutagenesis are listed in Table 4.1. After PCR, DpnI was added into the reaction mixture to digest the wild-type plasmids used as the template. 2 µl reaction mixture containing mutated sufR gene was directly used for transformation of the E. coli strain DH10B. All mutations were verified by DNA sequencing conducted at Nucleic Acid
Facilities of Pennsylvania State University. Double mutants were created by PCR using primers containing one of the mutation and plasmid template containing the other mutation.

Overexpression and purification

The wild-type and mutant SufR proteins were expressed in *E. coli* BL21(DE3) and purified as described in Chapter 2, except that the column for size exclusion chromatography was changed to Sephacryl S-300. Fractions containing purified SufR protein were collected and stored at −80°C in the presence of 20% (v/v) glycerol until further use.

Reconstitution of iron-sulfur cluster in the SufR protein

Solubilization of the inclusion bodies with urea under aerobic conditions leads to disassembly of any iron-sulfur clusters that may be present in the SufR protein. To reconstitute an anticipated iron-sulfur cluster, the apo-SufR protein was treated with ferric chloride, sodium sulfide, and 2-mercaptoethanol according to a method devised for PsaC (Mehari et al., 1991). 50 mM Tris/HCl buffer (pH 8.3) was prepared by heating it to boil and then cooling it to 45°C with constant purging of nitrogen gas during the entire process. The buffer was then transferred quickly into anaerobic chamber (Coy Products, Grass Lake, MI) and allowed to further cool to the room temperature. For reconstitution studies, 15 mg SufR protein was added into 50 ml 50 mM Tris/HCl buffer (pH 8.3) containing 9 mM FeCl₃, 9 mM Na₂S, and 10 mM 2-mercaptoethanol. The mixture was kept under anaerobic condition at 4°C for no more than 16 hours. The reconstituted SufR protein was concentrated by
ultrafiltration to 2.5 ml and loaded into a PD-10 column (Amersham Pharmacia Biotech, Piscataway, NJ) to remove free iron. The concentration of SufR holo-protein was measured and adjusted to 20 mg/ml. The SufR protein was distributed into 400 µl aliquots in 1.5 ml Eppendoff tubes, capped and wrapped with parafilm, frozen in liquid nitrogen, and transferred out of the anaerobic chamber and stored at -80°C until further use. For EPR studies, the reconstituted SufR protein was reduced with 20 mM sodium dithionite after adjusting the pH to 10.5 by addition of anaerobic 1M 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer or 1M glycine buffer, pH 10.5. To reconstitute the $^{57}$Fe-S cluster for Mössbauer studies, one-half of the amount of Fe in the standard reconstitution was used (4.5 mM compared to 9 mM). About 20 µl of 240 mM $^{57}$FeSO$_4$ was added to each vial containing ~50 ml of the reconstitution mixture. All other procedures were the same for EPR studies.

Preparation of $^{57}$Fe-enriched ferric sulfate solution

$^{57}$Fe (18 mg; 0.315 mmol; >95% isotopic enrichment) was converted to $^{57}$FeSO$_4$ by dissolving the metal foil in 0.8 ml 1 M H$_2$SO$_4$ at 100°C in a silicone oil bath. After the $^{57}$Fe had completely dissolved, saturated sodium bicarbonate was added to the $^{57}$Fe solution to adjust the pH to ~6.0. The final concentration of $^{57}$Fe was 0.239 M. An appropriate amount of this $^{57}$FeSO$_4$ solution was used directly for Fe-S cluster reconstitution.
Spectroscopic methods

UV-visible absorption spectra were recorded using GENESYS™ 10 series UV-visible recording spectrophotometer (Thermo Spectronic, Rochester, NY). The EPR spectrum was recorded using a Bruker ECS106 EPR X-band (9.2 GHz) spectrometer operating with an ER 4012 ST resonator and an Oxford liquid helium cryostat. The microwave frequency was determined with a Hewlett-Packard 5340A frequency counter. To measure the spin concentration in the reconstituted wild-type SufR protein, cupric sulfate of different concentrations was used as the standard. EPR signals of the cupric sulfate and the reconstituted SufR protein were recorded under exactly the same conditions except that microwave power was reduced to non-saturating conditions. The spin concentration of the SufR protein was calculated by comparing its double integrated EPR signal intensity against a standard curve of double integrated intensity of cupric ion. Mössbauer spectra were recorded on spectrometer from WEB research (Edina, MN) operating in the constant acceleration mode in a transmission geometry. Spectra were recorded with the temperature of the sample maintained at 4.2 K. For low-field spectra, the sample was kept inside a SVT-400 dewar from Janis (Wilmington, MA), and a magnetic field of 40 mT was applied parallel to the beam. For high-field spectra, the sample was kept inside a 12SVT dewar (Janis, Wilmington, MA), which houses a superconducting magnet that allows for application of variable magnetic fields between 0 and 8 T parallel to the beam. The isomer shifts quoted are relative to the centroid of the spectrum of a metallic foil of Fe at room temperature. Data analysis was performed using the program WMOSS from WEB research.
**Gel filtration chromatography under anaerobic condition**

Many DNA-binding proteins form homodimers as the active form. Immunoprecipitation analysis did not detect any protein that co-precipitated with the SufR protein (data not shown). To determine whether SufR (mutants and wild-type) is a homodimeric protein, the reconstituted SufR proteins were subjected to gel filtration analysis under anaerobic conditions. The experiment was conducted using an Akta FPLC system adapted to an anaerobic chamber at the Fermentation Facility of Pennsylvania State University. A Superose 6 column equilibrated with 50 mM Tris/HCl buffer (pH 8.3) was applied at a flow rate of 0.5 ml/min. The Tris/HCl buffer was prepared in a same manner as for Fe-S cluster reconstitution. Carbonic anhydrase, bovine serum albumin, α-amylase, and cytochrome c were used as standards. Blue dextran was used to determine the void volume of the column.

**Non-denature PAGE**

A native protein gel was prepared according to Laemml (1970) except that SDS was not added into all solutions. An 8% separating gel was overlaid with a 4% stacking gel. About 30 µg protein was loaded onto each lane. Carbonic anhydrase, bovine serum albumin, and cytochrome c were used as the protein standards. Electrophoresis was run at 4°C in a cold room at 120 volts for 5 hours. The gels were stained with Comassie Brilliant Blue.
**Protein quantitation**

The method for measuring protein concentrations was as described in Chapter 2. Because of possible overestimation of protein concentration when bovine serum albumin is used as standard (Li et al., 1991), IgG was used to make the calibration curve.

**Chemical analysis**

The Fe content was measured by the method of Beinert (1978) with a minor modification. Ferene (5,5'(3-(2-pyridyl)-1,2,4-triazine-5,6 diyl)-bis-2-furansulfonic acid) was used as the coloring reagent instead of bathophenanthroline disulfonate. Iron was quantitated by measuring the absorbance at 593 nm, and the absorbance was compared to a standard curve made with FeCl₃ solutions at different concentrations from 100 μM to 40 mM. The labile sulfide content was measured using a procedure from Golbeck and Kok (1978). Na₂S was used as a standard solution and the absorbance was measured at 595 nm.
RESULTS

Purification and characterization of the SufR-217 protein

All the wild-type and mutant SufR proteins reported in this study are listed in Table 4.2. As described in Materials and Methods, a shorter SufR protein (SufR-217, 217 amino acids) from *Synechocystis* sp. PCC 6803 was obtained by truncation of the N-terminal 24 amino acid residues from the mis-annotated SufR-240 protein (240 amino acids). Similar to the SufR-240 protein, very little truncated SufR-217 protein was present in the soluble fraction. Instead, the majority of the protein was expressed as inclusion bodies, which were light brown in color, indicating that the protein might harbor an iron-sulfur cluster. The inclusion bodies were solubilized in 50 mM Tris/HCl buffer (pH 8.3) containing 8 M urea, and 10 mM dithiothreitol (DTT), and the truncated SufR-217 protein was purified by size exclusion chromatography. After purification, the SufR-217 protein fractions were colorless. The SufR-217 protein had an apparent mass of ~24 kDa on SDS-PAGE, and it migrates faster than the previously-prepared SufR-240 protein (Figure 4.1A). This measured mass is in close agreement with the predicted mass of 24.6 kDa determined from the DNA sequence, suggesting that the N-terminal 24 amino acids were successfully truncated. In addition, truncation of the highly hydrophobic N-terminal 24 amino acid sequence facilitated purification by size exclusion chromatography. The purity of the truncated SufR-217 protein was higher than the SufR-240 protein after passage through the Sephacryl S-300 column (data not shown).
UV-visible absorption spectroscopy of the wild-type SufR-240 protein

The SufR-240 protein was used for Fe-S cluster reconstitution before the truncated SufR-217 protein became available. The UV-visible spectrum of the reconstituted wild-type SufR-240 protein shows absorbance in the UV and visible region, with maxima at 280, 320 and 411 nm (Figure. 4.2A). The peaks at 320 nm and 411 nm are typical of the broad S -> Fe charge-transfer transitions found in iron-sulfur proteins. The A_{411}/A_{280} ratio of 0.29 is within the usual range for a typical iron-sulfur protein (Aspinwall et al., 1997; Ikeda et al., 1998). The absence of absorption peaks in the 450 and 550 nm regions is more characteristic of a [4Fe-4S] cluster than a [2Fe-2S] cluster (Duin et al., 1997; Ugulava et al., 2001). Similar to other iron-sulfur proteins, the spectrum lost about one-half of its amplitude after reduction with sodium hydrosulfite. When air-oxidized, the spectrum lost most of its absorbance in the 320 to 420 nm region (Figure. 4.2A, inset), suggesting that the iron-sulfur cluster is oxygen-sensitive.

EPR spectroscopy of the wild-type SufR-240 protein

Figure 4.2B shows the low-temperature, X-band EPR spectrum of the reconstituted SufR-240 protein after chemical reduction with dithionite. The axial spectrum of the reduced sample, with a low-field peak at g = 2.02 and a high-field trough at g = 1.89, is characteristic of proteins that contain an iron-sulfur cluster. The spectrum and the high-spin relaxation rate implied from the microwave power and temperature dependence (Figure. 4.2B, inset) and the lower amplitude of the signal at temperatures above 20 K (Figure. 4.2B, inset) are consistent with the presence of a [4Fe-4S] cluster. The cluster is stable for days in
either the oxidized or reduced state when the protein is maintained under anaerobic conditions. The truncated, wild-type SufR-217 protein shows a very similar EPR spectrum (compare Figure 4.2B and Figure 4.4 & 4.5). Indeed, their EPR properties are indistinguishable from each other, indicating that removal of N-terminal 24 amino acid sequence has no effects on the EPR spectrum of the iron-sulfur cluster.

*Mössbauer spectra of the wild-type SufR-217 protein*

Mössbauer spectra of the oxidized SufR-217 protein are presented in Figure 4.3. The spectrum in Figure 4.3A was recorded at 4.2 K in a low magnetic field of 40 mT applied parallel to the $\mathbf{g}$ radiation. It exhibits a quadrupole doublet, with apparent quadrupole splitting, $\Delta E_Q = 1.16$ mm/s, and isomer shift, $\delta = 0.44$ mm/s. The spectrum in Figure 4.3B was recorded at 4.2 K in a high magnetic field of 8 T applied parallel to the observed $\mathbf{g}$ radiation. The solid line overlaid with the experimental spectrum is a computer simulation with the parameters $\Delta E_Q$ and $\delta$ obtained from Figure 4.3A as well as asymmetry parameter $h = 0.64$ determined from Figure 4.3B and assuming that the environments of all iron sites are diamagnetic ($S = 0$). The theoretical curve and the experimental data are in good agreement.

The values of isomer shift and quadrupole splitting as well as the diamagnetism are typical of $[4\text{Fe-4S}]^{2+}$ clusters. Therefore Mössbauer spectroscopy clearly indicates that reconstituted SufR is a $[4\text{Fe-4S}]^{2+}$ protein in its oxidized form. It can be reduced to $[4\text{Fe-4S}]^{1+} (S = 1/2)$ by dithionite as shown by EPR spectroscopy. Mössbauer spectra also indicated that ca $91 \pm 3\%$ of the total iron in the reconstituted SufR protein sample is in the
form of a [4Fe-4S]$^{2+}$ cluster. Therefore, both the oxidized (Mössbauer) and reduced (EPR) form of the SufR protein harbors a [4Fe-4S] cluster.

**EPR characterization of the mutant SufR proteins**

To investigate further the ligands involved in Fe-S cluster ligation and to confirm that a [4Fe-4S] cluster is present in SufR, we introduced Cys->Ser substitutions to each of the four cysteine residues and Cys->Gly substitutions in positions Cys191 and Cys206. In some cases, two cysteine residues were substituted for either Ser or Gly to construct double mutants. As a result, a total of eight mutants were engineered, C164S (C1S), C177S (C2S), C191S (C3S), C206S (C4S), C191G (C3G), C206G (C4G), C177S/C206S (C2S/C4S), and C191G/C206G (C3G/C4G) (see Table 4.2). The reason to choose Ser as the replacement amino acid residue is its structural similarity to Cys and that Ser can potentially provide an oxygen ligand to Fe-S clusters *in vivo* or *in vitro*. The reason to choose Gly is that it may disrupt the ability of SufR to reconstitute Fe-S cluster.

The EPR spectra were recorded with microwave powers ranging from 0.1 mW to 126 mW at temperatures from 4 K to 45 K. As shown in Figure 4.4, all the reconstituted mutant SufR proteins, except for the C177S/C206S double mutant, show a nearly axial lineshape of an Fe-S cluster with different g-tensors. C177S displays an $S = 1/2$ EPR signal ($g = 2.05, 1.84$) with the greatest anisotropy among all mutants. C206S ranks second with respect to unusual g-tensor ($g = 2.05, 1.86$). C164S has an EPR signal ($g = 2.05, 1.89$) with linewidths comparable to those of the wild-type SufR protein. Its g-value anisotropy is smaller than C177S and C206S, but is still significantly larger than that of the wild-type.
C191S exhibits a complex EPR signal with g values of 2.02, 1.96, 1.93 and 1.92. In all the Ser mutants except C191S, the EPR spectra extend to both lower fields and higher fields compared to the wild-type protein. The EPR signal of C191S falls within the same field range as the wild-type. The two Gly mutants also contain an Fe-S cluster. The EPR signal of C206G is obviously shifted to lower fields compared to the wild-type. Its g-value anisotropy is comparable to that of C164S. Similar to C191S, C191G exhibits a g-value anisotropy and lineshape similar to the wild-type. Interestingly, the spectrum of the C191G/C206G double mutant is almost superimposable with that of C206G. By comparison, the dithionite-reduced C177S/C206S double mutant is EPR silent. In general, the g-tensor of the Gly mutants is more similar to the wild-type than the Ser mutants. This can be understood if the 2-mercaptoethanol serves as a ‘rescue’ ligand, substituting for a Cys at the mutated position, as is the case for the reconstituted PsaC mutant proteins (Jung et al., 1996). In general, mutations that occurred at Cys177 and Cys206 result in more variations in spectra properties than the wild-type. Mutations with substitution at Cys164 and Cys191 exhibit similar EPR signals to the wild-type. All of the mutants have fairly equivalent, rapid-spin relaxation properties consistent with a [4Fe-4S] cluster (see Figure 4.5). In particular, [4Fe-4S] clusters show the strongest signals at 10-20 K and are usually unobservable above 45 K. In addition, all of the mutants except the C177S/C206S double mutant show a minor population of a [3Fe-4S] cluster in oxidized samples (data not shown). The spin concentrations of the [3Fe-4S] clusters are negligible, however, compared to those of the [4Fe-4S] clusters.
The ratio of [4Fe-4S] cluster to reconstituted SufR protein

Concentrations of iron and the labile sulfide in the reconstituted SufR proteins were measured. The numbers of irons and the sulfides per polypeptide (Table 4.3) were calculated based on quantitation of the SufR protein by using IgG as the standard. The iron and labile sulfide data are in good agreement for every SufR protein. The numbers of the irons and labile sulfides in the wild-type SufR protein suggest one [4Fe-4S] cluster per two polypeptides. With the exception of the C199G mutant, all other mutant proteins contain less iron and labile sulfide than the wild-type on a per polypeptide basis. The SufR proteins with mutations at Cys199 have more iron and labile sulfide than other mutants, and the double mutant proteins contain significantly less iron and labile sulfide than the single mutants. These results clearly indicate that substitutions of the cysteine residues affect Fe-S cluster reconstitution in these mutant SufR proteins, and replacement of Cys199 with either Ser or Gly has a less detrimental effect than mutations at other positions. The reconstituted C177S/C206S mutant protein has no detectable labile sulfides, which is consistent with the finding that it is EPR silent. Using cupric sulfate as the standard, spin quantitation experiments detected approximately 0.27 spins per polypeptide of wild-type SufR protein, which equals to about one iron and one sulfide per polypeptide (Figure 4.6). This number is one-half of that obtained from the iron and sulfide analysis. It is very likely that either the reconstituted wild-type SufR protein was not completely reduced by dithionite under our experimental conditions or that one-half of the proteins failed to reconstitute.
The SufR protein can form a homodimer

The predicted molecular mass of the SufR-217 protein is 24.6 kDa. As shown by gel filtration analysis (Figure 4.7 and Table 4.4), within error, peaks of the UV at 280 nm corresponding to dimeric SufR-217 protein were observed in the wild-type as well as the Ser mutant SufR-217 proteins. Protein aggregation apparently occurred in all the Gly mutant proteins, as revealed by the large, early peaks compared to none or very weak later peaks. A small amount of protein aggregation was also observed in the Ser mutants. In contrast, both the apo- and holo- wild-type SufR-217 proteins did not form aggregates. The reconstituted wild-type and C191S SufR-217 proteins formed more tetramers or hexamers than dimers. The wild-type apo-protein was found to only exist in a dimeric form. Interestingly, the wild-type holo-SufR-217 run slightly slower than the apo-protein, which is contrary to what is expected if solely based on their predicted molecular masses. A similar phenomenon was also observed for the dimeric C191G mutant protein. Thus, the shape (Stokes radius) of the holo-protein might be different that the apo-protein. Although the vast majority of either apo- or holo- C191G protein aggregated, a small but significant amount was still observable in the dimeric form. The apo-C191G dimer has a calculated mass of 54.2 kDa vs. 47.9 kDa for the holo-C191G dimer. This result suggests that formation of [4Fe-4S] cluster may make the SufR protein more compact in structure.

A dimeric SufR protein was also observed on non-denaturing PAGE gels. As indicated by Figure 4.1B, two bands formed on a native protein gel. The intensity of the upper band is much stronger than the lower band, indicating that under our experimental
conditions, the SufR protein exists mainly in the dimeric form. This result is consistent with the gel filtration experiment.
DISCUSSION

*SufR is a [4Fe-4S] protein*

The reconstituted recombinant SufR protein harbors an iron-sulfur cluster, as shown by optical absorption and EPR spectroscopy. The EPR spectra and spin relaxation properties are consistent with its identification as a [4Fe-4S] cluster. Mössbauer experiments further confirm that SufR is a [4Fe-4S]^{2+/1+} protein. An amino acid substitution study combined with EPR spectroscopy demonstrates that each mutant has a different g-tensor than the wild-type. These differences seem to be related more to the position than to the replacement amino acid. Substitutions at Cys177, Cys206, and Cys164 affect the EPR signals more than those at Cys191. Both Cys191S and Cys191G exhibit very similar resonances compared to the wild-type. Larger variations would be expected in a double mutant than a single mutant if both cysteine residues in the wild-type protein provide ligands to the Fe-S cluster. Contrary to this expectation, C206G and C191G/C206G have almost identical EPR spectra. All together, these data suggest that the third cysteine residue at the CX_{12}CX_{13}CX_{14}C motif in SufR has a minimal influence on EPR signal, and thus may not provide a ligand to the [4Fe-4S] cluster. This interpretation is consistent with the fact that the third cysteine residue is only conserved among cyanobacteria. If this were the case, how could SufR coordinate a [4Fe-4S] cluster that requires four ligands? There are two possibilities: 1) one [4Fe-4S] cluster is bridged between two subunits in a SufR dimer; or 2) one [4Fe-4S] cluster per sufR monomer is ligated by a mixed-ligand system, either 3 Cys, 1 Oxygen, or 3 Cys, 1 Nitrogen. For the first possibility, SufR has been shown to be able to form a dimer by gel filtration analysis performed under anaerobic conditions. Data from chemical analysis and spin
quantitation studies do not exclude this possibility. The SufA protein of *Synechocystis* sp. PCC 6803 contains three cysteines conserved among homologs in all organisms and two additional cysteines conserved within oxygenic photosynthetic organisms. A substitution study indicates that only the two cysteine residues in the C-terminal CGCS motif provide ligands to the [2Fe-2S] cluster (Wollenburg et al., 2003). Since four ligands are needed to coordinate a [2Fe-2S] cluster, and since SufA can form dimer, it is proposed that the SufA protein binds one [2Fe-2S] cluster per dimer. For the second possibility, the best candidate for the non-cysteine ligand would be a glutamate. Evolution has chosen cysteine as the most common ligand for Fe-S cluster coordination. Even so often, aspartate, serine, and histidine are used as alternative ligands. Aspartate can provide a carboxyl oxygen ligand, such as the PfFd ferredoxin protein of *Pyrococcus furiosus* (Conover et al., 1990). Serine contains a hydroxyl side group that is suitable for a ligand to an iron, such as the P-cluster of nitrogenase. However, because of the potential redox-dependent ligand protonation that can occur in serine coordination, [4Fe-4S] clusters with serine coordination have never been found *in vivo* (Brereton et al., 1999). A nitrogen in the imidazole ring of histidine can also provide a ligand, as in the Rieske protein (Link et al, 1992), the distal [4Fe-4S] clusters in a NiFe hydrogenase (Volbeda et al., 1995), and some Fe hydrogenases (Peters et al., 1998). In some instances, such as in aconitase, oxygen ligation can be provided by hydroxide, water, or aconitate. There are both conserved aspartate and histidine residues within \(CX_{12}CX_{13}CX_{14}C\) motif in cyanobacterial SufR, but none of them are conserved in other organisms. A glutamate downstream of the second cysteine by two amino acids is noticeably conserved in most organisms (data not shown), with a few variations by either
aspartate or histidine, both of which are well known to be able to provide ligands to Fe-S clusters. The amino acid residue at this position is the best candidate for the fourth ligation to the Fe-S cluster in SufR. Despite the fact that no glutamate ligation has ever been reported in a native protein, subsite-differentiated [4Fe-4S]\(^{2+}\) clusters ligated by three Cys with a glutamate providing the fourth ligand have been synthesized (Weigel et al., 1991).

EPR spectroscopy can determine the existence of Fe-S clusters and it can differentiate the types of Fe-S clusters, such as [4Fe-4S] and [2Fe-2S]. However, the method does not unambiguously establish the type of cluster ligation. For [2Fe-2S] clusters, the \(g\)-values and anisotropy of the reduced cluster are primarily determined by the ligand field at the Fe(II) site of the localized valence (Fujinaga et al., 1993; Bertrand et al., 1985; Werth et al., 1990). For [4Fe-4S] clusters, the properties of [4Fe-4S]\(^{2+}/1+\) clusters can be best understood in terms of pairwise Fe interactions. The \(S = 0\) ground state of the oxidized state results from antiferromagnetic interaction between two valence-delocalized Fe\(^{2.5+}\) pairs, and the \(S=1/2\) or \(3/2\) ground state of the reduced state results from antiferromagnetic interaction between the valence-delocalized (Fe\(^{2.5+}\)) and all ferrous (Fe\(^{2+}\)) pairs. In this respect, the only difference between [2Fe-2S] and [4Fe-4S] clusters is that the added electron is localized over a pair of Fe atoms upon reduction of [4Fe-4S]\(^{2+}\) \(vs.\) a single Fe upon reduction of [2Fe-2S]\(^{2+}\). Therefore, substitution of the cysteines that ligate nonreducible Fe (for [2Fe-2S] cluster) or Fe pair (for [4Fe-4S] cluster) may lead to minimal changes and misinterpretation of the data. Indeed, there are now several examples in which cysteine to serine mutations involving cysteines that ligate the nonreducible Fe site of a [2Fe-2S] cluster result in no significant changes in the EPR properties (Fujunata et al., Werth et al., 1990; 1993; Xia et
Moreover, it is found that cysteine ligand changes by either alanine or serine cause the AvFdI protein to recruit a nearby non-ligand cysteine as an alternative thiolate ligand to its [4Fe-4S] cluster (Martin et al., 1990; Shen et al., 1995). There are also cases in which a non-ligand amino acid mutation leads to an increased stability of Fe-S cluster, such as Leu28->His in FNR (Bates et al., 2000), Asp37->Ala in truncated NifU containing only N-terminal domain (Yuvaniyama et al., 1999), and Asp37->Ala of yeast Isu1p (Wu et al., 2002). Thus, we need to be cautious in concluding that the third cysteine is not involved in ligating the [4Fe-4S] cluster in the SufR protein if the data are solely based on a single mutant. That is why we introduced both Ser and Gly mutations into C191, and why we also constructed the C191G/C206G double mutant. The similarity of the EPR properties between C191S and C191G and between C206G and C191G/C206G helps to support and strengthen the argument, but does not constitute proof.

It was found that the Gly mutants have an EPR spectrum more similar than the Ser mutants compared to the wild-type. This may be caused by the different properties of the two amino acids. Serine is very similar to cysteine in structure, size and hydrophobicity. Thus, serine substitution is expected to cause the least conformational change in the protein. Serine could also provide an oxygen ligand to the Fe-S clusters. Although serinate ligation to Fe-S clusters is very rarely found in native proteins, NMR and X-ray crystallographic data have clearly indicated serinate Fe ligation in almost every structurally characterized Fe-S protein in which a coordinating cysteine has been replaced by a serine without loss of the Fe or Fe-S cluster (Xiao et al., 1998; Hurley et al., 1997; Babini et al., 1996; Calzolai et al., 1997). In contrast, because glycine cannot provide ligand to Fe-S clusters, a thiolate ligand,
in the case of our study provided by 2-mercaptoethanol, may provide an external ‘rescue ligand’ in the Gly mutants. This issue has been addressed in PsaC by $^{19}$F-labeled NMR spectroscopy (Antonkine M., Ph. D. thesis). It is thus very likely that the thiol-ligated Fe-S cluster (3Cys, 1thiol) vs. the serinated Fe-S cluster (3Cys, 1Oxy) constitutes the difference in EPR properties between the Gly mutants and Ser mutants of SufR.

[4Fe-4S] clusters that exist in [4Fe-4S] ferredoxins are usually coordinated by three cysteine ligands from a highly conserved CXXCXXC motif. The fourth cysteine ligand is distantly situated from these three cysteines. PsaC that contains 2 [4Fe-4S] clusters, $F_A$ and $F_B$, has two CXXCXXCXXXCP motifs. Coordination of the two [4Fe-4S] clusters follows the same rule, i.e., the first three cysteines in one motif together with the fourth cysteine in the second motif provide ligands to one Fe-S cluster (Golbeck, 1999). This rule appears to also direct the ligation of the [4Fe-4S] cluster in iron-sulfur flavoprotein (Isf) from *Methanosarcina thermophila*. This protein has only one CXXCXXCX$_4$C motif that is in high identity with the CXXCXXCX$_{4-7}$C motif among its homolog proteins. Two other cysteine residues in this protein do not participate in Fe ligation. It is thus proposed that two [4Fe-4S] clusters are bridged between two subunits of an Isf dimer, with the first three cysteines in the motif from one subunit and the fourth cysteine in the motif from the second subunit ligate to one [4Fe-4S] cluster (Leartsakulpanich et al., 2000). Finally, endonuclease III and MutY contain a redox-inert [4Fe-4S]$^{2+}$ cluster coordinated by a compact cysteine motif CX$_6$CX$_2$CX$_5$C (Porello et al., 1998; Prince et al., 1993). The cysteine motif CX$_{12}$CX$_{13}$CX$_{14}$C in SufR thus represents a novel method to coordinate a [4Fe-4S] cluster.
The SufR protein may utilize an iron-sulfur cluster to sense iron levels or stress

It must be kept in mind that the iron-sulfur cluster was inserted \textit{in vitro} into the \textit{E. coli}-expressed apo-protein; hence, the possibility exists that SufR does not harbor an iron-sulfur cluster \textit{in vivo} or that it harbors a different type of cluster (\textit{i.e.} a [2Fe-2S] cluster) when functioning within the cyanobacteria. Nevertheless, given the function of the proteins encoded by the \textit{sufBCDS} operon, as was discussed in Chapter 2, it is reasonable to assume that an iron-sulfur cluster would bind to SufR and serve as a sensor of oxidative stress that is commonly encountered by cyanobacteria. It is proposed that SufR indirectly senses the levels of iron-sulfur clusters in the cells through its own unstable iron-sulfur cluster; when this cluster is present, SufR binds to its operator and functions as a regulatory repressor of the \textit{sufBCDS} operon. The oxygen-sensitivity of the iron-sulfur cluster on the SufR protein may limit the lifetime of the active state, thereby allowing the poise of a quasi steady-state population that could respond rapidly as the level of oxidative stress rises or falls.

Other regulatory proteins are known to fill a similar role. In a study with \textit{E. chrysanthemi}, Nachin and colleagues found that the \textit{suf} operon might participate in a SoxR-dependent response to oxidative stress (Nachin et al., 2001). SoxR is a transcription activator and contains a helix-loop-helix motif that confers sequence-specific DNA-binding capability (reviewed by Pomposiello et al., 2001). SoxR in \textit{E. coli} is a homodimer and contains one [2Fe-2S] cluster per monomer. It has been shown that the iron-sulfur cluster is not required for SoxR to bind to the promoter of the \textit{soxS} gene, but only the form of SoxR that contains the 2$^+$ state iron-sulfur cluster can lead to the initiation of transcription of the \textit{soxS} gene (Hidalgo et al., 1997). SoxR senses reversible oxidation/reduction of the iron-
sulfur cluster, a one-electron redox system (Gaudu et al., 1996). A SoxR homolog, a MerR-like protein, can be identified from searches in genomic sequences of *Synechocystis* sp. PCC 6803 (Kaneko et al., 1996), *Synechococcus* sp. PCC 7002 (Genbank accession number AY375041), and *Anabaena* sp. PCC 7120 (Kaneko et al., 2001). Whether or not this merR-like gene participates in redox regulation of the suf operon in cyanobacteria will be investigated in future studies. The IscR protein of *E. coli* also functions as a repressor of the *iscRSUA* operon because deletion strains of *iscR* exhibit increased expression of this operon. IscR isolated anaerobically from a bacterial expression system contains a [2Fe-2S]^{1+} cluster that appears to be important for IscR function. It has been proposed that IscR may function in an autoregulatory mechanism that senses the iron-sulfur cluster assembly status of cells (Schwartz et al., 2001). The presence of a [4Fe-4S] cluster in SufR and the presence of a [2Fe-2S] cluster in IscR constitute significant differences between these two proteins, even though both function as iron-sensing transcriptional regulators. If the [4Fe-4S] cluster were indeed present *in vivo*, SufR would represent an entirely new subclass of regulator.
REFERENCES:


*Klebsiella pneumoniae* and *Azotobacter vinelandii*: NifL, transducing two 

Schwartz, C. J., J. L. Giel, T. Patschkowski, C. Luther, F. J. Ruzicka, H. Beinert, and 
expression of *Escherichia coli* genes encoding Fe-S cluster assembly proteins. *Proc Natl Acad Sci USA* 98:14895-14900.

Shen, B., D. R. Jollie, T. C. Diller, C. D. Stout, P. J. Stephens, and B. K. Burgess. Site-
directed mutagenesis of *Azotobacter vinelandii* ferredoxin I: cysteine ligation of the 
[4Fe-4S] cluster with protein rearrangement is preferred over serine ligation. *Proc Natl 
Acad Sci USA*. 92:10064-68.

single turnover of biotin synthase: destruction of a [2Fe-2S] cluster accompanies 

Volbeda, A., M. H. Charon, C., Piras, E. C. Hatchikian, M. Frey, and J. C. Fontecilla-
Camps. 1995. Crystal structure of the nickel-iron hydrogenase from *Desulfovibrio 

Werth, M. T., G. Cecchini, A. Manodori, B. A. C. Ackrell, I. Schroder, R. P. Gunsalus, 
and M. K. Johnson. 1990. Site-directed mutagenesis of conserved cysteine residues 
in *Escherichia coli* fumarate reductase: modification of the spectroscopic and 


Table 4.1 Sequences of oligonucleotides used for site-directed mutagenesis and truncation of the SufR-240 protein.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>S88EcutF</td>
<td>5’- CTTCTCCTGTTGTTGCATATGACCCTCAGTTCTTC-3’</td>
</tr>
<tr>
<td>S88EcutR</td>
<td>5’- GAAGAAACTGAGGGTCATATGCAACAACAGGAGAAAG-3’</td>
</tr>
<tr>
<td>C1SF</td>
<td>5’- TCGGAGCACCACAGTGCATCGCCGATG-3’</td>
</tr>
<tr>
<td>C1SR</td>
<td>5’- CATCGGCGATCGCACTGTGTTGCTCCG-3’</td>
</tr>
<tr>
<td>C2SF</td>
<td>5’- TACCCCACCGTTAGTGCCATGAGTTGG-3’</td>
</tr>
<tr>
<td>C2SR</td>
<td>5’- CCAACTCATGGCCACTAACGTTGGGT-3’</td>
</tr>
<tr>
<td>C3SF</td>
<td>5’- CTATTCTGCCGGACAGTGCCATCGAAAG-3’</td>
</tr>
<tr>
<td>C3SR</td>
<td>5’- CTTTCGATGGCACTGTCCGCCAAGAATAG-3’</td>
</tr>
<tr>
<td>C4SF</td>
<td>5’- GGAACACACCAGTGGTTATTTGATC-3’</td>
</tr>
<tr>
<td>C4SR</td>
<td>5’- GATCAAATAACCACCTGTTGTTTCC-3’</td>
</tr>
<tr>
<td>C3GF</td>
<td>5’- ATTCTGCGGAGGCTGCCATCGAAAG-3’</td>
</tr>
<tr>
<td>C3GR</td>
<td>5’- CTTTCGATGCCACGGTCCGCCAAGATT-3’</td>
</tr>
<tr>
<td>C4GF</td>
<td>5’- ATGGGGAACACACCGGTGTTATTTGATCC-3’</td>
</tr>
<tr>
<td>C4GR</td>
<td>5’- GGATCAAATAACCACCGGTGTTCCCAT-3’</td>
</tr>
</tbody>
</table>
Table 4.2 List of the recombinant wild-type and mutant SufR proteins used in this report

<table>
<thead>
<tr>
<th>Recombinant proteins</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SufR-240 (original wild-type SufR protein)</td>
<td>Contains 240 amino acid residues as annotated in Cyanobase. Used for antibody production, PCR template for truncation of the N-terminal 24 amino acid residues, and Fe-S cluster reconstitution (its UV-visible and EPR spectra are presented in Figure 4.2 and Figure 4.4 respectively).</td>
</tr>
<tr>
<td>SufR-217 (truncated, wild-type SufR)</td>
<td>Contains 217 amino acid residues. Used as PCR template for cysteine substitutions and Fe-S cluster reconstitution.</td>
</tr>
<tr>
<td>C164S (C1S)</td>
<td>The cysteine residue at the 164th position (or the 1st cysteine in the cysteine motif) of SufR-217 protein is replaced with a Ser.</td>
</tr>
<tr>
<td>C177S (C2S)</td>
<td>The cysteine residue at the 177th position (or the 2nd cysteine in the cysteine motif) of SufR-217 protein is replaced with a Ser.</td>
</tr>
<tr>
<td>C191S (C3S)</td>
<td>The cysteine residue at the 191st position (or the 3rd cysteine in the cysteine motif) of SufR-217 protein is replaced with a Ser.</td>
</tr>
<tr>
<td>C206S (C4S)</td>
<td>The cysteine residue at the 206th position (or the 4th cysteine in the cysteine motif) of SufR-217 protein is replaced with a Ser.</td>
</tr>
<tr>
<td>C191G (C3G)</td>
<td>The cysteine residue at the 191st position (or the 3rd cysteine in the cysteine motif) of SufR-217 protein is replaced with a Gly.</td>
</tr>
<tr>
<td>C206G (C4G)</td>
<td>The cysteine residue at the 206th position (or the 4th cysteine in the cysteine motif) of SufR-217 protein is replaced with a Gly.</td>
</tr>
<tr>
<td>C177S/C206S (C2S/C4S)</td>
<td>The cysteine residues at the 177th and 206th positions (or the 2nd and 4th cysteines in the cysteine motif) of SufR-217 protein are replaced with a Ser.</td>
</tr>
<tr>
<td>C191G/C206G (C3G/C4G)</td>
<td>The cysteine residue at the 191st and 206th position (or the 3rd and 4th cysteines in the cysteine motif) of SufR-217 protein are replaced with a Gly.</td>
</tr>
</tbody>
</table>
Table 4.3 The number of iron and sulfide per polypeptide of reconstituted SufR protein

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>C164S</th>
<th>C177S</th>
<th>C191S</th>
<th>C191G</th>
<th>C206S</th>
<th>C177S/C206S</th>
<th>C191G/C206G</th>
<th>C206G</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron</td>
<td>1.4±</td>
<td>1.3±</td>
<td>1.8±</td>
<td>2.5±</td>
<td>1.3±</td>
<td>0.5±</td>
<td>0.7±</td>
<td>1.7±</td>
<td>2.2±</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.05</td>
<td>0.07</td>
<td>0.06</td>
<td>0.06</td>
<td>0.01</td>
<td>0.06</td>
<td>0.07</td>
<td>0.12</td>
</tr>
<tr>
<td>Sulfide</td>
<td>1.4±</td>
<td>1.9±</td>
<td>1.5±</td>
<td>2.2±</td>
<td>1.1±</td>
<td>n/d</td>
<td>1.0±</td>
<td>1.1±</td>
<td>2.1±</td>
</tr>
<tr>
<td></td>
<td>0.42</td>
<td>0.24</td>
<td>0.37</td>
<td>0.03</td>
<td>0.16</td>
<td>0.29</td>
<td>0.26</td>
<td>0.38</td>
<td></td>
</tr>
</tbody>
</table>

*: n/d means not detectable
Table 4.4: Calculated molecular weights corresponding to UV-absorption peaks of the mutant and the wild-type SufR-217 proteins. The SufR proteins are: C1S (C164S), C2S (C177S), C3S (C191S), C4S (C206S), C3G (C191G), C4G (C206G), C2S/C4S (C177S/C206S), and C3G/C4G (C191G/C206G). WT means wild-type protein, and apo- and holo- mean SufR proteins without and with Fe-S clusters respectively.

<table>
<thead>
<tr>
<th>SufR proteins</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
<th>Peak 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-SufR WT</td>
<td></td>
<td></td>
<td></td>
<td>50,151</td>
</tr>
<tr>
<td>Holo-SufR WT</td>
<td>97,727</td>
<td>48,221</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holo-C1S</td>
<td>1.042e+07</td>
<td>58,675</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holo-C2S</td>
<td>9.633e+06</td>
<td>58,675</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo-C3G</td>
<td>1.105e+07</td>
<td>54,246</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holo-C3G</td>
<td>9.583e+06</td>
<td>1.151e+06</td>
<td>97,153</td>
<td>47,938</td>
</tr>
<tr>
<td>Holo-C3S</td>
<td>8.234e+06</td>
<td>123,672</td>
<td>50,184</td>
<td></td>
</tr>
<tr>
<td>Holo-C4S</td>
<td>9.583e+06</td>
<td>504,976</td>
<td>65,618</td>
<td></td>
</tr>
<tr>
<td>Holo-C4G</td>
<td>1.002e+09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holo-C3G/C4G</td>
<td>8.563e+06</td>
<td>2.538e+06</td>
<td>83,530</td>
<td></td>
</tr>
<tr>
<td>Holo-C2S/C4S</td>
<td>9.263e+06</td>
<td>46,365</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 4.1 A) Comparison of the size of purified recombinant proteins of the SufR-240 (lane 1) and SufR-217 (lane 2) proteins. The purity of SufR-217 is very high after reconcentration and passage over a PD-10 column. B) Probable dimeric SufR-217 protein on non-denaturing protein gel as shown by the upper arrow. The lower arrow points to probable monomeric SufR protein. 30 μg (lane 1) and 10 μg (lane 2) of the wild-type SufR-217 protein was loaded.

Figure 4.2 UV/visible absorption spectrum (A) and EPR spectrum (B) of the SufR-240 recombinant protein after reconstitution with iron, sulfide and 2-mercaptoethanol. The UV/visible absorption spectrum shows maxima at 280, 320 and 411 nm. The inset shows the loss of the absorbance of the oxidized protein upon exposure to 30 min (solid line), 60 min (dotted line), and 90 min (dashed line) of air. The EPR spectrum shows the axial lineshape of an iron-sulfur cluster, with a lowfield peak at $g = 2.02$ and a highfield trough at $g = 1.89$. The inset shows the signal intensity as a function of temperature and microwave power. EPR conditions: microwave frequency 9.4709 GHz, microwave power 40 mW, modulation amplitude 10 G, receiver gain $6.3 \times 10^4$, temperature 14 K. The spectrum represents an average of 4 scans.

Figure 4.3 Mössbauer spectra of $^{57}$Fe-enriched wild-type SufR-217 protein recorded at 4.2 K in a low magnetic field of 40 mT (A) and a high magnetic field of 8 T (B) applied parallel
to the radiation. The solid line in (B) is an $S = 0$ simulation using the parameters $E_Q = 1.16$ mm/s, isomer shift, $\Delta = 0.44$ mm/s, and asymmetry parameter $\delta = 0.64$.

**Figure 4.4** EPR spectra of reconstituted eight mutant and wild-type SufR-217 proteins after reduction with dithionite. Their EPR spectra are as marked in the figure. The SufR proteins are: C1S (C164S), C2S (C177S), C3S (C191S), C4S (C206S), C3G (C191G), C4G (C206G), C2S/C4S (C177S/C206S), and C3G/C4G (C191G/C206G), WT means wild-type protein. All EPR spectra were recorded under the same conditions as described in Figure 1.

**Figure 4.5** Power and temperature dependence of the EPR signals of the mutant and wild-type SufR-217 proteins. The proteins are as marked in the figure and as described in the text and in Figure 4.4.

**Figure 4.6** Spin quantitation of the reconstituted wild-type SufR-217 protein using cupric sulfate of different concentrations as the standard. The EPR spectra were recorded at 14 K and 20 K with microwave powers ranging from 100 mW to 160 mW. The double integrated intensity was plotted against microwave power square root. Each line is as marked in the figure. The lines of SufR protein fall between lines of 0.5 mM and 1.0 mM cupric sulfate and are very close to those of 0.5 mM cupric sulfate at both 14 K and 20 K.

**Figure 4.7** Gel filtration analysis of the reconstituted mutant and wild-type SufR-217 proteins under anaerobic conditions. A) standard proteins. The inset shows the standard line
of logMW against elution volume. Protein standards used in this experiment are: carbonate anhydrase (blue line), cytochrome c (red line), bovine serum albumin (black line), and lysozyme (pink line). B) SufR proteins. The calculated molecular weights corresponding to the UV absorption peaks are listed in Table 4.4. The proteins are as marked in the figure and as described in the text and Figure 4.4. apoWT and apoC3G are unreconstituted wild-type and C3G (C199G) mutant SufR proteins respectively.
Figure 4.1

A

B
Figure 4.2

A

B

Absorbance

Temperature, K

Magnetic Field (G)

Wavelength (nm)

Intensity

Magnetic Field (G)

Wavelength (nm)

Absorbance

Wavelength (nm)
Figure 4.3
Figure 4.4
Figure 4.5
Figure 4.6

![Graph showing double integrated intensity against (Power)$^{1/2}$ with different concentrations of SufR at 14K and 20K.]
Figure 4.7

A

![Graph A](image)

B

![Graph B](image)

\[ y = -0.4728x + 4.2697 \]
Chapter 5. *slr0148*, an ORF involved in an operon spanning from *slr0144* through *slr0151*, is the *fdx* Homolog of the ISC Fe-S Cluster Assembly Pathway in *Synechocystis* sp. PCC 6803

**ABSTRACT**

The open reading frame, *slr0148*, was identified to encode an Fdx homolog of the ISC Fe-S cluster assembly pathway in *Synechocystis* sp. PCC 6803 based on amino acid sequence similarity and the finding that the recombinant protein can coordinate a [2Fe-2S] cluster. To demonstrate further that *slr0148* codes for the Fdx component of the ISC machinery in *Synechocystis* sp. PCC 6803, the *slr0148* gene was insertionally inactivated and growth phenotypes of the *slr0148* mutant were compared with those of the *slr0387* and *sll0704* inactivation mutants under standard, high light, low temperature, and iron limitation conditions. There are a total of four *nifS*-like homologs in *Synechocystis* sp. PCC 6803. *slr0387* and *sll0704* are two *iscS* homologs; the other two are *slr0077* (*sufS*) and *slr2143*. Their transcription under various growth conditions and oxidative stress conditions was investigated. The four *nifS*-like genes were differentially expressed under various conditions, clearly indicating that each individual NifS-like protein alone or in combination functions under certain stress conditions. It is previously shown that the transcription of the *sufBCDS* genes was up-regulated under oxidative stress and iron limitation conditions in *Synechococcus* sp. PCC 7002. This result was confirmed in *Synechocystis* sp. PCC 6803. *slr0148* is part of a large gene cluster extending from *slr0144* through *slr0152*. RT-PCR data indicated that this gene cluster forms an operon spanning from *slr0144* through *slr0151*. Transcription of genes of this operon was down-regulated in response to high light, low
temperature, H$_2$O$_2$ treatment, and iron starvation, although there were a few variations in regard to certain genes under certain conditions. The function of the overall gene cluster might be related to Photosystem I assembly and/or turnover.
**INTRODUCTION**

Ferredoxins are a major group of Fe-S proteins. The first Fe-S protein ever found was a ferredoxin that functions in nitrogen fixation in *Clostridium pasteurianum* (Mortenson et al., 1962). These small acidic proteins can coordinate different kinds of Fe-S clusters with various functions, such as Fe(Cys)$_4$, [2Fe-2S], [3Fe-4S], and [4Fe-4S]. Rubredoxins have only one iron ligated by four cysteine residues and represent the simplest Fe-S proteins. [2Fe-2S] ferredoxins can be divided into two groups based on whether they function in photosynthesis or not (Matsubara and Saeki, 1992). The [2Fe-2S] ferredoxin acting as a terminal electron acceptor from PS I in spinach chloroplasts is the second Fe-S protein to have been identified (Tagawa and Arnon, 1962). Non-photosynthetic [2Fe-2S] ferredoxins are involved in various processes, such as halobacterial ferredoxin, *Rhodobacter* ferredoxin, *Clostridium* ferredoxin, and those in oxygenase systems (reviewed by Sticht and Rosch, 1999 and references therein). [3Fe-4S] and [4Fe-4S] ferredoxins include low potential proteins (−250 to −650 mV) and HiPIPs (high potential iron-sulphur proteins) (+50 to +500 mV). Low potential ferredoxins usually contain one or two [4Fe-4S] clusters, but there are ferredoxins with a single [3Fe-4S], such as *D. gigas* ferredoxin II (Cammack et al., 1977), or one [3Fe-4S] and one [4Fe-4S], such as 7-Fe Fd from *A. vinelandii* (Stout, 1989). HiPIPs are a class of small proteins containing only one [4Fe-4S]. Many organisms have multiple ferredoxins, and each of them has a particular role. *Synechocystis* sp. PCC 6803 contains at least ten ferredoxins that function in photosynthesis and nitrite/sulfite reduction. Many of them are hypothetical proteins with unknown functions. *slr0148*, an ORF in *Synechocystis* sp. PCC 6803 (Kaneko et al., 1996), encodes a protein that shows high sequence similarity
with the Fdx component of the ISC Fe-S cluster assembly pathway. An inactivation study demonstrated that the Fdx protein from *E. coli* is one of the most important components for Fe-S cluster biogenesis (Takahashi and Nakamura, 1999). Its homolog in yeast (Yah1p) is essential for cell viability (Barros et al., 1999). Depletion of Yah1p by regulated gene expression resulted in elevated Fe concentration in mitochondria (Lang et al., 2000). In *A. vinelandii*, attempts to construct a knock-out *fdx* mutant failed, indicating that *fdx* is essential in this diazotrophic bacterium (Zheng et al, 1998). The *A. vinelandii* Fdx protein was co-purified with a specific 7-Fe FdI mutant protein from *A. vinelandii* cells, and was renamed FdIV (its coding gene renamed *fdxD*) (Jung et al., 1999a). It was later realized that the protein level of FdIV was up-regulated in this mutant strain in response to functional disruption of FdI, which allowed the co-purification of FdIV with FdI. FdIV is a [2Fe-2S] protein with a redox potential of −340 mV. Interestingly, the *fpr* gene (which encodes NADPH:ferredoxin reductase (Isas et al., 1995)) has the same expression profile as *fdxD* either in the *fdxA* (encoding the FdI protein) deletion mutant of *A. vinelandii* or under oxidative stress. This result suggests the involvement of an Fdx reductase-Fdx system in an electron transfer step during Fe-S cluster biogenesis or repair. *slr0148* is found in a large gene cluster spanning *slr0144* through *slr0152* in *Synechocystis* sp. PCC 6803 (Figure 5.2) (Kaneko et al., 1996). *Thermosynechococcus elongatus* contains homologs to all of the genes in this cluster (Nakamura et al., 2002). These homologs are also grouped into a gene cluster, but *slr0149* and *slr0150* are situated elsewhere in the genome of *Thermosynechococcus elongatus*. *Synechococcus* sp. PCC 7002 (Genbank accession number AY375041) has homologs to *slr0144, slr0147, slr0148, slr0149*, and *slr0150*, which are
organized differently from their homologs in *Synechocystis* and *Thermosynechococcus*. Homologs to *slr0145* and *slr0146* are missing in *Synechococcus* sp. PCC 7002. Other cyanobacteria, e.g., *Anabaena* sp. PCC 7120, lack the entire gene cluster. Recent microarray analyses indicated that this gene cluster is coordinately repressed in response to cold (Suzuki et al., 2001), high light (Hihara et al., 2001), oxidative stress, and iron deficiency conditions (Singh et al., 2004). Here, we report that *slr0148* encodes an Fdx homolog of the ISC Fe-S cluster assembly machinery based on an amino acid sequence alignment. EPR spectroscopy indicated that the recombinant Slr0148 protein is capable of binding a [2Fe-2S] cluster. To establish further that *slr0148* is the real *fdx* homolog, growth of the *slr0148* mutant was compared with that of the two *iscS*-like inactivation mutants under various conditions, and the expression levels of all four *nifS*-like homologs were investigated by RT-PCR analysis. Transcription of the genes in this operon under stress conditions was also examined by RT-PCR. Our studies show that *slr0148* is part of a large operon spanning from *slr0144* to *slr0151*. 
MATERIALS AND METHODS

Growth of Synechocystis sp. PCC 6803

Growth of the Synechocystis sp. PCC 6803 wild-type and mutant strains and chromosomal DNA preparation were described in chapter 2. The slr0387 and sll0704 inactivation mutants were kindly provided by Prof. Bollinger’s Laboratory at The Pennsylvania State University. The temperature for cold treatment was 22°C. The light intensity for high light treatment was 250 $\text{mE} \cdot \text{M}^{-2} \cdot \text{S}^{-1}$. For iron starvation, ferrous ammonium citrate was not added to the growth medium. Oxidative stress conditions were induced by the addition of 5 mM H$_2$O$_2$ into the growth medium. Cells grown for RNA isolation under low temperature, high light, oxidative stress, or iron limitation conditions were treated for 20 min, 1 hour, 30 min, and overnight, respectively, similar to the conditions used in microarray analyses (Hihara et al., 2001; Singh et al., 2004; Suzuki et al., 2001)

Construction of the plasmids for mutagenesis

The slr0148 gene and flanking regions were amplified from the chromosomal DNA of Synechocystis sp. PCC 6803 by PCR using primers 6M0148F and 6M0148R (Table 1). The resulting 1400-bp fragment was digested with EcoRI and XbaI and cloned into the plasmid pUC19 cleaved with the same enzymes, creating the plasmid pUC0148. To construct insertional mutant, an aphII gene derived from the plasmid pRL161 by digestion with HincII was inserted into the unique SmaI restriction site within the slr0148 gene sequence in the plasmid pUC0148, yielding the plasmid pUCK0148. The insertion was verified by cleavage with PstI and SphI.
Transformation of cyanobacteria and Selection

The *Synechocystis* sp. PCC 6803 wild-type cells were grown to OD$_{730}$ = 0.6-0.8. Cells were collected by centrifugation at 4000 g at room temperature for 5 min, resuspended in the original growth medium, and adjusted to OD$_{730}$ = 2.5. Concentrated *Synechocystis* sp. PCC 6803 cells (1 ml) were mixed with 5-10 μg pUCK0148 plasmid DNA and bubbled slowly for 3-4 hours. Cells (200 μl) were streaked onto sterile filter membranes on top of agar plates. The plates were placed under standard light conditions for 2 days. The filter membranes with cells on the surface were transferred onto new BG11 plates containing 50 μg/ml kanamycin. Antibiotic-resistant colonies were selected under reduced light conditions.

Construction of expression plasmids

The open reading frame *slr0148* was amplified from the chromosomal DNA of *Synechocystis* sp. PCC 6803 using PCR primers 6E0148F and 6E0148R (Table 1). An NcoI and a BamHI restriction site were introduced into the 5’- and 3’- primers respectively. The plasmid used for the expression of the S1r0148 protein was constructed by cloning the *slr0148* gene into pET3d and pET32a. After verification by DNA sequencing that no mutations were introduced into the *slr0148* gene, the expression plasmid was transformed into *E. coli* strain Bl21(DE3) for protein overproduction. DNA sequencing was carried out at the Nucleic Acid Facility of The Penn State University.
Methods for overexpression and purification of the Slr0148 protein, protein quantitation, Fe-S cluster reconstitution, EPR spectroscopy, RNA isolation, and RT-PCR analysis were as described in Chapter 2.
RESULTS

slr0148 is the cyanobacterial homolog to fdx in the isc operon.

A BLAST search against Cyanobase (http://www.kazusa.or.jp/cyano) using the amino acid sequence of AvFdIV identified two potential homologs. One is slr0148 in Synechocystis sp. PCC 6803 (Kaneko et al., 1996), the other is tlr2302 in Thermosynechococcus elongatus BP-1 (Nakamura et al., 2002) (Figure 5.1). A third fdx homolog was also found in Synechococcus sp. PCC 7002 (Genbank accession number AY375041). These three fdx homologs show 90% identity in their deduced amino acid sequences and 34% identity with AvFdIV. This similarity is much lower compared to the Fdx homologs from other organisms, such as E. coli and Yersinia pestis, which show 81% identity with AvFdIV. A fourth fdx homolog from Synechococcus sp. WH 8102 (Palenik et al., 2003) has even lower sequence similarity. Cyanobacterial Fdx homologs contain four conserved cysteine residues that are present in all the Fdx homologs. The first three cysteines reside in an adrenodoxin-like cysteine motif, i.e., C-X$_2$-[STAQ]-X-[STAMV]-C-[STA]-T-C-[HR] (Grinberg et al., 2000), with some variations in each cyanobacterial Fdx homolog. By contrast, this cysteine motif in the Fdx homologs from other organisms is in consensus with the adrenodoxin motif. The fourth cysteine is in a [SW]-R-L-A-C-Q-[AT] motif that is well conserved in all of the Fdx homologs. Either the cyanobacterial Fdx homologs have expanded the consensus adrenodoxin-like motif, or these differences reflect functional variations. One major difference in the Fdx homologs between cyanobacteria and other organisms is that the cyanobacterial Fdx homologs have an obvious 50 amino-acid C-terminal extension.
Properties of the Slr0148 protein

The overexpressed Slr0148 protein in *E. coli* forms red/brown colored inclusion bodies, indicating that it might be an Fe-S protein. Interestingly, the recombinant Slr0148 protein purified by gel filtration chromatography formed two bands with a ratio of 1:2 on SDS-PAGE gels (Figure 5.3). Since an Fe-S cluster would have been destroyed during solubilization of the inclusion bodies, neither of the two bands could represent the holo-form of Slr0148. This was further confirmed by boiling the Slr0148 protein in the presence of EDTA for 30 min, a method commonly used for preparing apo-ferredoxins (Nishio and Nakai, 2001). The same double bands still appeared on the protein gel. It is not rare for ferredoxins to migrate abnormally on SDS-PAGE gels. The *A. vinelandii* FdI protein runs at about twice its predicted size on SDS-PAGE gels (Jung et al., 1999b), and the *A. vinelandii* FdIV protein runs faster than its native protein (Jung et al., 1999a). This phenomenon was also observed in other small acidic proteins (Böhme et al., 1987; Pueyo et al., 1992). Recombinant proteins that form two bands on SDS PAGE gels have been documented for the hydrogenosomal [2Fe-2S] ferredoxin from *Trichomonas vaginalis* (Vidakovic et al., 1996), the OmpA protein from *E. coli* (Hendennach et al., 1975; Schnaitman, 1974), and the IscU protein from *Thermotoga maritima* (Mansy et al., 2002). The odd behavior of these proteins on SDS-PAGE gels is due to the fact that they bind SDS in two stoichiometries and therefore run as a doublet.

As presented in Figure 5.4A, the reconstituted recombinant Slr0148 protein in the presence of 10 mM dithionite shows a nearly axial EPR spectrum with *g* values of 2.02 and 1.89 at the lowfield and highfield respectively, which is typical of the proteins containing an Fe-S cluster. Figure 5.4B shows the temperature and power dependence of its EPR spectra.

205
The EPR signal is still observable at temperatures over 100 K. These slow spin relaxation properties are characteristic of a [2Fe-2S] cluster. Thus, our EPR data clearly indicate that Slr0148 is a [2Fe-2S] protein, which is consistent with reports on Fdx homologs from other organisms.

**A comparison of growth between the slr0148 inactivation mutant and wild-type**

*Synechocystis sp. PCC 6803*

slr0148 in *Synechocystis* sp. PCC 6803 was inactivated by inserting an *aphII* gene either parallel or antiparallel to the transcription direction of *slr0148* (Figure 5.5A). Complete segregation was confirmed by PCR using the chromosomal DNAs prepared from the wild-type and the *slr0148* transformants as the template (Figure 5.5B). All of the mutant strains showed no appreciable growth phenotype different from the wild-type under standard growth conditions. The growth rate of the *slr0148* mutant was similar to that of the wild-type, with a doubling time of 21.5 and 20.8 hours respectively (Figure 5.6A). By comparison, the growth rate of both the wild-type and the *slr0148* mutants was significantly slower at low temperatures than under normal growth conditions. However, growth of the *slr0148* mutant was more severely retarded than the wild-type as reflected by their doubling times, i.e., 37.5 and 80.3 hours respectively (Figure 5.6C). Under high light conditions, both the wild-type and the *slr0148* mutant grew much faster than under normal light conditions, but the growth rate of the *slr0148* mutant was slower than the wild-type (Figure 5.6D). In general, the *slr0148* mutant grew much slower under either low temperature or high light
conditions compared to the wild-type. When subjected to Fe starvation, the \textit{slr0148} mutant grew much faster than the wild-type (Figure 5.6B).

\textit{slr0148 involved in an operon spanning from slr0144 to slr0151}

\textit{slr0148} is situated in a gene cluster extending from \textit{slr0144} to \textit{slr0152}. The distance between each two adjacent genes from \textit{slr0144} to \textit{slr0150} is no longer than 55 bp. \textit{slr0144} and \textit{slr0145} overlap by 3 bp. This organization strongly suggests that the genes in this cluster form an operon. To test this hypothesis, 5’-primers of \textit{slr0143} through \textit{slr0150}, i.e., 6RT0143F, 6RT0144F, 6RT0145F, 6RT0146F, 6RT0147F, 6RT0148F, 6RT0149F, 6RT0150F, and 6RT0150F were combined with 3’-primers of \textit{slr0144} through \textit{slr0152} respectively, i.e., 6RT0144R, 6RT0145R, 6RT0146R, 6RT0147R, 6RT0148R, 6RT0149R, 6RT0150R, 6RT0151R, and 6RT0152R to form primer pairs for amplifying DNA fragments consisting of sequences of any two adjacent genes by RT-PCR. DNA fragments with size of 1.79 kb, 1.18 kb, 0.92 kb, 1.17kb, 0.95 kb, 0.8 kb, 0.8 kb, and 1.27kb corresponding to \textit{slr0143_slr0144}, \textit{slr0144_slr0145}, \textit{slr0145_slr0146}, \textit{slr0146_slr0147}, \textit{slr0147_slr0148}, \textit{slr0148_slr0149}, \textit{slr0149_slr0150}, and \textit{slr0150_slr0151} were amplified (Figure 5.7). The DNA band corresponding to \textit{slr0143_slr0144} is much weaker compared to other bands. This result clearly indicates that this gene cluster consists of one operon spanning from \textit{slr0144} through \textit{slr0151}, and possibly also including \textit{slr0143}. 
Transcription of slr0148 and genes involved in the operon under various stress conditions

The expression of slr0148 and the genes involved in the operon in Synechocystis sp. PCC 6803 cells grown under high light conditions, low temperature conditions, in medium with addition of H₂O₂, or iron limitation conditions was investigated by RT-PCR analysis. As shown in Figure 5.8, compared to standard growth conditions, transcription of slr0144 through slr0148 was down-regulated at low temperature, while the transcription levels, except for slr0148, remained almost the same under iron limitation conditions. Expression levels of slr0145, slr0146, slr0147, and slr0148 were also reduced under oxidative stress conditions (H₂O₂ treatment). In contrast, no obvious changes in the mRNA level could be observed for these four genes under high light conditions. Although contained in the same operon, the slr0144 gene was differentially expressed from the others under high light conditions and H₂O₂ treatment: its mRNA level was significantly lowered under high light conditions, but remained almost unchanged by H₂O₂ treatment. Transcription of slr0149 and slr0150 did not exhibit changes as significant as that of the first five genes in the operon under any growth conditions. The mRNA level of slr0149 and slr0150 had an observable decrease only under oxidative stress conditions (H₂O₂ treatment).

Transcription of nifS-like genes under various stress conditions

There are four nifS-like homologs in Synechocystis sp. PCC 6803. Two of them, slr0387 and slr0704 show high degree of similarity to iscS. A third one, slr2143, was identified by purification of its gene product from cyanobacterial cell lysates. The fourth one, slr0077, is an sufS homolog (refer to Chapter 1). These nifS-like genes in cyanobacteria
are believed to function under different metabolic conditions. To address this issue, and to determine which one functions with Slr0148 in the ISC Fe-S cluster assembly pathway, their expression levels under various stress conditions were examined by RT-PCR analysis. As shown in Figure 5.9, under oxidative stress and iron limitation conditions, transcription of all the nifS-like genes was up-regulated. The slr0077 gene showed most significant increase in mRNA level by H$_2$O$_2$ treatment among the four nifS-like homologs. slr0387 and slr2143 showed the largest increase under iron limitation conditions. Under high light and low temperature conditions, transcription levels of the slr0077, slr0704, and slr2143 genes were up-regulated. By contrast, slr0387 did not show any changes in mRNA levels. Expression levels of slr0704 and slr2143 were more elevated in response to high light and low temperature than under oxidative stress and iron limitation conditions. The mRNA level of slr0077 at low temperature was about half that under high light conditions. In general, these four nifS-like genes were differentially expressed, and none of them exhibited the same transcription profile under our experimental conditions.
DISCUSSION

Is slr0148 the real fdx homolog?

Slr0148 was identified to be an Fdx component of the ISC machinery in *Synechocystis* sp. PCC 6803 mainly based on its similarity with Fdx from *E. coli* and *A. vinelandii* and its ability to coordinate a [2Fe-2S] cluster. The finding that the four *nifS*-like genes are differentially expressed under various growth conditions prompted us to make a comparison of growth rates of the two *iscS*-like inactivation mutants and the *slr0148* disruption mutant. The results, that these mutants displayed similar growth phenotypes (Figure 5.10), provide further evidence that Slr0148 is an Fdx homolog of the ISC Fe-S cluster assembly machinery. The question is why *slr0148*, together with the remainder of the operon, exists only in a few cyanobacterial strains. In comparison, homologs to *iscS, iscA,* and *nifU* are present in all the cyanobacterial strains whose genomes have been sequenced. As discussed in the introduction, the Fdx-Fdx reductase system is crucial for Fe-S cluster assembly, and in some organisms, is even essential for cell survival. There are no reports showing that Fdx is missing from the ISC pathway in any organism. Hence, it would be appropriate to assume that Fdx homologs do exist in other cyanobacteria. Note that the similarities between the cyanobacterial Fdx homologs and the Fdx homologs from other organisms are relatively low. The 50 amino-acid C-terminal extension in Slr0148 makes this difference even larger. However, these differences may suggest that Slr0148 participates in two bioprocesses, one being the ISC-mediated Fe-S cluster assembly, and the other related to the operon spanning from *slr0144* through *slr0151*. Many ferredoxins have functions in more than one bioprocesses, such as the [2Fe-2S] ferredoxin that accepts electrons from PS I.
during photosynthesis. It can provide electrons for NADP$^+$ reduction, nitrate reduction, and sulfate reduction (Knaff and Hirasawa, 1991). Given this assumption, the C-terminal extension of Slr0148 might be involved in an interaction with the proteins encoded by other genes in the operon. On the other hand, cyanobacterial strains lacking slr0148 homologs may use other ferredoxin-like proteins to provide electrons needed during the Fe-S cluster assembly process. Cyanobacteria contain multiple ferredoxin-like proteins, many of which are not assigned any function. For example, Synechocystis sp. PCC 6803 contains an ORF, ssl3044, which encodes a putative ferredoxin protein with 99 amino acids. This ORF was identified by a BLAST search against the Synechocystis sp. PCC 6803 genome using the cysteine motif $\text{CX}_5\text{CX}_2\text{C}$. An amino acid alignment indicates that Ssl3044 shows significant similarity (30 identity) with the Fdx protein from E. coli (data not shown). A BLAST search against Cyanobase using the deduced amino acid sequence of Ssl3044 found homologs in most cyanobacterial strains. The matched amino acid length, high similarity with the Fdx protein of E. coli, and wide occurrence among cyanobacteria make Ssl3044 another suitable candidate for the Fdx component of the ISC machinery.

Nonessential $\text{slr0148 (fdx)}$ strengthens argument that ISC is a minor pathway in cyanobacteria

Our gene inactivation study showed that $\text{slr0148}$ is not essential for the growth of Synechocystis sp. PCC 6803. The $\text{slr0148}$ inactivation mutant did not show any phenotype under standard growth conditions. This result is consistent with the mutagenesis study conducted on other ISC components in cyanobacteria, such as $\text{sll0704}$ and $\text{slr0387}$ (Figure
two *iscS* homologs, and *slr1417* and *slr1565*, two *iscA* homologs (unpublished data). These four ORFs are dispensable in *Synechocystis* sp. PCC 6803 (*iscS*) or in *Synechococcus* sp. PCC 7002 (*iscA*) under normal growth conditions. Strains lacking any one of these genes showed almost no phenotype under standard growth conditions. These results suggest that unlike other organisms, such as *E. coli* and *A. vinelandii*, the ISC machinery is not a major Fe-S cluster assembly pathway in cyanobacteria. *Synechocystis* sp. PCC 6803 contains homologs of all the *isc* genes except *iscU*. There is only one ORF, *ssl2667* whose gene product shows homology to the C-terminal domain of NifU. Surprisingly, *ssl2667* could not be completely inactivated in *Synechocystis* sp. PCC 6803, implying that it must participate in an essential Fe-S cluster assembly pathway in this cyanobacterium (Nakai et al., 2002). *A. thaliana* contains five putative *nfu* homologs. Three are predicted to be targeted into the chloroplast. Their localization in the chloroplast was recently confirmed by GFP-fusion analysis (Leon et al., 2003). As mentioned in Chapter 1, the SUF Fe-S cluster assembly pathway is essential in cyanobacteria and *A. thaliana*, as demonstrated by the mutagenesis studies conducted on *sufB*, *sufC* and *sufS*. Mutants of these three *suf* genes could not be completely segregated. Also, it is very likely that the SUF machinery is the only Fe-S cluster assembly pathway in chloroplasts (refer to Chapter 2). These studies strongly suggest that Ssl2667 (Nfu) might be a component of, or interact with, the SUF machinery.

*The four nifS-like homologs are transcribed differentially under various stress conditions*

Among the four NifS-like homologs in cyanobacteria, Slr0077 has been clearly identified as the gene that codes for the SufS protein that participates in the SUF Fe-S
cluster assembly pathway of *Synechocystis* sp. PCC 6803 (Tirupati and Bollinger, personal communication). Slr0387 and Sll0704 are identified as encoding IscS-like proteins based on amino acid sequence similarity and the cysteine desulfurase activity of the recombinant proteins. A fourth candidate, Slr2143, was identified to be a NifS-like protein by purification from the cyanobacterial cell lysates followed by biochemical characterization (Leibrecht and Kessler, 1997). Only slr0077 has been shown to increase its expression level under oxidative stress and iron limitation conditions (Chapter 2). The physiological roles of the remaining three genes are unknown. This is the first report to show that these four nifS-like homologs are differentially expressed under high light, low temperature, oxidative stress, and iron limitation conditions, and that transcription of slr0077 also responds to high light and low temperatures. Integration of our RT-PCR and growth data provided us with an opportunity to gain further insight into the ISC Fe-S cluster assembly pathway. Under standard growth conditions (Figure 5.10A), the three insertional inactivation mutants, slr0148, slr0387, and sll0704, had the same growth rate as the wild-type (compare Figure 5.6A and Figure 5.10A). Although sll0704 was highly expressed under high light conditions, its inactivation did not result in a more severe growth retardation than the mutations in slr0148 and slr0387 that were constitutively transcribed (Figure 5.10D). This is possibly because of the complementation made by slr0077 and slr2143, whose transcription was also significantly up-regulated under high light conditions. The largest difference in growth was under low temperature conditions (Figure 5.10C). The slr0148 and sll0704 mutants showed very similar growth rates, but the growth rates were significantly lower than that of the slr0387 mutant, suggesting that sll0704 may be the leading player in Fe-S
cluster assembly under low temperature conditions. This was further supported by RT-PCR data showing that the transcription levels of \textit{sll0704} were the most elevated. \textit{slr2143} may also play an important role in response to low temperatures, because its expression was up-regulated in a manner similar to that of \textit{sll0704}. Under iron limitation conditions (Figure 5.10B), all three mutants grew faster than the wild-type, and the growth rate of the \textit{slr0148} mutant was much higher than that of the \textit{slr0387} and \textit{sll0704} mutants, implying that \textit{slr0148} might encode the common Fdx protein for both IscS-like homologs. In chapter 2, it was shown that SufR also regulates the transcription of \textit{slr0387} in an opposite manner to \textit{slr0077}. However, under these stress conditions, \textit{slr0387} and \textit{slr0077} were not expressed in a similar pattern, indicating that other transcription factors may also regulate their expression. A strong candidate would be \textit{slr0846} in \textit{Synechocystis} sp. PCC 6803. Its gene product shows 60% similarity with the IscR protein from \textit{E. coli} (http://www.kazusa.or.jp/cyano). Interestingly, the three cysteine residues that coordinate a [2Fe-2S] cluster in the IscR protein are missing in Slr0846 and other cyanobacterial IscR homologs. It is thus reasonable to assume that Slr0846 does not respond to oxidative stress; rather, this task is accomplished through SufR. If true, it can be further assumed that holo-SufR acts as the repressor of the \textit{suf} operon, and that apo-SufR is the activator of \textit{slr0387}. In the absence of the SufR protein (the \textit{sufR} null mutant), transcription of \textit{slr0077} would be up-regulated, and \textit{slr0387} would be down-regulated. This prediction is consistent with the RT-PCR data presented in Figure 2.8. Under oxidative stress conditions (H₂O₂ treatment), the protein level of holo-SufR decreases and apo-SufR increases, and as a result, transcription of both \textit{slr0077} and \textit{slr0387} are elevated as indicated in Figure 5.9. In this model, Slr0846 (the
IscR homolog) may function under other stress conditions. Unfortunately, no slr2143 inactivation mutant is available thus far. An investigation of the growth phenotype of an slr2143 mutant would definitely shed more light on the presence of the multiple nifS-like genes in cyanobacteria.

**slr0148 involved in an operon with unknown functions**

Our RT-PCR data shows that slr0148 is found in a large operon consisting of at least eight genes. Transcription of the genes in this operon was found by microarray analyses to be repressed in response to low temperature, high light, Fe deficiency, or oxidative stress (Hihara et al., 2001; Singh et al., 2004; Suzuki et al., 2001). Our RT-PCR data showed some variations from these microarray analyses. For example, under high light conditions, only the transcription of slr0144 was significantly down-regulated in our experiment. Under oxidative stress conditions, slr0144 showed the least changes in mRNA levels, while it was one of the highest in the microarray analysis. The transcription of the operon was not repressed under high light and Fe-limiting conditions as significantly as indicated by microarray analyses (Hihara et al., 2001; Singh et al., 2004). These variations may be caused by the differences in the treatment of cells. For example, the cells were grown in the standard growth conditions and were then subject to iron-starvation for overnight in this report, while in microarray analysis, the cells were firstly iron-starved for several days and then supplied with Fe (Singh et al., 2004). The largest difference is that these genes were not coordinated as closely as suggested by the microarray analyses under certain stress conditions. The slr0149 and slr0150 genes did not show any significant transcriptional
changes under any growth conditions in this report, suggesting that these two genes may have their own promoter(s) and thus be differentially transcribed than the other genes in the operon, and/or that their mRNAs are processed and more stable than mRNAs of the other genes.

Despite these differences, our RT-PCR data confirmed the involvement of this gene cluster in response to stress conditions. However, the functions of virtually all of the genes in this operon are unknown. A conserved protein domain search through NCBI gives us some useful information on their potential function. For example, Slr0144 is predicted to be a hydrocarbon binding protein containing V4R (4-vinyl reductase) domain. This motif is a small molecular binding domain that may bind hydrocarbons. This domain is present in a protein involved in chlorophyll biosynthesis and in a regulator of the phenol catabolic pathway (Anantharaman et al., 2001). The 4-vinyl reductases involved in chlorophyll biosynthesis link the divinyl to monovinyl chlorophyll biosynthetic routes (Kolossov et al., 2001). Slr0147 also contains a V4R domain. slr0144 and slr0147 are the only two ORFs whose gene products contain the V4R domain in Synechocystis sp. PCC 6803 (Singh et al., 2004). They share 44% similarity in amino acid sequence. slr0146 and slr0149 also exhibit ~39% similarity in their deduced amino acid sequences. Their gene products are predicted to be phycobilisome-like proteins that contain bilin-binding motifs. Slr0145 shows some similarity with the sterol regulatory element binding protein (SREBP) cleavage-activating protein (SCAP). Slr0150 is a ferredoxin-like protein. Slr0151 contains multiple putative domains, such as KOG4648 that contains LRR repeats with an unknown functions; KOG0547, a domain in translocase of outer mitochondrial membrane complex, which
functions in intracellular trafficking, secretion, and vesicular transport; KOG0376, a serine-threonine phosphatase 2A domain; TPR (tetratricopeptide repeat domain) that is involved in a variety of functions including protein-protein interactions; and an Hsp70-interacting domain. The last gene, slr0152, encodes a putative serine-threonine protein kinase (PknB). Taken together, this gene cluster encodes proteins that could function in signaling (Slr0151 and Slr0152), electron transfer (Slr0148 and slr0150), pigment binding (Slr0146 and Slr0149), and a membrane-associated complex (Slr0151). Because so many genes apparently work together under various stress conditions, it is reasonable to assume that they are involved not only in signaling but also in downstream processes that help cells adapt or adjust to different environments. To elucidate how these functions are co-coordinated in response to various stress conditions would be a challenging task. However it would not be a surprise if they were found to be involved in photosystem I assembly and/or turnover. Under stress conditions the whole gene cluster was repressed, transcription of PS I genes was also reduced, and in PS I-less cyanobacterial mutants, this gene cluster was also down-regulated (Singh et al., 2004). Elucidation of the function of this operon would also help understand why it only exists in a few cyanobacterial strains.

**slr0148 functions in response to low temperature condition**

Our RT-PCR data shows that transcript levels of slr0148 decrease significantly under low temperature conditions. Transcription of the PS I genes was also down-regulated (Suzuki et al., 2001). PS I contains three [4Fe-4S] clusters. A reduction in the amount of PS I complexes reflects a decreased requirement for Fe-S clusters in these cells. However, the
parallel down-regulation of the transcription of slr0148 may not be related to its function as a component of the ISC pathway, but rather be related to the coordinated repression of the operon extending from slr0144 to slr0151. In other words, changes in the expression of the part of slr0148 that is the component of the ISC pathway may be covered by the part of slr0148 with function related to the operon and thus may not be discernable, as the expression level of the whole operon is much higher than the four nifS-like genes (compare Figure 5.8 and Figure 5.9) and the two functions of Slr0148 may respond to the same stress conditions. Interestingly, fdx is up-regulated along with hscB and hscA, in E. coli under cold shock (Kiley, University of Wisconsin, in Marburg Meeting of Fe-S proteins, Germany). E. coli uses the ISC machinery as the major Fe-S assembly pathway. Transcription of the whole iscSUA-hscBA-fdx gene cluster is negatively regulated by the [2Fe-2S] IscR protein. DNA microarray analysis also demonstrated that transcription of the isc operon was up-regulated under oxidative stress conditions. By contrast, cyanobacteria use SUF as the major pathway for Fe-S cluster assembly, their isc genes do not form an operon, and their IscR homologs do not contain cysteine residues. Based on these differences, it is likely that cyanobacteria and E. coli apply different strategies in coping with various stress conditions.
REFERENCES:


Table 5.1 Primers and their sequences used for RT-PCR analysis, mutagenesis of slr0148 in *Synechocystis* sp. PCC 6803, and cloning of slr0148 for protein overexpression in *E. coli*.

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FIGURE LEGENDS

Figure 5.1 Amino acid alignment of Slr0148 from *Synechocystis* PCC. 6803, Tlr2302 from *Thermosynechococcus elongatus*, SYNW1981 from *Synechococcus* WH 8102, and an Fdx homolog from *Synechococcus* sp. PCC 7002 with fdx homologs from *A. vinelandii* and *E. coli*. Cyanobacterial Fdx homologs have an obvious 50 amino-acid extension at the C-terminus. The four conserved cysteines in all Fdx homologs are marked by a red letter C.

Figure 5.2 Gene organization of the gene cluster where slr0148 is involved in *Synechocystis* sp. PCC 6803, *Thermosynechococcus* elongatus, and *Synechococcus* sp. PCC 7002.

Figure 5.3 Purified recombinant Slr0148 protein forms two bands on SDS-PAGE gels. Arrows show the molecular weights of protein markers (Bio-Rad low range protein standard).

Figure 5.4 EPR spectra of the recombinant Slr0148 protein after reconstitution with iron, sulfide and 2-mercaptoethanol. Panel A shows the EPR spectra of Slr0148 at 50 K and microwave power of 40 mW. Panel B shows the signal intensity as a function of temperature and microwave power.

Figure 5.5 Insertional inactivation of the slr0148 gene in *Synechocystis* sp. PCC 6803. A) Restriction maps of DNA fragments from the wild-type and the slr0148 inactivation mutants; B) PCR analysis of segregation of the slr0148 gene insertional inactivation. The
primers are as indicated by the arrows in the lower panel of Figure B. The leftmost lane contains DNA molecular weight markers. Lanes 1 and 5 are the wild-type, lanes 2, 3, and 4 are three *slr0148* inactivation mutants in which the *aphII* gene was inserted parallel (lane 2 and 3) or antiparallel (lane 4) to the transcriptional direction of *slr0148*. All three mutants have been completely segregated.

**Figure 5.6** Growth curves of the *slr0148* inactivation mutant (///) and wild-type *Synechocystis* sp. PCC 6803 under: A) standard growth conditions; B) iron limitation conditions; C) low temperatures; or D) high light conditions. Green triangle represents the *slr0148* inactivation mutant, and red square the wild-type.

**Figure 5.7** Co-transcription of *slr0143* through *slr0151* detected by RT-PCR. Figure A shows the primer pairs. The 5’-primers of *slr0143* through *slr0150*, i.e., 6RT0143F, 6RT0144F, 6RT0145F, 6RT0146F, 6RT0147F, 6RT0148F, 6RT0149F, 6RT0150F, and 6RT0150F were combined with the 3’-primers of *slr0144* through *slr0152* respectively, i.e., 6RT0144R, 6RT0145R, 6RT0146R, 6RT0147R, 6RT0148R, 6RT0149R, 6RT0150R, 6RT0151R, and 6RT0152R to form primer pairs for amplification of DNA fragments consisting of sequences of any two adjacent genes. In Figure B, lanes from left to right are: M) molecular weight DNA marker; 1) *slr0143_slr0144*; 2) *slr0144_slr0145*; 3) *slr0145_slr0146*; 4) *slr0146_slr0147*; 5) *slr0147_slr0148*; 6) *slr0148_slr0149*; 7) *slr0149_slr0150*; 8) *slr0150_slr0151*; 9) *slr0150_slr0152*. 
Figure 5.8 Transcription levels of slr0144, slr0145, slr0146, slr0147, slr0148, slr0149, slr0150 and rnpB (which encodes the RNA component of RNase P, control) in *Synechocystis* sp. PCC 6803 cells growing under various conditions. Lanes from left to right are: 1) standard growth conditions; 2) high light conditions; 3) low temperature; 4) growth under standard growth conditions in the presence of 5 mM H$_2$O$_2$; 5) growth under iron limitation conditions.

Figure 5.9 Differential expression of the four *nifS*-like genes in *Synechocystis* sp. PCC 6803 growing under various growth conditions. 1) standard growth conditions; 2) high light treatment for one hour; 3) low temperature treatment for 20 minutes; 4) 5 mM H$_2$O$_2$ treatment for 30 minutes; 5) iron starvation for overnight; 6) standard growth conditions as in lane 1, except that the amount of RNA used was twice of that used in lane 1 for RT-PCR. *rnpB* serves as the control.

Figure 5.10 Growth curves of the slr0148, slr0387, and sll0704 inactivation mutants of *Synechocystis* sp. PCC 6803 under: A) standard growth conditions; B) iron limitation conditions; C) low temperature; or D) high light. Blue diamond represents slr0148 mutant; red square slr0387 mutant; and green triangle sll0704 mutant.
### Figure 5.1

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Figure 5.2

*Synechocystis* sp. PCC 6803

- Slr0144
- Slr0145
- Slr0146
- Slr0147
- Slr0148
- Slr0149
- Slr0150
- Slr0151
- Slr0152

*Thermosynechococcus* BP-1

- Tlr2299
- Tlr2299
- Tlr2230
- Tlr2301
- Tlr2302
- Tll0854
- Tlr1236
- Tlr2303
- Tlr2304

*Synechococcus* sp. PCC 7002

- slr0150-like
- CheY-like
- slr0148-like
- slr0144-like
- Ser/Thr
- slr0149-like
- slr0147-like
- slr0440-like
Figure 5.3
Figure 5.4

A

B

Temperature, K

126mW Fdx
100mW Fdx
80mW Fdx
40mW Fdx
20mW Fdx
10mW Fdx
1mW Fdx
0.1mW Fdx
Figure 5.5

A

B
Figure 5.6

A

B

C

D

Time (hours)

OD at 730 nm

Time (hours)

OD at 730 nm

Time (hours)

OD at 730 nm

Time (hours)
Figure 5.7

A

B

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- **A**
  - Labeled regions: 1F, 2F, 3F, 4F, 5F, 6F, 7F, 8F/9F
  - Genes: slr0143, slr0144, slr0145, slr0146, slr0147, slr0148, slr0149, slr0150, slr0151, slr0152

- **B**
  - Labeled regions: M, 1, 2, 3, 4, 5, 6, 7, 8, 9
  - Markings: 2000, 1500, 1000, 800, 600
Figure 5.8
Figure 5.9

1  2  3  4  5  6

slr0077

slr0387

sll0704

slr2143

rnpB
Figure 5.10
Chapter 6. Concluding Remarks

The work presented in this dissertation expands our understanding of the Fe-S cluster assembly in cyanobacteria, especially the regulation of the Fe-S cluster assembly in PS I. It is concluded that unlike many other organisms that use ISC as the major Fe-S cluster assembly pathway, in cyanobacteria, SUF is the major Fe-S cluster assembly pathway. Cyanobacteria have a specific transcriptional repressor, SufR to regulate the expression of suf genes, through which SufR regulates PS I assembly/biogenesis. SufR also positively regulates the transcription of the slr0387-like gene (iscS homolog), indicating that SufR controls a regulon and may also participate in the regulation of the ISC pathway. It is shown that SufR is a [4Fe-4S] protein by EPR and Mössbauer spectroscopy. SufR may use the [4Fe-4S] cluster as a sensor to regulate the expression of the regulon. The SufR protein is distributed in the cytosol and thylakoid membranes and may assume a dual function. More extensive work is needed to describe its complete function, such as its DNA-binding ability, the assembly of [4Fe-4S] cluster in living cells, and the function of its membrane-associated form. Further studies are currently underway in Professor Golbeck’s laboratory.

The up-regulation of sufS vs. the down-regulation of the iscS-like genes, especially the slr0387-like gene, in a sufR null mutant of Synechococcus sp. PCC 7002 suggests that the SUF and ISC pathways are coordinately controlled in cyanobacteria. Further study shows that the four nifS-like homologs are differentially expressed under various stress conditions. However, both iscS-like genes are not transcribed in an opposite manner to the slr0077-like gene (sufS-like) as was true in the sufR null mutant, clearly indicating that other factors are involved in the regulation of their expression. The responsible factors remain to
be identified. An ORF, *slr0846* in *Synechocystis* sp. PCC 6803 would be a strong candidate, because its gene product shows high sequence similarity with IscR from *E. coli*. However, the deduced amino acid sequence of Slr0846 does not contain cysteine residues that can coordinate a [2Fe-2S] cluster similar to the IscR protein from *E. coli*. Based on these data, it is hypothesized that SufR senses stress conditions and regulates transcription of both *suf* and *iscS* genes in cyanobacteria in such a way that holo-SufR binds to the operator of *suf* genes, and apo-SufR binds to the operator of *iscS*. This hypothesis, together with the role of IscR homolog in regulation of the *iscS*-like genes in cyanobacteria, needs to be further investigated.

Mutagenesis studies on the *iscS*-like, *iscA*-like, and *fdx*-like genes confirm that ISC is not essential in cyanobacteria. It is an enigma as to why cyanobacteria would then maintain two *iscS*-like homologs. Physiological studies indicate that their inactivation mutants only show differences in growth under low temperatures. But their transcription is different under other more stressful conditions. Our explanation is that the other two *nifS*-like homologs come into play and thus compensate for the differences. Clearly, the interplay among these four *nifS*-like homologs needs to be further investigated in order to understand fully Fe-S cluster assembly in cyanobacteria. To add more complications to the ISC pathway, the *fdx* homolog is found only in a few cyanobacterial strains (such as *slr0148* in *Synechocystis* sp. PCC 6803), and it is involved in a large operon that is also missing in some other cyanobacteria. The function of the entire gene cluster is unknown, but is seemingly related to PS I assembly and/or turnover. The possible multiple functions of the Fdx protein encoded by *slr0148*, the further identification of *fdx* homologs in cyanobacteria,
and the function of the gene cluster slr0148 involved are challenging issues for studies on the Fe-S cluster assembly in PS I of cyanobacteria.
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PUBLICATIONS

Wang, T., G. Shen, R. Balasubramanian, D. A. Bryant, and J. H. Golbeck. slr0148, an ORF involved in an operon spanning slr0144 through slr0151, is the fdx homolog of ISC Fe-S cluster pathway in *Synechocystis* sp. PCC 6803. (In Preparation)

Wang, T., R. Balasubramanian, G. Shen, C. Krebs, D. A. Bryant, and J. H. Golbeck. A [4Fe-4S] Cluster Is Coordinated by Four Cysteine Residues in a Unique CX_{12}CX_{13}CX_{14}C Motif in SufR Protein, a Transcription Repressor of *sufBCDS* Operon in Cyanobacteria. (In Preparation)
