SufR Coordinates Two $[4\text{Fe}-4\text{S}]^{2+,1+}$ Clusters and Functions as a Transcriptional Repressor of the sufBCDS Operon and an Autoregulator of sufR in Cyanobacteria*

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Gaozhong Shen‡, Ramakrishnan Balasubramanian‡, Tao Wang‡, Yingxian Wu‡, Lee M. Hoffart‡, Carsten Krebs‡, Donald A. Bryant‡, and John H. Golbeck‡§

From the Departments of‡Biochemistry and Molecular Biology and§Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

The sufR gene encodes a protein that functions as a transcriptional repressor of the suf regulon in cyanobacteria. It is predicted to contain an N-terminal helix loop helix DNA binding motif and a C-terminal Fe/S binding domain. Through immuno blotting assays of cell extracts, the sufR product in Synechocystis sp. PCC 6803 was shown to have a mass of $\sim 25$ kDa. This indicates that the second ATG in the open reading frame is the correct start codon and that sufR encodes a protein of 216 amino acids (SufR216) rather than the originally predicted 240 amino acids. Recombinant SufR216 harbored $[4\text{Fe}-4\text{S}]^{2+,1+}$ clusters, which were present in a mixture of S = 1/2 and 3/2 ground spin states, and the holoprotein was a homodimer, containing 3.7 of non-heme iron and 3.5 labile sulfides per monomer. Thus, two $[4\text{Fe}-4\text{S}]^{2+,1+}$ clusters are coordinated by each SufR216 homodimer. SufR216 bound to two DNA sequences in the regulatory region between the divergently transcribed sufR gene and the sufBCDS operon, and its binding affinity depended on the presence and redox state of the $[4\text{Fe}-4\text{S}]^{2+,1+}$ clusters. A high affinity binding site, which controls sufBCDS expression, and a low affinity binding site, which controls sufR expression, were identified. The SufR binding sites, which are separated by 26 base pairs, each contain a perfect inverted repeat, CAAC$_{N_{-}}$GTTG, and are highly conserved in cyanobacteria. The Fe/S protein SufR thus functions both as a transcriptional repressor of the sufBCDS operon and as an autoregulator of sufR.

Iron-sulfur (Fe/S)$^2$ clusters are highly versatile prosthetic groups found in a wide variety of proteins in all living organisms. They perform indispensable functions including electron transfer, redox sensing, gene regulation, catalysis, and maintenance of protein structure (1–3). In recent years, significant progress has been made in understanding the mechanism of Fe/S cluster assembly and in identifying proteins that are involved in the transfer of nascent Fe/S clusters to target apoproteins (4 – 6). These advances have led to the construction of models for Fe/S cluster assembly and have defined research aimed at elucidating both general and stress-related Fe/S cluster assembly mechanisms (5, 7, 8).

Cyanobacteria have two major Fe/S cluster assembly systems, denoted ISC and SUF (9 – 12). In nitrogen-fixing bacteria, including cyanobacteria, an additional NIF system is required specifically for the assembly of Fe/S clusters destined for the nitrogenase enzyme (4 – 6, 13, 14). The function of the proteins encoded by the suf and isc regulons have been studied using reverse genetics in the cyanobacteria Synechococcus sp. PCC 7002 and Synechocystis sp. PCC 6803 (9, 15, 16). Unlike genes in the isc regulon, many of the genes in the suf regulon are essential in cyanobacteria. For example, both of the cysteine desulforase genes, iscS1 and iscS2, can be inactivated, whereas the suff gene cannot (9). Additionally, analogs of the suf system are localized in the plant chloroplasts, which do not have the ISC system. These observations imply that biogenesis and maintenance of the Fe/S clusters, including those required in the photosynthetic machinery, are primarily produced by the Suf system in oxygenic photosynthetic organisms.

The biogenesis of Fe/S clusters is a highly regulated process. Several cyanobacterial genes, including suf (15, 16), sufA and iscA (10), and nfu (11), have been proposed to encode proteins that participate in the regulation and/or bioassembly of Fe/S clusters. The sufR gene (sll0088 in Synechocystis sp. PCC 6803) is an open reading frame located directly upstream of the conserved sufBCDS operon in most sequenced cyanobacterial genomes (15, 16). It has been shown to function as a negative regulator of the suf regulon in response to redox stress, oxidative stress, and iron stress (15). The SufR protein is highly conserved and contains two interesting features: 1) an N-terminal domain containing a helix loop helix motif characteristic of DNA-binding proteins, and 2) C-terminal domain containing four conserved Cys residues that may provide ligands to an Fe/S cluster. The four Cys residues are separated by 12, 13, and 14 amino acid residues, respectively; if these Cys residues could be shown to provide the ligands for an Fe/S cluster, this conserved $\text{CX}_{12}\text{CX}_{13}\text{CX}_{14}\text{C}$ arrangement would be both new and unique (Synechococcus sp. PCC 6803 also contains a Cys residue near the N terminus at position 15, but this Cys is not present in 12 other cyanobacteria whose genomes have been sequenced).
Characterization of the SufR Transcriptional Repressor

TABLE 1

List of the oligonucleotides used for generating the SufR216 variant (pET24a/sufR), and the site-directed mutants of sufR

| Construct | Primer name | Sequence |
|-----------|-------------|----------|
| pET24a/sufR | sufRNdelF | 5’-CTCTCTCTTGTTGGTCTATACCCCTCAATTC-3’ |
| C164S | C177S | 3’-GAGAGAGACCAACACGTCTACATGGCTGGATCAAGAAG5’ |
| C191S | C206S | 5’-TCGCCGGATCGCTCCATGAAAG-3’ |
| suf-P-DNA1 | suf-P-DNA1F | 3’-CATTCTGCCGAGTCCGCACTCGGTACATGCTC-3’ |
| suf-P-DNA2 | suf-P-DNA2F | 5’-GTTTTAACAGTGGTTGGAGAAGC-3’ |
| Primer extension | PEf | 5’-ATGTTGCGACAGTGCGATC-3’ |
| | PER1 | 5’-TCGACCCAGGGGAGTACAG-3’ |
| | PER2 | 5’-GGCCATATATCGTAACTACCCCTAG-3’ |

Based on a comparison of the transcript levels of suf genes in the wild-type and suf null-mutant strains of Synechococcus sp. PCC 7002, it was proposed that the sufR product functions as a transcriptional repressor of the suf regulon (15). The identity and properties of the Fe/S cluster, the identification of operator sequences, and the mechanism by which the sufR product regulates the suf genes are investigated in this paper.

EXPERIMENTAL PROCEDURES

Generation of the Expression Construct for a Variant of SufR with 216 Amino Acids—Based on a comparison of the predicted amino acid sequences in several cyanobacteria, the sufR gene in Synechocystis sp. PCC 6803 is likely to encode a protein that contains 216 amino acids (SufR216) rather than the 240 amino acids (SufR240) that were originally predicted (www.kazusa.or.jp/cyanobase). To obtain SufR216, an NdeI restriction site was introduced at the second ATG codon of the predicted open reading frame by PCR using the pET24a/sll0088 plasmid (15) as the template and sufRNdel primers (Table 1). A 72-bp DNA sequence at the 5’ end of the annotated sll0088 gene was deleted by incubating the PCR product with the restriction enzyme NdeI. The truncated plasmid (renamed pET24a/sufR) was transformed into BL21(DE3) for overproduction of SufR216.

Generation of Cys to Ser Variants of SufR216—Using the primers listed in Table 1, PfuUltra™ HotStart DNA Polymerase (Stratagene, La Jolla, CA) was used to generate the mutated sufR gene with codon changes at specific sites. After the PCR, DpnI was added into the reaction mixture to digest the wild-type plasmid that was used as the template. The reaction mixture (2 μl) containing the mutated sufR gene was used to transform Escherichia coli strain DH10B. DNA sequencing was performed to verify the sequence of all plasmids used for protein production.

Reconstitution of the Iron-Sulfur Cluster with 57Fe—The Fe/S cluster in recombinant SufR216 was reconstituted as described (15). To incorporate 57Fe for Mössbauer studies, 4.5 mM 57Fe was used in the reconstitution protocol. In particular, 57Fe (18 mg; 0.315 mmol; >95% isotopic enrichment) was converted to 57FeSO4 by adding the metal to 1 M H2SO4 (0.8 ml) and heating to 100 °C in a silicon oil bath. After the conversion to 57FeSO4 was complete, the pH of the solution was adjusted to ~6.0 by the addition of saturated sodium bicarbonate. All other procedures were identical to those previously described (15, 18).

Protein Purification and Quantitation—SufR216 was over-produced in E. coli strain BL21(DE3) and purified using a procedure similar to that described for SufR240 (15). The protein was purified by size exclusion chromatography using Sephacryl S-300, and the purity of the protein was verified by analytical PAGE in the presence of SDS. The protein concentration was determined using the dye-binding method of Bradford (19). Because of the known problems in determining protein concentration using bovine serum albumin as the standard, IgG was also used. Quantitative amino acid analyses (University of Iowa, Amino Acid Facility) of SufR216 permitted a correction factor (0.94 for IgG, and 0.47 for bovine serum albumin) to be applied when the protein concentration was estimated by the dye-binding method.

Protein Electrophoresis and Immunoblotting—Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described (20). PAGE of native proteins was performed using the same buffer system as for SDS-PAGE except that SDS was omitted. Purified protein (30 μg total) was loaded onto each lane. The electrophoresis was performed at 120 V for 5 h. The gels were stained with Coomassie Brilliant Blue (R-250).

Low Temperature EPR Spectroscopy—EPR spectra were recorded at cryogenic temperatures with a Bruker ECS106 X-band (9.2 GHz) spectrometer that was operated with an ER 4012 ST resonator and an Oxford liquid helium cryostat. The microwave frequency was determined with a Hewlett-Packard 5340A frequency counter. Spin quantitation of the S = 1/2 ground state resonances was carried out under nonsaturating conditions of temperature and microwave power using paramagnetic Cu(II) standard solutions.

Mössbauer Spectroscopy—Mössbauer spectra were recorded on spectrometers from WEB Research (Edina, MN), which were operated in the constant acceleration mode in transmission geometry. Spectra were recorded with the temperature of

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the sample at 4.2 K maintained by a liquid helium cryostat. For low-field spectra, the sample was kept inside an SVT-400 Dewar (Janis, Wilmington, MA), and a magnetic field of 53 mT was applied parallel to the γ-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 fields between 0 and 8 T parallel to the γ-beam. The isomer shifts quoted are relative to the centroid of the spectrum of a metallic foil of iron at room temperature. Data analysis was performed using the program WMOSS from WEB Research (Edina, MN).

**Optical Spectroscopic Determination of Fe/S Cluster Content**—The cluster concentration in the chemically reconstituted SufR216 sample was estimated by measuring the absorbance of the as-reconstituted sample (with [4Fe-4S]$_2^{2+}$ clusters) at 410 nm. The molar extinction coefficient was estimated as 15,000 M$^{-1}$ cm$^{-1}$ per [4Fe-4S]$_2^{2+}$ cluster, a typical value for ferredoxins that contain [4Fe-4S]$_2^{2+}$ clusters (21).

**Chemical Analysis of Iron, Sulfide, and Protein**—The chemically reconstituted SufR216 sample was washed twice with 50 mM Tris-HCl buffer, pH 8.3, and concentrated to remove free iron and sulfide prior to analysis. The iron content of holo-SufR216 was measured using the method of Beinert (22) except that ferrene was used as the detection reagent instead of bathophenanthroline disulfonic acid. The concentration of iron was estimated by measuring the absorbance of the product, iron-ferrene complex at 593 nm, which was compared with a standard curve prepared from dilutions of fresh ferrous ammonium sulfate solutions. The acid-labile sulfide content of holoSufR216 was measured using a previously described procedure (23). A freshly opened vial of Na$_2$S was used to prepare standard solutions, and the absorbance of the product, methylene blue, was measured at 670 nm. For iron, sulfide, and protein analyses, two independent preparations of SufR216 were assayed to ensure reproducibility. Six dilutions of each SufR216 sample were measured relative to the standard curves of iron (ferrous ammonium sulfate), sulfide (sodium sulfide), and protein (dye binding using bovine serum albumin as standard). This results in a standard curve for each sample. The slopes of the sample and the standards are compared to obtain the iron, sulfide, and protein concentrations. Hence, the value obtained is an average of the estimate from each dilution, which corresponds to obtaining the average of the average and calculating the standard deviation of the average. The high linearity obtained ($R^2$ is very close to 1) indicates the low error for each data point. This lowers the standard deviation of the final result to 0.7 to 1% for each determination. The largest systematic error corresponds to the quantitative protein analysis (see above), which was used to calibrate the dye binding assay used to determine the protein concentration. It was measured for three independent samples of SufR$_{216}$ and the average value was used (see Table 2).

**Anaerobic Gel Filtration Chromatography**—The SufR$_{216}$ apo- and holo-proteins were analyzed by size exclusion chromatography using an Äkta fast protein liquid chromatography system (Amersham Biosciences), which was located in an anaerobic chamber (Coy Products, Grass Lake, MI). A Superose 6HR column was equilibrated with a buffer containing 50 mM Tris-HCl buffer, pH 8.3, and applied at a flow rate of 0.5 ml min$^{-1}$. Tris-HCl buffer, pH 8.3, was prepared similarly to the buffer used in the Fe/S cluster reconstitution protocol (15). Carbonic anhydrase, alcohol dehydrogenase, bovine serum albumin, β-amylase, and cytochrome c were used as molecular mass standards. Blue dextran was used to determine the void volume of the column.

**DNase I Footprinting**—For DNase I footprinting reactions, a shorter 250-bp fragment was used (suf-P-DNA2). This fragment was amplified using suf-P-DNA2 primers listed in Table 1. $^{32}$P labeling of the DNA fragment was performed using [γ-$^{32}$P]ATP (GE Healthcare) and polynucleotide kinase (Promega, Madison, WI) following standard procedures. All binding reactions were performed in a manner similar to the gel mobility shift reactions except that they were performed aerobically using argon-purged, degassed binding buffers. The footprinting reactions were performed using the protocol described in Ref. 24. DNA corresponding to 13,000 cpm was used for each binding reaction. The DNase I digestions were performed by incubating the reactions with 0.003–0.0008 units of DNase I µl$^{-1}$ for 2 min at 37 °C, and the reactions were quenched by the addition of a buffer containing 20 mM EDTA, 0.1% SDS, and 80% formamide. The fragments were separated on a 6% (w/v) denaturing acrylamide gel containing 8 M urea. Sequencing markers were prepared using a Sequenase kit (U. S. Biosciences). After electrophoresis, the gels were dried and exposed to a phosphorimaging screen, which was scanned by a Typhoon 8400 phosphorimager (GE Healthcare).

**Gel Mobility Shift Assays**—For the electrophoretic mobility shift assays, a 456-bp fragment (suf-P-DNA1) spanning the intergenic region between sufR and sufB was amplified from the genome of Synechocystis sp. PCC 6803 using suf-P-DNA1 primers (see Table 1). The binding of SufR$_{216}$ to suf-P-DNA was carried out in an anaerobic chamber. The reactions were performed in a buffer containing 100 mM NaCl, 5 mM MgCl$_2$, 5% glycerol, and 2.0 mM Tris-HCl, pH 8.0. To test the binding properties of SufR$_{216}$, three different SufR$_{216}$ proteins were prepared as: 1), apo-SufR$_{216}$ (without Fe/S clusters); 2) holo-SufR$_{216}$ (with as-reconstituted Fe/S clusters); and 3), red-SufR$_{216}$ (with reconstituted Fe/S clusters followed by chemical reduction using sodium hydrosulfite). The presence of, and the redox state of, the Fe/S clusters in SufR$_{216}$ were verified by optical absorption spectroscopy. Varying molar ratios of protein to DNA were used to assess the binding affinity of SufR$_{216}$. The mixtures were incubated for 30 min at room temperature. The reaction mixtures were separated in an anaerobic chamber by electrophoresis at constant voltage (30 V) on a 3% (w/v)
agarose gel prepared in 40 mM Tris acetate buffer, pH 8.3, 1 mM EDTA containing 5 μg of ethidium bromide ml⁻¹.

RESULTS
Identification of the in Vivo Start Codon for sufR in Synechocystis sp. PCC 6803—In Synechocystis sp. PCC 6803, the sufR product is encoded by open reading frame sll0088. According to Cyanobase (www.kazusa.or.jp/cyanobase), two closely spaced ATG start codons are present near the 5’ end of the annotated sll0088 DNA sequence. Depending upon which ATG is the correct start codon, sll0088 thus predicts a protein of either 240 (SufR240) or 216 (SufR216) amino acids. Based on a comparison of the orthologous protein sequences from several other cyanobacterial species (15), the second ATG rather than the first ATG seems more likely to be the correct start codon. The sufR gene encoding SufR216 was overexpressed in E. coli. The resulting SufR216 inclusion bodies were solubilized in urea, and the apo-protein was refolded and purified by size exclusion chromatography. As shown in Fig. 1A, the purified SufR216 apoprotein has an apparent mass of ~25 kDa on SDS-PAGE, which is in good agreement with the calculated mass of 24.6 kDa. Using immunoblotting analysis, the electrophoretic mobility of SufR that was synthesized in Synechocystis sp. PCC 6803 cells (Fig. 1B, lane 2) matched that of recombinant SufR216 (Fig. 1B, lane 3) and was demonstrably different from that of recombinant SufR240 (Fig. 1B, lane 1). These results strongly suggest that the second ATG codon in open reading frame sll0088 is the correct start codon for translation of sufR in Synechocystis sp. PCC 6803.

Reconstituted SufR216 Harbors an Oxygen-labile Fe/S Cluster—SufR240 harbors an Fe/S cluster when the recombinant apoprotein is reconstituted in vitro with inorganic reagents (15). Reconstituted SufR216 similarly shows a broad absorbance between 400 and 600 nm that is characteristic of the S → Fe charge transfer band of an Fe/S protein (data not shown). The Fe/S cluster is sensitive to dioxygen, as shown by the loss of the absorption centered around 410 nm, which diminished to about 25% of its original value in 1 h when the cuvette was opened to atmospheric oxygen (data not shown). The low-temperature EPR spectrum of the as-reconstituted sample showed no resonances around g = 2, thereby indicating that non-reduced SufR216 does not contain Fe/S clusters with a S = 1/2 ground state ([2Fe-2S]⁺, [4Fe-4S]⁺, or [3Fe-4S]⁺). The low temperature EPR spectrum of the chemically reduced SufR216 (Fig. 2, top trace) has partially resolved rhombic symmetry of a S = 1/2 ground state system with apparent g values of 2.02, 1.92, and 1.87. When the spectra of SufR240 (15) and SufR216 (this work) are overlaid, the g values of the principal components are
very similar (in fact, the g values of the low field peak at 2.02 are identical), but the line widths of the individual g components of SufR216 are broader than those of SufR216. We suspect that there is additional conformational flexibility in SufR240 as a result of the 24 additional amino acids and that these additional conformations are frozen at low temperature. The rapid spin relaxation properties of SufR240 as indicated by the maximum signal amplitude between 10 and 15 K, and the rapid decline in signal amplitude above 25 K, are more typical of a [4Fe-4S] cluster than a [2Fe-2S] cluster.

Reconstituted SufR216 Harbors a [4Fe-4S]2+ Cluster—The identity of the Fe/S cluster in SufR216 was verified by Mössbauer spectroscopy. The spectrum of 57Fe-reconstituted SufR216 was recorded at 4.2 K in a 53-mT external magnetic field oriented parallel to the γ-ray beam (Fig. 3A). The Mössbauer spectrum is dominated by a quadrupole doublet with parameters typical of a [4Fe-4S]2+ cluster: an isomer shift δ = 0.44 (2) mm/s; a quadrupole splitting parameter ΔE_Q = 1.12 (2) mm/s; and a width Γ = 0.40 mm/s. The quadrupole doublet accounts for 94 ± 3% of the total intensity of the spectrum. In addition, there is a small peak at +2.8 mm/s, which is at the position that is typical of the high-energy line of a high spin ferrous species coordinated by hard N/O ligands. The low energy line of the peak overlaps with the low energy line of the quadrupole doublet of the [4Fe-4S]2+ cluster. The ground state of a [4Fe-4S]2+ cluster is diamagnetic, and we have experimentally verified this by recording a spectrum in an externally applied 8-T magnetic field (Fig. 3B). The solid line is a spin Hamiltonian simulation assuming a diamagnetic (S = 0) ground state, and the asymmetry parameter η = 0.6. The excellent agreement between the experimental and simulated spectra corroborate the assignment of the quadrupole doublet to a [4Fe-4S]2+ cluster.

ApoSufR216 and HoloSufR216 Exist as Homodimers—Transcriptional regulators often function as homo-oligomeric proteins. When apoSufR216 was subjected to non-denaturing PAGE under aerobic conditions, its rate of migration suggested that the protein was an oligomer rather than a monomer (data not shown). Analysis of gel exclusion chromatography data, which was performed under anaerobic conditions, showed that apoSufR216 eluted as a protein with a molecular mass of ~50 kDa, indicating that apoSufR is entirely dimeric. Similarly, gel exclusion chromatography showed holoSufR216 to be a dimeric protein with a molecular mass of ~50 kDa; however, an additional, small peak at ~95 kDa indicated that holoSufR216 can also form tetramers (data not shown).

The SufR216 Homodimer Harbors Two [4Fe-4S]2+ Clusters—The ratio of the [4Fe-4S]2+ cluster to protein was determined by measuring the non-heme iron, acid-labile sulfide, protein, and [4Fe-4S]2+ cluster content of holoSufR216 (Table 2). On average, holoSufR216 contained 3.7 non-heme iron atoms and 3.5 acid-labile sulfides per protein monomer. This observation, together with the finding by Mössbauer spectroscopy that 94 ± 3% of the iron is in the form of [4Fe-4S]2+ clusters, indicated that there is one [4Fe-4S]2+ cluster per protein monomer. This argument is further supported by the optical spectroscopic measurement of the content of [4Fe-4S]2+ clusters, which showed an average of 0.94 clusters per protein monomer (Table 2). The analytical data therefore indicated that the [4Fe-4S]2+ cluster binding sites were nearly completely occupied in reconstituted SufR216. The most straightforward interpretation of these data is that each SufR216 homodimer harbors two [4Fe-4S]2+ clusters.

Cys164, Cys171, and Cys206 Provide Ligands to the [4Fe-4S]2+ Cluster—The C-terminal domain of SufR216 contains four Cys residues at positions 164, 177, 191, and 206 that are predicted to provide ligands to the [4Fe-4S]2+ cluster (15). Cys → Ser variants were generated at each Cys residue using the oligonucleotides listed in Table 1. Due to their different side chains, the replacement of Cys by Ser should provide a suitable ligand to the iron while resulting in a different electronic environment for the [4Fe-4S]2+ cluster (25). The low temperature EPR spectrum of the as-reconstituted C164S, C177S, and C206S variants showed a weak set of resonances around g = 2, characteristic of [3Fe-4S]1+ clusters. These resonances, which correspond to less than 5% of the total spin population, were missing in the C191S variant and in the wild-type. After chemical reduction (Fig. 2), the C164S, C177S, and C206S variants showed broad, nearly axially symmetric resonances around g = 2 that were significantly different from those of the wild-type protein. Nevertheless, all three variants exhibit rapid relaxation properties as judged from the temperature dependence consistent with the presence of a [4Fe-4S]1+ cluster. In contrast, the
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EPR spectrum and the relaxation properties of the C191S variant were identical to those of the wild-type protein. Thus, the most straightforward interpretation of these data is that Cys\textsuperscript{164}, Cys\textsuperscript{171}, and Cys\textsuperscript{206}, but not C191S, provide the ligands to the \([4Fe-4S]\)\textsuperscript{2+} cluster(s) in the SufR\textsubscript{216} homodimer. Because a \([4Fe-4S]\)\textsuperscript{2+} cluster requires four ligands, these results imply that the missing ligand to the \([4Fe-4S]\)\textsuperscript{2+} cluster of wild-type SufR\textsubscript{216} is provided by either a non-Cys residue or by a small molecule such as H\textsubscript{2}O.

The \([4Fe-4S]\)\textsuperscript{2+} clusters in SufR\textsubscript{216} exist in S = 1/2 and 3/2 Ground Spin States—Ferredoxins that contain \([4Fe-4S]\)\textsuperscript{2+} clusters with ligands other than Cys frequently display ground spin states other than S = 1/2 (25–27). If the \([4Fe-4S]\)\textsuperscript{2+} clusters in SufR\textsubscript{216}, are exclusively in the ground spin state of S = 1/2, then there should exist one spin per protein monomer in the g = 2 region, but if higher ground spin states are also represented, then there should exist less than one spin per protein monomer in the g = 2 region. Using Cu\textsuperscript{2+} as the standard, it was shown that 0.27 spins per protein monomer are present in the g = 2 region in holoSufR\textsubscript{216} (data not shown). Because the analytical measurements described above indicated that the \([4Fe-4S]\)\textsuperscript{2+} cluster binding sites are nearly completely filled, and because optical absorbance measurements centered at 410 nm additionally indicated that the \([4Fe-4S]\)\textsuperscript{2+} clusters can be completely reduced with sodium hydrosulfide (data not shown), evidence of resonances derived from higher (S > 1/2) ground spin states was sought to account for the missing spins.

As shown in Fig. 4, at low temperatures and high microwave powers, a broad and intense EPR signal arises between g = 5 and 6 along with the known set of resonances around g = 2. The former are similar to those of the \([4Fe-4S]\)\textsuperscript{1+} cluster in the Fe protein of Azotobacter vinelandii nitorgenase, which exists in two conformations having S = 3/2 and S = 1/2 ground spin states (28). Excluding those between g = 5 and 6, and around g = 2 (which appear distorted due to saturation and possibly the presence of additional resonances from the S = 3/2 spin system), no other resonances from Fe/S clusters are present. Thus, at low temperatures, the \([4Fe-4S]\)\textsuperscript{1+} clusters in holoSufR\textsubscript{216} are present exclusively as a mixture of ground spin states S = 1/2 and S = 3/2.

**Mapping the 5' End Point of the sufr and sufb Transcripts**—The 5' end points of the transcripts from the sufbCDS operon and the sufr gene were determined by primer extension analyses. Using the radiolabeled oligonucleotide 5'-AATTTCGGAGACCGATGGC-3', the major 5' end point of sufr transcripts was mapped to 122 nucleotides upstream from the translation start codon of the sufr\textsubscript{216} gene (noted as the bold "T" in Fig. 5). Using the radiolabeled oligonucleotide 5'-TCAGACCCAGG1GG1AGAG-3', the major 5' end point of the sufbCDS transcript was mapped to 74 nucleotides upstream from the translation start codon of the sufb gene (noted as the bold T in Fig. 5).

**DNase I Footprinting of HoloSufR\textsubscript{216} Bound onto Upstream Sequence of the sufr Operon**—DNase I footprint analysis was employed to identify specific DNA sequences to which SufR\textsubscript{216} binds in the intergenic region, denoted sufr-P-DNA2, between the divergently transcribed sufr gene and the sufbCDS operon. The DNase I footprint of holoSufR\textsubscript{216} containing \([4Fe-4S]\)\textsuperscript{2+} clusters and bound to sufr-P-DNA2, is presented in Fig. 6. Although one region of protection from DNase I digestion by SufR\textsubscript{216} could be distinguished at equal concentrations of protein and DNA (compare lanes 2 and 3), a larger molar excess of protein to DNA clearly revealed a second region of protection. Thus, it appears that holoSufR\textsubscript{216} binds to two distinct sequences (marked A and B in Fig. 6) with different affinities. Region B is more protected from DNase I digestion than is Region A. The operator sequences contain two perfect inverted repeats that are separated by 26 bp (see Fig. 5). Each inverted repeat is composed of the sequence CAAC-N\textsubscript{6}-GTGG. The second invert repeat also includes an additional 5 bases that form a larger perfect inverted repeat (TAAAACAAC-N\textsubscript{6}-GTGGTTTTA). It is possible that the existence of the conserved flanking sequences of the CAAC-N\textsubscript{6}-GTGG motif in the second binding site (Region B) is responsible for the difference in binding affinity of holoSufR\textsubscript{216}. As shown in Fig. 7, the inverted repeats to which SufR bind are highly conserved in the regulatory regions of the sufr operons in the genomes of sequenced cyanobacteria. Modeling of the DNA fragment (Fig. 6) showed an inherent curvature (31); the space-filled segments in the DNA model indicate the inverted repeat regions.

**Binding of HoloSufR\textsubscript{216} to sufb-P-DNA1 Depends on the Redox State of the [4Fe-4S]\textsuperscript{2+} Clusters**—The binding of holoSufR\textsubscript{216} to sufb-P-DNA1 was further analyzed by electrophoretic mobility shift assays using three different forms of SufR: apo-SufR\textsubscript{216} (devoid of Fe/S clusters), holoSufR\textsubscript{216} (with \([4Fe-4S]\)\textsuperscript{2+} clusters), and reduced holoSufR\textsubscript{216} (with \([4Fe-4S]\)\textsuperscript{1+} clusters).

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3 We employ the nomenclature SufR\textsubscript{340} to denote the mis-annotated protein, SufR\textsubscript{216} to describe the specifically analyzed protein encoded by the sufr gene in Synechocystis sp. PCC 6803, and SufR to describe the transcriptional repressor in general.
As shown in Fig. 8, the electrophoretic mobility of the suf-P-DNA1 fragment was shifted by apoSufR216 when compared with no additions or to a bovine serum albumin control. This result suggests that apoSufR216 has a weak but detectable binding affinity for suf-P-DNA1. A similar shift occurred with holoSufR216 but a second, more slowly migrating complex can clearly be seen. This band increased in intensity at the expense of the lower mass band until saturation was achieved at 0.9 mM holoSufR216. To determine whether the redox state of the Fe/S cluster affected the binding affinity of holoSufR216, the [4Fe-4S]2+/3+ cluster was reduced with sodium hydrosulfite prior to the mobility shift assay. As shown in Fig. 8, the binding affinity of reduced holoSufR216 was similar to that of apoSufR216. These results indicate that holoSufR216 binds to the DNA fragment that contains promoters for sufB and indicated as the corresponding −10 and −35 sequences. The transcription start site of the sufR gene is 133 nucleotides upstream of the ATG codon and the transcription start site of the sufB gene is 74 nucleotides upstream of the sufR ATG codon.

**DISCUSSION**

The C-terminal Domain of SufR and Its Role in Binding a [4Fe-4S]2+/3+ Cluster—Proteins that contain [4Fe-4S]2+/3+ clusters are typically ligated by four Cys residues, although there are instances of naturally occurring and variant proteins in which an oxygen from the side chain of Ser (29, 30), Asp (26, 27, 31, 32), or H2O/OH− (33), or a nitrogen from a His (34) can serve as one of the ligands. The highly conserved CX13CX13CX14C sequence in the C-terminal domain of cyanobacterial SufR16 represents a potentially novel [4Fe-4S]2+/3+ cluster binding motif. However, our study of SufR216 variants show that despite the presence of four conserved Cys residues in each monomer, only three are involved in ligating the [4Fe-4S]2+/3+ cluster. The fourth ligand remains unidentified, but it is likely to be oxygen from either a small molecule such as water, or an amino acid with an appropriate side chain. It is highly unlikely to be Cys12 because this residue is only present in *Synechococcus* sp. PCC 6803 and because transcription factors that contain metal binding sites such as NikR (35), ZntR, and CueR (36) have distinct domain structures in which the DNA binding domain is separate from the metal binding domain. Structure-based sequence alignments of SoxR (which contains a [2Fe-2S] cluster) to metallo-regulatory proteins indicate that distinct domains also exist for Fe/S cluster coordinating regulatory proteins (36). This assessment is strongly supported by the finding that the [4Fe-4S]1+/2 cluster in SufR216 exists in a mixture of S = 1/2 and 3/2 ground spin states. Ferredoxins that contain [4Fe-4S]1+/2 clusters with a ligand structure other than four Cys residues often show ground spin states other than S = 1/2. *Pyrococcus furiosus* ferredoxin, which contains one [4Fe-4S]2+/3+ cluster, is ligated by three Cys residues and one Asp residue and exists in a spin mixture of S = 3/2 (80%) and S = 1/2 (20%) ground states (26). Ferredoxin III from *Desulfovibrio africanus* contains two [4Fe-4S]2+/3+ clusters, one of which is ligated by four Cys residues and the other of which is ligated by three Cys residues and one Asp residue. This ferredoxin contains approximately equal proportions of a [4Fe-4S]1+ cluster with an S = 1/2 ground state and a [4Fe-4S]1+ cluster with an S = 3/2 ground spin state (27). A spin state crossover from S = 1/2 to higher spin states occurs when the second Cys in the CXXCXXCXXXCP motif of PsaC is changed by directed mutagenesis to either Ser or Asp (25). In a satisfying example of symmetry, when the Asp in ferredoxin III from *D. africanus* is changed to a Cys, the ground spin state crosses over from S = 3/2 to S = 1/2 (37). The presence of a non-Cys residue

**FIGURE 5. The intergenic region between the sufR and sufB genes.** The boxed sequence represents the operator region that is bound by SufR216 and protected from DNase I digestion. The transcription start sites are indicated by bold text and increased larger size. Invert repeats that can be identified within the operator region are indicated by arrows and by the bold. Putative σ70 promoter regions are underlined and indicated as the corresponding −10 and −35 sequences. The transcription start site of the sufR gene is 133 nucleotides upstream of the ATG codon and the transcription start site of the sufB gene is 74 nucleotides upstream of the sufR ATG codon.
is also consistent with the observation that the third Cys residue of this motif is not conserved in SufR homologs that occur in non-photosynthetic bacteria (Fig. 9). With the single exception of the SufR homolog from Vibrio cholerae, there is no conserved Cys in the third position or in any other nearby position in predicted SufR-like proteins from non-photosynthetic bacteria. The identity of the fourth ligand to the \([4\text{Fe-4S}]^{1+}\) cluster in cyanobacterial and proteobacterial SufR remains unknown, although one potential candidate is the nearly conserved Glu residue that follows two residues after the second conserved Cys residue (note that a His residue occurs in this position in SufR from Corynebacterium glutamicum ATCC 13032).

The transcription factors FNR, SoxR, and IscR also employ Fe/S clusters as sensors (1), but there does not appear to be a shared motif of either structure or function among these proteins and SufR. Nevertheless, SufR shares some functional and structural features with the oxygen regulator FNR in Bacillus subtilis (38). Both have a helix loop helix domain at the N terminus and a sensory domain with conserved Cys residues at the C terminus, and both sense redox changes via the presence of a \([4\text{Fe-4S}]^{2+}\) cluster. The absorption spectra of the holoproteins show a ratio of \(A_{420}/A_{280} = 0.42\) for FNR and 0.33 for SufR. Considering that the molar extinction coefficient at 280 nm for SufR is 1.33 times that of FNR (calculated on a per protein basis using ProtParam), the protein to cluster ratios for FNR and SufR are nearly identical (1:1.01). One \([4\text{Fe-4S}]^{2+}\) cluster per protein monomer is known to be present in FNR (38), hence, this analysis is entirely consistent with our assessment that one \([4\text{Fe-4S}]^{1+}\) cluster per protein monomer is present in SufR. The similarities between SufR and FNR can be extended to the ligands for the \([4\text{Fe-4S}]^{2+}\) cluster. Both proteins are proposed to contain three Cys ligands and one non-Cys ligand to the \([4\text{Fe-4S}]^{2+}\) cluster, although the identity of the non-Cys ligand is unknown in both SufR and FNR. It is interesting that at least two of these features are not shared by FNR from E. coli, which has an N-terminal, rather than a C-terminal, extension rich in Cys residues, and a dimeric oligomeric state that is dependent on the presence of the Fe/S cluster (39). However, the sequence identity between B. subtilis FNR and E. coli FNR is only 19.6%, which suggests that these proteins may be evolutionarily unrelated.

SoxR, an oxygen-sensing protein, contains two \([2\text{Fe-2S}]\) clusters ligated by four Cys residues in the N-terminal domain of the protein. The protein has transcription activity only when the \([2\text{Fe-2S}]\) clusters are oxidized, yet apo-SoxR, which is homodimeric, binds to the SoxS promoter with unchanged affinity (40). IscR, a transcriptional regulator that controls the function of the isc regulon in E. coli, contains an oxygen-sensitive \([2\text{Fe-2S}]\) cluster (whether two Fe/S clusters are present in the IscR homodimer has not yet been ascertained). Even
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1. The cysteine-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 sequence that was drawn from sequence analysis of SufR from Synechococcus sp. PCC 7002 and published sequences of the Synechococcus elongatus sp. PCC 7120, 29413 (Anabaena variabilis ATCC 29413), and 29133 (Nostoc punctiforme ATCC 29133). The arrows indicate the regions of invert repeats that can be identified within the consensus sequence. A sequence logo representation of the consensus sequence is also indicated.

2. Binding of SufR216 to the suf-P-DNA1 fragment by electrophoretic mobility shift assay. Lane 1, suf-P-DNA1, DNA only (control 1); lane 2, suf-P-DNA1 fragment with 0.45 mM oxidized holoSufR216 protein; lane 3, suf-P-DNA1 fragment with 0.9 mM oxidized holoSufR216 protein; lane 4, suf-P-DNA1, DNA fragment with 1.8 mM oxidized holoSufR216 protein; lane 5, the suf-P-DNA1, DNA fragment with 1.8 mM reduced holoSufR216 protein; lane 6, the suf-P-DNA1, DNA fragment with 1.8 mM apo-SufR216 protein; lane 7, the suf-P-DNA1, DNA with addition of 0.45 mM bovine serum albumin (control 2); and lane 8, DNA fragment size markers.

3. Sequence alignment of the operator regions of suf DNA from 6803 (Synechocystis sp. PCC 6803), 7002 (Synechococcus sp. PCC 7002), 6301 (Synechococcus sp. PCC 6301), 7942 (Synechococcus elongatus 7942), 7120 (Nostoc sp. PCC 7120), 29413 (Anabaena variabilis ATCC 29413), and 29133 (Nostoc punctiforme ATCC 29133). The arrows indicate the regions of invert repeats that can be identified within the consensus sequence. A sequence logo representation of the consensus sequence is also indicated.

4. The N-terminal Helix Loop helix Motif and Its Role in Binding DNA — The N-terminal helix loop domain of SufR consists of an α-helix, a loop turn, and a second α-helix that is also termed the DNA “recognition” helix (15). The role of the C-terminal domain in transcriptional regulators is to mediate protein dimerization (42, 43). As is the case with transcriptional regulators such as SoxR, and SmtB, the formation of a protein dimer is essential to the function of DNA-binding proteins. We found that the oligomerization state of SufR, as well as SufR binding to DNA, was independent of the presence or absence of the Fe/S clusters. Its ability to function as a transcriptional regulator therefore does not depend on a monomer to dimer transition but is instead dictated by whether the Fe/S clusters are present and by whether the Fe/S clusters are reduced or oxidized. The 86-bp operator region to which SufR binds exhibits a remarkable sequence similarity across widely divergent species of cyanobacteria. This observation suggests that the mechanism of gene regulation by SufR is highly conserved.

The basic residues in the helix loop helix domain of SufR define its specificity and affinity for DNA binding. Using electrophoretic mobility shift assays and DNase I footprinting, we have shown a specific interaction of SufR with the DNA between sufR and sufB, and we have identified two SufR binding sites within the promoter regions for the sufBCDS operon and the sufR gene. Although both binding sites possess identical motifs (CAAC-N6-GTTG), they exhibit different apparent binding affinities for SufR. The binding site with higher affinity is adjacent to the sufBCDS operon, and the lower affinity binding site is adjacent to the sufR gene. It is likely that the binding affinity is enhanced at the higher affinity site by the extended palindromic sequences that flank the (CAAC-N6-GTTG) motif. Based on the results of mapping of the transcriptional...
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starting points of the sufBCDS operon and the sufR gene, a possible mechanism can be envisioned for DNA binding and gene regulation by SufR. The higher affinity binding site is located between the −35 and −10 motifs of the sufBCDS promoter (Fig. 5). The binding of SufR to the operator of the sufBCDS operon is expected to block access of RNA polymerase and repress the transcription of the sufBCDS genes. The second lower affinity binding site of SufR216 is located about 20 nucleotides beyond the −35 region of sufR promoter. We propose that the binding of SufR to two sites with different affinities is the key for controlling the expression of the sufBCDS operon and the sufR gene. If the concentration of SufR with bound Fe/S clusters is sufficiently high, signaling an adequate capacity to produce Fe/S clusters, then repression of sufBCDS transcription would occur. If the level of apoSufR were to increase due to oxidative stress, iron limitation, or other environmental factors, or if the redox state of SufR with bound Fe/S clusters were to change, then derepression of the sufBCDS operon would occur, and the cell would thereby increase its capacity for Fe/S cluster biogenesis. Repression of both sufBCDS and sufR would occur only when the SufR levels were high and the population of SufR proteins contained a sufficient loading of Fe/S clusters.

Comparison to Previous Studies of SufR—During the preparation of this manuscript, a publication from Seki et al. (44) appeared that reported the presence of two sufR promoters for the sufBCDS operon in Synechocystis sp. PCC 6803: P1, which is regulated by light, and P2, which is constitutive. Using RNA isolated from the Synechocystis sp., PCC 6803 strain that is currently maintained in our laboratory, we could only identify one promoter for the sufBCDS operon and only one promoter for the sufR gene in repeated and reproducible primer extension assay experiments. Neither of these initiation start sites corresponds to P1 or P2 reported by Seki et al. (44). To avoid any artifacts due to chromosomal DNA contamination, the purity of RNA that was used in our primer extension analysis was verified by PCR assays using primers for amplification of the sufR gene and the region between the sufR and sufB genes. Although no evidence of the P2 promoter could be found in our analyses using the RNA isolated from our laboratory strain, DNase I footprint analysis consistently mapped the SufR216 binding site of higher affinity to the sequence between the −10 and −35 elements, which were deduced from the transcription initiation site that we mapped for the sufBCDS operon (see Fig. 5). Considering the transcription of the sufR gene, it is necessary to point out that the start codon of sufR was not mapped correctly in Fig. 1B in Seki et al. (44). As shown by the immunoblotting assays presented in this paper, SufR is expressed in Synechocystis sp. PCC 6803 as a protein of 216 amino acids rather than 240 amino acids. The second SufR binding site with lower affinity is located just upstream from the location of the putative −35 element of the sufR promoter as mapped by our primer extension analysis. Indeed, this finding may explain our early observation that expression of sufR was not obviously regulated by iron stress or oxidative stress (data not shown) and apparently exhibited a different regulatory response from the expression of the sufBCDS operon.

Summary and Future Perspectives—In summary, we have shown that Synechocystis sp. PCC 6803 SufR is a homodimer of 216 amino acids (per monomer) containing two [4Fe-4S]2+ clusters in a mixture of S = 1/2 and 3/2 ground spin states. Consistent with its role as a transcriptional regulator, the intergenic region between sufR and the sufBCDS operon was shown to contain two SufR binding sites with different affinities for SufR. One of the most interesting findings is that transcriptional regulators that are also Fe/S proteins frequently contain non-Cys ligands to the Fe/S clusters. The ability of these proteins to regulate transcription depends on the presence or absence of the Fe/S clusters, which by definition must be sensitive to oxygen or otherwise labile. We hypothesize that the non-Cys ligand is precisely the structural feature that confers a high degree of lability to the Fe/S clusters. That DNA bending may be involved in SufR regulation is hinted at by the presence of two binding sites with inverted repeats, by the large distance (26 nucleotides) between these sites, and by the finding that SufR can form tetramers. It is possible that binding of SufR at the two sites leads to the bending of the DNA region in the vicinity of the sufBCDS and sufR promoters, which results in coordination of the regulation of the sufBCDS operon and the sufR gene. Future studies with SufR will be aimed at identifying the non-Cys ligand to the Fe/S cluster, at testing the proposal that a Fe/S cluster ligated by four Cys residues would confer greater stability, and at characterizing a possible interaction between the low affinity and high affinity SufR binding sites in the regions between the sufBCDS operon and the sufR gene.

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