The Protein Complex Composed of Nickel-binding SrnQ and DNA Binding Motif-bearing SrnR of Streptomyces griseus Represses sodF Transcription in the Presence of Nickel*

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Nickel-responsive transcriptional repression of sodF, which codes for iron- and zinc-containing superoxide dismutase of Streptomyces griseus, was mediated through an operator (−2 to +15) spanning over the 5′ end (+1) of the transcript. Two open reading frames, SrnR (12,343 Da) and SrnQ (12,486 Da), with overlapping stop-start codons were identified downstream from sodF and found responsible for the repression of sodF. The deduced amino acid sequence of SrnR revealed a DNA binding motif and showed homology to the transcriptional regulators of ArsR family, whereas SrnQ did not show any similarity to any known proteins. When srnRQ DNA was maintained in trans in S. griseus on a multicopy plasmid, sodF transcription was highly repressed by nickel, but neither srnR nor srnQ alone showed the effect. Consistently, the sodF transcription of srnR-interrupted mutant was no longer repressed by nickel, which was complemented only with srnRQ DNA. Nickel-dependent binding of SrnR and SrnQ to the sodF operator DNA was observed only when the two proteins were provided together. The maximum protein-DNA interaction was shown when SrnR and SrnQ were present in one-to-one stoichiometric ratio. The two proteins appear to constitute an octamer composed of four subunits of each protein. SrnR directly interacted with SrnQ, and the protein interaction did not require nickel. The confirmation of SrnQ was changed upon nickel binding, which was in the ratio of one Ni²⁺ ion per protein molecule. A model is proposed in which SrnR of the protein complex senses nickel and subsequently enhances the DNA binding activity of SrnR through the protein-protein interaction.

Aerobic organisms have acquired a specific mechanism to protect themselves from reactive oxygen species (ROS) such as superoxide radical (O₂⁻) and hydrogen peroxide (H₂O₂), which can be generated from incomplete reduction of dioxygen during respiration. The ROS may cause oxidative damage to DNA, protein, and lipid (1). The Fenton reaction generating hydroxy radicals by reduction and oxidation of Fe ions in the presence of ROS has been proposed as a mechanism for cell damage (2). Major defense system against ROS comprises superoxide dismutase (SOD), catalase, and peroxidase, which concertedly convert the ROS into O₂ and H₂O (3). The SOD is distinguished into several types according to their active metals (2). The SOD containing either manganese (Mn-SOD) or iron (Fe-SOD) has been found in the cytoplasm of prokaryotic cells. The copper- and zinc-containing SOD (CuZn-SOD) has been described in the periplasm of several bacteria (4–6). In addition, two novel SODs containing nickel (Ni-SOD) or both iron and zinc (FeZn-SOD) have been characterized as cytoplasmic enzymes of Streptomyces griseus and Streptomyces coelicolor (7, 8).

The Ni-SOD and FeZn-SOD are found as homotetramers of approximately 13- and 22-kDa subunits, respectively, in S. griseus (7) and S. coelicolor (8). The expression of sodN coding for Ni-SOD of S. coelicolor (9) and S. griseus (10) increased in the presence of nickel, whereas their FeZn-SOD (sodF) expressions were regulated through the repression of sodF transcription in the presence of nickel (10, 11). Previously, we found that the nickel-responsive transcriptional repression of sodF of S. griseus was exerted through an inverted-repeat sequence (TTGCAAN-TGCAA) overlapping the 5′ end (+1, G) of sodF transcript (11). In addition, nickel-dependent interaction between cell extracts and the sodF operator DNA was confirmed by gel-mobility shift assay (11), suggesting the presence of trans-acting regulatory protein(s) responsible for the repression. To the best of our knowledge, a nickel-responsive transcriptional regulator has not been described in Gram-positive bacteria, whereas a transcriptional regulator, NikR, has been found for the repression of nik operon coding for ATP-dependent nickel transport system of Escherichia coli when nickel is provided in excess (12). An inverted-repeat sequence in the promoter of nik operon was proposed as a potential NikR-binding site (13, 14). No sequence homology was found between the inverted-repeat sequence of E. coli nik operon and the operator of S. griseus sodF gene.

In this study, we identified srnRQ coding for two small proteins mediating nickel-responsive transcriptional repression of S. griseus sodF (srn stands for sodF repression by nickel). They are located immediately downstream from sodF. SrnR and SrnQ appear to form an octameric complex composed of four subunits of each protein and then bind to the sodF operator in a nickel-responsive manner. We propose that DNA binding motif-bearing SrnR is a repressor, whereas nickel-binding SrnQ is a co-repressor. A hypothetical model for the
repression of sodF transcription by the SrnR/SrnQ complex is presented.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—**S. griseus ATCC 23345 was grown at 28 °C in YMED or YMPE medium as described previously (15, 16). S. griseus protoplasts were prepared with a modification previously (15, 17) and used for transformation with plasmid DNA, which was isolated from E. coli SURE strain (Stratagene) (18). Transformants were selected on R2YE agar plate (15). Ampicillin, streptomycin (Sm), spectinomycin (S), kanamycin (Km), and apramycin (Ap) (final concentrations of 50, 25, 25, and 50 µg/ml, respectively) were used when E. coli was cultured. Antibiotic-resistant genes Km, Apr, and thiosrepton were used at 5, and 20 µg/ml, respectively, when appropriate for S. griseus culture.

**Nucleotide Sequence Accession Number—**Nucleotide sequence of 0.86-kb Smal/SalI DNA (Fig. 1), which includes srnRQ of S. griseus, has been assigned to GenBank70 under the accession number AF1418663.

**DNA Mutagenesis and Plasmid Construction—**The initiation codon of SrnR and SmQ were mutated by PCR including overlap extension as described previously (19). The 1.0-kb PstI/SalI DNA fragment containing srnRQ (Fig. 1) was cloned into pBS (Stratagene) to generate pBSRQ and used as a DNA template. The primer 5'-TCGACCCGTTGGATACTCA-3' (A of S. griseus ATG codon was replaced by T) was used for the disruption of initiation codon of SrnR, whereas the SrnQ initiation codon was mutated using the primer 5’-CTCTCATGATGGCTGTCGT-3’ (AG was substituted for GA corresponding to the third (G) and first base (A) of the first and second codons of SrnQ, respectively, leaving the SrnR stop codon, originally TGA, as TAG). The PCR products were confirmed for the mutations through the DNA sequence analyses followed by digestion with PstI and SalI and cloning into PstI/SalI sites of pPDF666 (20) to generate pFDRQ and pFDRQ-q carrying the mutations in srnR and srnQ, respectively. Plasmid pFDRQ, as a derivative of pPDF666, contained the same 1.0-kb PstI/SalI DNA including the wild-type srnRQ (Fig. 1).

Plasmid pJK365 derived from a low copy number plasmid pXE4 (21) has the sodF regulatory DNA from −365 to +59 (+1, 5’ end of sodF transcript), which was transcriptionally fused to promoterless xye DNA (11), pMMU (5') to +9) carries the same sodF DNA as in pJK365 with the exception that a 5-bp nonspecific DNA was inserted between the left and right halves of the dyad symmetry (11). All plasmids contain transcription-translation stop Ω DNA (Smr/Spr) (22) upstream from the sodF DNA to block any fortuitous transcriptional read-through originated from the vector DNA.

**Catechol Dioxygenase Activity—**S. griseus containing xye fusion plasmids were cultured for 48 h on an YEME agar plate. Cells were broken by sonication, and catechol dioxygenase activity was measured with cell extracts as described previously (21).

**Detection of SOD Activity, Preparation of SOD, and Western Immunoblot Analysis—**Activity staining of SOD in a native polyacrylamide (10%) gel was performed as described previously (22). The FeZn-SOD of S. griseus was purified and used as an immunogen to raise antisera as described previously (12). The fusion proteins SrnR and SrnQ after the removal of GST by Factor Xa (New England Biolabs) digestion. The purified proteins were assessed for the purity and size by SDS-PAGE (12% polyacrylamide). The fusion proteins were purified from the cell lysate using glutathione-Sepharose 4B (Amersham Biosciences) followed by elution and concentration of native SrnR and SrnQ after the removal of GST by Factor Xa (New England Biolabs) digestion. The purified proteins were assessed for the purity and size by SDS-PAGE (15% polyacrylamide) (data not shown). The purified SrnR and SmQ were used as immunogens to raise antisera as described previously (7).

**Gel-mobility Shift Assay—**The DNA fragments to be run for the assay were labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega). The DNA probes (×105 cpm) were incubated at 25 °C with the purified proteins of SrnR and SmQ in the binding reaction buffer as described previously (11, 31). After a 10-min incubation with or without nickel, the reaction mixture was examined using 5% non-denaturing polyacrylamide gel as described previously (11). The results provided in this work are typical ones of at least three to four independent experiments showing virtually the same patterns.

**Far-Western Immunoblot Analysis—**Expressed SrnR and SmQ in E. coli—pBSRQ has an insert of the 1.0-kb PstI/SalI DNA containing srnRQ (Fig. 1) in the same orientation as lac promoter of pBSR, pBSR ‘Q’ and pBSRQ ‘Q’ harbored the same DNA as in pBSRQ with the exception of mutations in initiation codon of SmQ and SrnQ, respectively. Plasmids pBSRQ, pBSR ‘Q’, and pBSRQ ‘Q’ were transformed into E. coli DH5a. Expression of SmQ and SrnR was induced either in YMPE or YMED media as described previously (6.0–0.7. SrnR and SmQ were separated using Tricine-SDS-PAGE (26). The separating gel of 16.5% T, 6% C was overlaid with a 4% T, 3% C stacking gel (T denotes the total percentage concentration of both acrylamide and bisacrylamide, and C is the percentage concentration of bisacrylamide relative to the total concentration).
membranes were treated with SrnQ-specific mouse antibodies in the same buffer, reacted with goat anti-mouse IgG conjugated with horseradish peroxidase, and detected with an ECL detection system (Amer sham Biosciences).

Analytical Gel-filtration Chromatography—The complex of SrnR-SrnQ was identified through analytical Sephadex G-100 (Sigma) column (1.2 × 20.0, inner diameter × height in cm). The column buffer was 50 mM Tris-HCl, pH 7.5, containing 100 mM KCl (33). SrnR and SrnQ (60 μg each) were incubated at 25 °C for the protein interaction in the binding reaction buffer, which is the same as that used in the gel-mobility shift assay. The incubation continued with or without nickel (20 μM) for 10 min, and the samples were subjected to the column chromatography, which was run at room temperature. The A600 of the eluted fractions was measured, and SrnR and SrnQ in elutes were quantified using Tricine-SDS-PAGE (26).

CD Spectroscopy—Either SrnR or SrnQ (15 μM) was incubated at 25 °C in 10 mM Hepes, pH 7.6, containing 100 mM NaCl (14) with or without nickel (20 μM). The CD spectra of the proteins were obtained in wavelength range between 200 and 250 nm at 0.1-nm intervals with J-720 CD spectropolarimeter (Jasco). Five spectra with a resolution of 1 nm, a scan speed of 50 nm/min, and a response time of 1 s were averaged. The mean residue ellipticity was calculated using the molecular mass of each protein.

Nickel Binding Assay—Nickel binding of SrnQ and SrnR was assayed by atomic absorption spectrophotometry following equilibrium dialysis. 2-ml solution of SrnQ or SrnR (6.4 μM) was dialyzed against 0.5-liter buffer (10 mM Hepes, pH 7.6, 100 mM NaCl (14) containing different amounts of NiCl2 at 4 °C for 48 h. Unbound nickel was then removed by dialysis in ~500-fold volume of the 10 mM Hepes, pH 7.6, at 4 °C for 6 h. Metal content was determined with an AA-880 mark II atomic absorption (flame) spectrophotometer from Thermo Jarrel Ash. Each reading reported by the instrument was an average of three determinations. Bovine serum albumin was used as a control.

RESULTS

Nickel-dependent Expression of *S. griseus* FeZn-SOD Was Severely Repressed by the Multicopy Presence of the 1.0-kb DNA Downstream from sodF—The activity and protein level of FeZn-SOD were found much more repressed by nickel when the 1.0-kb PstI/SalI DNA (Fig. 1) downstream from sodF was maintained in multiple copies in cells (Fig. 2, pFDRQ). *S. griseus* harboring pFDRQ containing the sodF downstream DNA was grown on YEME agar plates supplemented with nickel up to 5 μM, and the FeZn-SOD activity and the protein level were measured at 48 h after inoculation. Although the FeZn-SOD expression of *S. griseus* having vector DNA, pFD666, decreased in proportion to nickel concentration (Fig. 2, pFD666), the presence of pFDRQ in repeated analyses always lowered the enzyme expression to a greater extent to display the activity (Fig. 2A) and the protein level (Fig. 2B) that are barely detectable at 5 μM nickel.

The sodF Downstream DNA Contains Two ORFs, SrnR and SrnQ—A sequence analysis of the sodF downstream DNA revealed two ORFs, which are oriented in the same direction as sodF, and started at 160 and 501 bp downstream from the SodF stop codon, respectively (Fig. 1). The first ORF (SrnR) consisted of 114 deduced amino acids with molecular mass of 12,486 Da, whereas the second ORF (SrnQ) revealed 110 amino acids of 12,343 Da. The initiation codon of SrnQ overlaps the stop codon of SrnR. A comparison of amino acid sequence between residues 12 and 82 of SrnR with data base revealed its homology to ArsR as a transcriptional repressor of transcription of hemolysin gene of *Vibrio cholerae* (34); 60% similarity (36% identity) to ArsR as a transcriptional repressor of arsenical resistance genes of *Staphylococcus xylosus* (35); 59% similarity (36% identity) to ArsR as a transcriptional repressor of the arsenic resistance genes of *Staphylococcus aureus* (36); 57% similarity (40% identity) to MerR acting as an activator-repressor protein involved in mercury resistance of *Streptomyces lividans* (37); and 52% similarity (42% identity) to a putative ArsR found in genome data base of *S. coelicolor* M145 at Sanger center (www.sanger.ac.uk/Projects/S_coelicolor/). Around the middle of SrnR was found a putative helix-turn-helix motif as depicted in Fig. 3. No protein displaying homology to SrnQ was found. SrnQ has remarkably high content (26%) of arginine.

![Diagram](image)
FeZnSOD expression of *S. griseus* containing *srnR* and *srnQ* in trans. *S. griseus* harboring pFD666, pFDRQ, pFDRQ, or pFDRQ was grown on YEEM agar plates supplemented with nickel (0, 0.1, or 5 µM) for 48 h. Cell extracts were prepared through sonication. Activity staining of SOD was done with 20 µg of protein/lane in 10% native polyacrylamide gel. A, Western immunoblot analysis of FeZnSOD was accomplished with 7 µg of protein/lane.

Both *SrnR* and *SrnQ* Are Required for the Repression of FeZn-SOD Expression in the Presence of Nickel—To determine which ORF(s) of the sodF downstream DNA mediates the enhanced repression of FeZn-SOD in the presence of nickel, *SrnR* and *SrnQ* were mutagenized through PCR. As illustrated in pFDRQ (Fig. 1), the initiation codon of *SrnR* was changed into TTG resulting in SrnR. The *SrnR* mutation might have polar effect on the expression of downstream SrnQ, so the 213-bp XmaIII-SrnQ DNA corresponding to the peptide from residues 9–78 of *SrnR* was in-frame deleted to generate pFDARQ (data not shown). In addition, plasmid pFDRQ was constructed to have a substitution of ATA for the initiation codon of *SrnQ*, but the *SrnR* stop codon, originally TGA, was still kept as TAG (Fig. 1).

The FeZn-SOD expression of *S. griseus* harboring either pFDRQ or pFDRQ in trans was compared with that of the control cells containing pFD666 in the presence of nickel (Fig. 2). No significant difference in FeZn-SOD expression was observed. *S. griseus* having pFDARQ showed the same results as those of the cells harboring pFDRQ (data not shown), suggesting no polar mutation for the expression of *SrnQ* by the *SrnR* mutation of pFDRQ. Thus, the results clearly indicate that *SrnR* and *SrnQ* are required together for the in trans effect of the downstream DNA in multicopies.

*SrnR* and *SrnQ* Are Expressed in *E. coli*—It was examined whether *srnR* and *srnQ* code for the polypeptides of deduced sizes. The proteins were expressed through IPTG induction from *E. coli* harboring pBSRQ, pBSRQ, or pBSRQ. The orientation of pBSRQ in these plasmids was the same as that of lac promoter, but their reading frames were shifted from that of the LacZ α-polypeptide of the plasmid. The Tricine-SDS-PAGE of the cell extracts of *E. coli* containing pBSRQ revealed two discrete polypeptides of ~12 kDa only after IPTG induction (Fig. 4, lane 4). The slow-moving band was regarded as SrnR, because it was still detected with pBSRQ (Fig. 4, lane 6). The other fast-moving one was concluded to be SrnR because it was observed with pBSRQ (Fig. 4, lane 8). The sizes of the expressed polypeptides were also similar to the deduced values of SrnR (12,343 Da) and SrnQ (12,486 Da). We did not believe that *E. coli* RNA polymerase(s) recognized the promoter for *srnR* and *srnQ*, because the polypeptides were not expressed when pBSRQ were oriented in opposite direction to the plasmid *lac* promoter (data not shown).

Multicopy Effect of *srnR* and *srnQ* on FeZn-SOD Expression Is Exerted at the Transcriptional Stage through the sodF Operator—It was determined whether the effects of *srnR* and *srnQ* in multicopies shown above was exerted at the level of sodF transcription, and if so, whether the sodF operator was involved. The sodF::xylE fusion plasmids, plJK365 (the wild-type operator) (Fig. 5) and pMin(+) (+9) (the mutated operator) (Fig. 5), were derived from pX4E, so that they can be compatible with pFD666 or its derivative, pFDRQ. The intro-
Transcription Repression of S. griseus sodF

The transcription of sodF was investigated in the presence or absence of nickel. The plasmid pIJK365, containing the sodF gene and its 256-bp upstream DNA, was transformed into cells, which had harbored either pIJK365 (left panel) or pIM5in (right panel). The transcription of sodF was measured by gel mobility shift assay. The wild-type sequence (Fig. 8A) or the mutated operator (Fig. 8B) resulted in no retardation (Fig. 8B). In contrast, the nickel-responsive repression was observed with the presence of SrnR and SrnQ. The reduction of the transcript level by pFDRQ was also observed without nickel addition, which is consistent with the observation that SrnR and SrnQ interact with the operator. However, SrnQ should be present due to the effect of overexpressed SrnR and SrnQ. The nickel-dependent binding of SrnR and SrnQ to the sodF promoter was demonstrated in gel mobility shift assay. The results were consistent with the observed effects by overexpressed SrnR and SrnQ.

Nickel-dependent Binding of SrnR and SrnQ to the sodF Operator Was Observed Only When Both Proteins Were Provided—SrnR and SrnQ were overexpressed as GST fusion proteins and purified from E. coli as described under "Experimental Procedures." The purified SrnR and SrnQ showed a molecular mass of ~12 kDa that were identified by SDS-PAGE (data not shown). The 113-bp Styl-BalI (Fig. 5) DNA containing either wild-type sequence (Fig. 5A) or the mutated operator (Fig. 5B) was used for gel-mobility shift assay. The wild-type DNA was retarded only when SrnR and SrnQ were provided together in the nickel-containing reaction mixture. The retarded band was intensified as nickel concentration increased (Fig. 5B). However, neither SrnR nor SrnQ alone shifted the DNA, even in the presence of nickel (Fig. 5A). On the other hand, the DNA including the mutated operator did not show any retardation (Fig. 5B), being consistent with the XylE activities of pIM5in (+8 +9) shown in Fig. 5. Thus, SrnR and SrnQ appear to exert the repressive effect in a nickel-responsive way through binding to the operator. Because SrnR has a DNA binding motif, it is expected that SrnR may directly interact with the operator. However, SrnQ should be present together for the repression.

The gel-mobility shift of the operator DNA was examined with the two proteins in different ratios. No retardation was observed without nickel addition, which is consistent with the lower XylE activity of pIM5in in the presence of pFDR (Fig. 5, left panel, Nickel 0). The nickel-responsive repression by pFDR Q and pFDRQ was not much different from that shown by pFD666 (Fig. 6). In addition, the half-life of sodF transcript of the cells containing pFD666 was ~13 min irrespective of the nickel treatment, which was not changed at all by the presence of srnR and srnQ (data not shown). Thus, srnRQ in multicopies highly repressed the sodF transcription in a nickel-dependent way.

Chromosomal Interruption of srnR Resulted in Deregressed Transcription of sodF Even in the Presence of Nickel—The chromosomal copy of srnR was interrupted with transcription-translation stop Ω DNA (KmR) (28) to confirm the in vivo effects of overexpressed SrnR and SrnQ. The interruption at EcoNI site of srnR (Fig. 7A), we believe, also resulted in a lack of srnQ expression because no XylE activity was observed with S. griseus containing srnR::xylE fusion construct with its 5'-DNA limited to the EcoNI site (data not shown), illustrating the dependence of srnQ expression on the regulatory DNA upstream from srnR. The genomic Southern analysis of srnR-interrupted mutant R45 (Fig. 7A) revealed the presence of 3.7-kb BamHI bands hybridized to both srnR (Fig. 7B, left panel) and the Ω DNA (Fig. 7B, right panel), confirming the correct arrangement of chromosomal interruption. Mutant R45 showed the F3n-SOD activities and sodF transcription (Fig. 7, C and D, respectively), which were no longer repressed by nickel. As expected, the mutation phenotype was complemented only when pFDRQ (Fig. 2) was introduced into R45 (data not shown). The results were consistent with the observed effects by overexpressed SrnR and SrnQ.

nickel when pFDRQ was contained in trans. Nickel 0 or 0.1 mM was added to the reaction mixture. The retardation was intensified as nickel concentration increased (Fig. 4B). However, neither SrnR nor SrnQ alone shifted the DNA, even in the presence of nickel (Fig. 4A). On the other hand, the DNA including the mutated operator did not show any retardation (Fig. 4B), being consistent with the XylE activities of pIM5in (+8 +9) shown in Fig. 4. Thus, SrnR and SrnQ appear to exert the repressive effect in a nickel-responsive way through binding to the operator. Because SrnR has a DNA binding motif, it is expected that SrnR may directly interact with the operator. However, SrnQ should be present together for the repression.

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consistently observed in the absence of one of the two proteins (Fig. 9, lanes 1 and 7). The amount of the binding complex reached the maximum level when SrnR and SrnQ were provided simultaneously at 10 μM each (lane 4). The signal intensity of the binding complex (C) was 2-fold higher than that of lanes 3 and 5 in which one of the two proteins was maintained at half-concentration (5 μM). Thus, the maximum interaction of

Fig. 7. Characterization of srnR disruptant R45. A, chromosomal disruption of srnR by Ω transcription-translation stop DNA (Km') (28). B, genomic Southern hybridization analysis of R45 and single cross-over (SCO) recombinant for comparison with wild type (WT). The chromosomal DNA was digested with BamHI and probed with PCR-amplified srnR DNA (left panel) or probed with 2.2-kb transcription-translation stop Ω DNA (Km') (right panel). C, the extracts from wild-type and R45 mutant, which had been grown in the presence of nickel (0, 5, or 20 μM), were used for the activity staining of SOD as described in Fig. 2. D, Northern (RNA) hybridization analysis of sodF transcript of wild type and R45. Total RNA was isolated from the cells harvested at 6 h after treatment of culture with nickel (0, 5, or 20 μM). The same RNA probe as used in Fig. 6 was used.

Fig. 8. Gel-mobility shift assay of sodF regulatory DNA (113-bp StyI/BalI in Fig. 5) with purified SrnR and SrnQ. Wild-type and mutated sequence of sodF operator are indicated below the gel. The mutated StyI/BalI DNA was prepared from pIM5in (lanes 1 and 3). Reaction mixtures containing 400 pmol (10 μM) of SrnR and/or SrnQ were incubated in the absence or presence of nickel (5, 10, and 20 μM). Wild-type DNA (A) or mutated DNA (B) was used as a probe. Free probe (FP) and binding complex (C) were indicated with arrows.

Fig. 9. Gel-mobility shift assay of the sodF regulatory DNA with SrnR and SrnQ in varying amounts. The wild-type DNA as used in Fig. 8 was incubated with the proteins in the presence of 20 μM nickel. The micromolar ratios of SrnR to SrnQ were 0:10 (lane 1), 2:10 (lane 2), 5:10 (lane 3), 10:10 (lane 4), 10:5 (lane 5), 10:2 (lane 6), and 10:0 (lane 7). Free probe (FP) and retarded complex (C) were indicated with arrows. The relative levels of C were quantified by densitometer scan of the resulting autoradiogram.

Fig. 10. Far-Western immunoblot analysis for the interaction between SrnR and SrnQ. Native SrnR (panel A) or the denatured SrnR (95 °C for 5 min in 0.2% SDS) (panel B) in TDTG buffer (32) were spotted onto nitrocellulose membrane in the absence (Nickel −) or the presence (Nickel +) of nickel. The nickel concentration was twice as much as the protein concentration. Spots 1 contained 2 pmol of SrnR, whereas spots 2 and 3 had 5 pmol of the proteins. The blots were incubated with native SrnQ (15 μM) followed by reaction with anti-SrnQ antiserum. Native and denatured SrnQ (panels A and B, spots 3, respectively) were used as positive controls, whereas the native and denatured bovine serum albumin (panels A and B, BSA, respectively) were included as negative controls. BSA, bovine serum albumin.
SnR and SnQ to the sodF operator appears to require both proteins in 1:1 ratio.

SnR and SnQ Directly Interact with Each Other, and the Interaction Does Not Require Nickel—Both native (Fig. 10A, spots 1 and 2) and SDS-heat-denatured SnR (Fig. 10B, spots 1 and 2) were spotted on nitrocellulose membrane, and the blots were incubated with native SnQ followed by treatment with anti-SnQ mouse antiserum, which was then detected using anti-mouse IgG conjugated with horseradish peroxidase. The two proteins in native forms interact with each other, even by direct binding, even in the absence of nickel (Fig. 10A, Nickel−, spots 1 and 2). Indifference in signal intensity between the applications of 2 and 5 pmol (spots 1 and 2, respectively) of SnR onto the membrane was probably attributed to the limiting concentration of SnQ (15 nM) used in binding. Denatured SnR did not interact with SnQ irrespective of nickel treatment (Fig. 10B, spots 1 and 2). The control immunoblots with SnQ in native and SDS-heat-denatured forms showed positive signals by anti-SnQ antiserum as expected (Fig. 10A, A and B, spots 3, respectively). Bovine serum albumin did not show any reaction (Fig. 10A, A and B, BSA), and no cross-reactivity was observed between SnR and anti-SnQ antiserum (data not shown). The analyses with SnQ on the membrane followed by sequential reactions with SnR, anti-SnR antiserum, and anti-mouse IgG-horseradish peroxidase displayed the same results (data not shown). From the results, it was evident that the interaction between the two proteins does not require nickel and that the protein-protein interaction can take place before binding to the operator DNA.

SnR and SnQ Constitute a Hetero-octameric Complex Composed of Each Protein in 1:1 Ratio—The formation of SnR-SnQ complex was confirmed through gel-filtration chromatography of Sephadex G-100, and its molecular weight was measured using the mass calibration curve drawn with the standard proteins (Fig. 11A). The chromatographic resolution of either SnR or SnQ alone showed a peak, which was estimated around 10–12 kDa (Fig. 11B, c and d). Nickel treatment to each protein prior to gel loading showed the same results (data not shown), which implied that SnR or SnQ by itself, does not form homomultimeric complex. The two proteins were incubated with or without nickel and then subjected to the column chromatography. The elution profiles revealed an identical peak corresponding to ~95 kDa regardless of the nickel treatment (Fig. 11B, a and b). The Tricine-SDS-PAGE of the 95-kDa fractions showed the two protein bands with equal intensity, which co-migrated with SnR and SnQ (Fig. 11C). Thus, SnR and SnQ appear to form an octameric complex composed of four subunits of each protein.

Nickel Binds to SnQ—The CD spectra of SnQ were largely changed after nickel treatment (Fig. 12A, left panel). However, the spectral patterns of SnR did not show any significant difference with respect to nickel (Fig. 12A, right panel). The spectral change of SnQ by nickel disappeared following incubation of the protein mixture with EDTA (50 mM) for 3 h at room temperature (data not shown). The results suggested that the nickel binding to SnQ causes the conformational change of the protein. Atomic absorption spectrophotometry of SnQ following equilibrium dialysis against nickel showed the metal-binding
capacity of the protein that saturates at 0.80 atom of nickel/molecule with an apparent $K_d$ of 0.65 M. Thus, the stoichiometric balance of at least 1:1 is expected for Ni$^{2+}$ ion bound to SrnQ. Other metals such as Zn$^{2+}$ and Cd$^{2+}$ were found in the ratio $0.04$ metal atom/protein molecule, and no binding signal was detected with other metal ions including Ca$^{2+}$, Mg$^{2+}$, Cu$^{2+}$, Mn$^{2+}$, and Co$^{2+}$ (data not shown). In addition, SrnR did not reveal any metal-binding capacity (data not shown). Thus, the nickel specifically binds to SrnQ, which appears to be in a stoichiometric balance of 1:1 (Ni$^{2+}$/SrnQ).

**DISCUSSION**

A highly aerobic organism, *S. griseus* contains Ni-SOD and FeZn-SOD for protection against oxidative damages by superoxide radicals. Ni-SOD activity increased after nickel treatment, whereas FeZn-SOD expression is repressed by nickel at the stage of transcription (10, 11). The transcriptional repression was exerted through an operator of sodF (11). The antagonistic production of the two SODs has been proposed as a regulatory circuit to keep the total SOD activity constant (10). In this work, we found two ORFs, SrnR and SrnQ, which are responsible for the nickel-responsive transcriptional repression of *S. griseus sodF*. When SrnR and SrnQ were maintained in multicopies in *S. griseus*, the Ni-SOD activity was not changed much, indicating that sodN expression is regulated differently from that of sodF.

The calculated pI of SrnQ, fairly rich (26%) in arginine, is 12.8, whereas that of SrnR is 7.0. The physiological implication of the basic pI of SrnQ is not known. The constitutive expression of *srnRQ* independent of the presence of nickel was identified using *srnR*:xyIE transcriptional fusion construct. This result explains the observation that the binding complex with the *sodF* operator DNA was still detected in gel-mobility shift assay when nickel was added to the cell extracts prepared from a nickel-deficient culture of *S. griseus*.

The gel-mobility shift band (Fig. 8) was compared with those observed with the cell extracts of *S. griseus*. The retarded band with the purified proteins exactly co-migrated with one (faster-moving band) of the two binding complexes with the cell extracts (11). The slower-moving band was not detected, even with up to five times more the amount of the purified proteins shown in Fig. 8, so the band might reflect additional binding of other proteins from cell extracts. The probe DNA contains the sodF promoter as well.

**Fig. 12. Nickel binding to SrnQ.** A, CD spectra of SrnQ (left panel) and SrnR (right panel) treated with or without nickel (20 μM). Proteins were used at 15 μM. B, nickel-binding saturation curve of SrnQ (6.4 μM). Nickel binding was determined by equilibrium dialysis against varying concentrations of nickel followed by atomic absorption spectrophotometry. ● SrnQ, ○ bovine serum albumin (negative control).

**Fig. 13. A model illustrating nickel-dependent repression of sodF transcription by the complex of SrnR-SrnQ.** The sodF operator was indicated with facing arrows (11). Nickel ion was shown as closed circles. R and Q denote SrnR and SrnQ, respectively.

The results shown in this work clearly suggest that SrnR interacts with SrnQ to form an octameric complex, which appears to be composed of four subunits of each protein (Fig. 13). The overlapping stop-start codons of SrnR and SrnQ may provide a balanced translation to maintain 1:1 stoichiometry of the two proteins. The protein interaction does not require nickel. Our current data explain that SrnQ of the complex is probably a co-repressor of SrnR, because it can hold nickel at a ratio of one Ni$^{2+}$/polypeptide and the ligand binding appears to change the conformation of SrnQ, which may in turn enhance the DNA
binding activity of SrnR through the protein-protein interaction. The complex then binds to the sodF operator for transcriptional repression (Fig. 13). A similar observation has been reported for the interaction between BarA repressor and BarX co-repressor of Streptomyces virginiae (38–40). They are needed together for binding to the operator site of barB-varS operon, which codes for a putative DNA-binding protein and virginiamycin S-specific transport protein, respectively. BarX was suggested to induce DNA binding ability of BarA by direct interaction.

A nickel-sensing transcriptional repressor NikR of E. coli has been reported as a direct sensor of nickel ion to negatively regulate nikABCDE expression by binding to an operator consisting of two 5′-GTATGA-3′ half-sites related by dyad symmetry and separated by 16 base pairs (14). Roughly, a one-nickel ion was proposed to bind to each NikR subunit of the acting dimeric complex (41). No homology between the primary structures of SrnR and NikR was found.

Taken together, the experimental results presented here provide an interesting example of transcriptional repression by the protein complex composed of the DNA binding motif-bearing repressor and nickel-binding co-repressor. The detailed nature of the protein-protein interaction between the repressor and co-repressor remains to be determined.

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