Streptomyces coelicolor SCO4226 Is a Nickel Binding Protein

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Introduction

Nickel is first demonstrated as a bacterial growth nutrient for Hydrogenomonas strains [1]. Since then studies on the physiological role of nickel have grown tremendously [2]. Nine types of enzyme, which utilize nickel as a catalytic cofactor have been identified, including hydrogenase, urease, superoxide dismutase (SOD), carbon monoxide dehydrogenase, acetyl-CoA decarboxylase/synthase, methyl-CoM reductase, glyoxylase I, acireductone dioxygenase and methylenediurease [3]. Nickel is also one of the most common contact sensitizers causing delayed-type hypersensitivity in humans [4]. Accordingly, the cellular immune response to nickel has become a model system for studying hapten-induced hypersensitivity in humans [4]. Therefore, microorganisms evolve a variety of permeases [8,10]. The nickel ABC transporter from E. coli consists of five proteins (NikA-E), which transports nickel by coupling ATP hydrolysis [11]. The nickel permease (HoxN) from Cupriavidus necator was observed to transport nickel [12]. After nickel is transported into the cells, the nickel regulator, exporter and/or storage proteins are responsible to maintain the intracellular homeostasis of nickel. The best-characterized nickel sensor/regulator is NikR, which was first identified in E. coli [11]. The binding of nickel in the C-terminal metal-binding domain of NikR may trigger the conformational changes of its DNA binding domain to facilitate the interaction with a 20-bp palindromic DNA of the nickel uptake operon NikABCDE [13]. In addition, Nickel storage proteins could bind multiple nickel ions via the histidine-rich N-terminus [14]. For example, HypB in Bradyrhizobium japonicum can bind nine nickel ions per monomer at the N-terminal histidine-rich region [15,16]. On the other hand, the exporters could pump superfluous nickel ions out of the cells [17]. For instance, the expression of a nickel exporter gene nreB was specifically induced by the presence of nickel and conferred nickel resistance in both Achromobacter xylosoxidans and E. coli [18].

Streptomyces, a genus of filamentous Gram-positive bacteria usually living in the superficial layers of the soil, are crucial for maintaining the microenvironment because of their broad range of metabolic processes and biotransformations [19,20]. Several nickel-related proteins have been proposed to be involved in nickel homeostasis and regulation to avoid its cytotoxic effects [21]. The nickel-responsive transcription factor Nur in Streptomyces coelicolor

Abstract

The open reading frame SCO4226 encodes an 82-residue hypothetical protein. Biochemical assays revealed that SCO4226 dimer binds four nickel ions. To decipher the molecular function, we solved the crystal structures of SCO4226 in both apo- and nickel-bound (Ni-SCO4226) forms at 1.30 and 2.04 Å resolution, respectively. Each subunit of SCO4226 dimer adopts a canonical ferredoxin-like fold with five β-strands flanked by two α-helices. In the structure of Ni-SCO4226, four nickel ions are coordinated at the surface of the dimer. Further biochemical assays suggested that the binding of Ni2+ triggers the self-aggregation of SCO4226 in vitro. In addition, RT-qPCR assays demonstrated that the expression of SCO4226 gene in S. coelicolor is specifically up-regulated by the addition of Ni2+, but not other divergent ions such as Cu2+, Mn2+ or Co2+. All these results suggested that SCO4226 acts as a nickel binding protein, probably required for nickel sequestration and/or detoxification.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. The coordinates and the structure factors of apo- and nickel-SCO4226 are available from the PDB database (accession numbers 4OI3 and 4OI6, respectively).

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has been proven to control the nickel homeostasis and anti-oxidative response through repressing the expression of a nickel-transporter gene cluster [22]. While the nickel-containing superoxide dismutases (NiSOD) have been identified in several Streptomyces species [23]. Moreover, addition of nickel ions can up-regulate the NiSOD gene and down-regulate the FeSOD gene in S. coelicolor [24]. However, the fine regulation of nickel homeostasis in S. coelicolor remains largely unclear. Here we showed that the 82-residue hypothetical protein SCO4226 is a nickel binding protein. Further structural and biochemical analyses revealed that SCO4226 could sequester the excess nickel ions via self-aggregation. These findings provided novel insights into the nickel response in Streptomyces.

Materials and Methods

Bacterial strains and culture conditions
S. coelicolor A3(2) strain was grown and maintained according to the standard procedures [25]. Gauze’s medium No. 1 and YEME medium (YEME medium with yeast extract (Difco), 3 g/L; Bacto Peptone (Difco), 5 g/L; malt extract (Difco), 3 g/L; sucrose, 340 g/L; and MgCl₂·6H₂O, 0.25 g/L) were used to cultivate the S. coelicolor A3(2) strain. Pre-germinated spores were inoculated into seed media and cultivated at 28°C for 24–48 hr. One percent (v/v) of the seed culture was inoculated to the main media and grown for 48 hr in YEME medium.

Cloning, expression, purification, and Se-Met labeling
The coding region of SCO4226 was cloned as described previously [26]. The recombinant plasmid was transformed into E. coli B334 (DE3). The cells were cultivated in seleno-Methionine (Se-Met)-Minimal medium (M9 medium with 1% glucose, 2 mM MgSO₄, 0.1 mM CaCl₂, 7.5 mg/mL FeSO₄, 1 µg/mL thiamine, 0.05 g/L for each amino acid except that methionine was replaced by 0.025 g/L Se-Met). The expression of the target protein was induced for 5 hr at 37°C with C by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM protein was induced for 5 hr at 37°C.

Dynamic Light Scattering Assays
Dynamic Light Scattering (DLS) assays were carried out on a DYNAPRO-99 (Wyatt Technology Corp., 6300 Hollister). The molecular size of the protein was determined by DLS using cylindrical light scattering cuvettes at 28°C. Determinations were conducted on an ALV/DLS/SLS-5022F spectrometer equipped with a multi-digital time correlation (ALV5000) and a cylindrical 22 mW He-Ne laser (λ₀ = 632 nm, Uniphase) as the light source. The intensity-intensity time correlation function $G(2)(t, q)$ was measured to determine the line-width distribution $G(2)$ in dynamic LLS. For diffusive relaxation, $\Gamma$ is related to the translational diffusion coefficient $D$ of the scattering object in dilute solution or dispersion by $D = (\langle \Gamma \rangle / 4 \pi n^2) C_{\text{SOD}} C_{\text{NiSOD}}$ and further to hydrodynamic radius $R_H$ from Stokes-Einstein equation: $R_H = k_B T / 6 \pi \eta D$, where $\eta$, $k_B$, and $T$ are the solvent viscosity, the Boltzmann constant and the absolute temperature, respectively. The hydrodynamic radius distribution $f(R_H)$ was calculated from the Laplace inversion of $G(2)(t, q)$ using the CONTIN program.
UV/visible spectroscopy

The chemical NiSO$_4$ and Ethylene Diamine Tetraacetic Acid (EDTA) were bought from Sangon, China. The turbidity of the protein solution towards NiSO$_4$ was measured at 28°C monitoring the change in absorbance at 340 nm in 30 min or more with the interval of 15 sec, using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Titrations of NiSO$_4$ (0–2 mM) against 10–50 μM SCO4226 were performed in the buffer of 0.1 M Tris-HCl, pH 7.5, 100 mM NaCl. Then EDTA was added to a final concentration of 5 mM in the turbidity solution. The data were recorded by the program Origin 7.5. The assays were performed in triplicates.

RNA isolation

The bacteria of S. coelicolor A3(2) strain were grown in the YEME media with the addition of varying concentrations (100, 200 and 300 μM) of NiCl$_2$, MnCl$_2$, CuCl$_2$ or CoCl$_2$. The strain with the addition of buffer alone was used as the control. All chemicals of ≥98% purity were purchased from Sangon, China. RNA was isolated from the mycelia of S. coelicolor A3(2) strains which was grown for 48 hr on YEME medium by using an Spin Column Bacterial total RNA Purification Kit and the suggested protocol of the manufacturer (Sangon). The genomic DNA was removed by incubating one unit of RNase-Free DNase I (Thermo) per 1 μg estimated DNA for 10 min at 37°C. The concentrations of RNA in different samples were calculated by determining the absorbance at 260 and 320 nm using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The quality of RNA sample was assessed by electrophoresis in a formaldehyde denaturing agarose gel stained with ethidium bromide. Preliminary time course studies (data not shown) indicated that transcript levels were highest in 48 hr RNA preparations.

Quantitative Real-time PCR (RT-qPCR) analysis

The cDNAs for RT-qPCR analyses were synthesized using PrimeScript RT reagent Kit (Perfect Real Time, TaKaRa Dalian Biotechnology Co., Ltd. Dalian, China). Quantitative Real-time PCR (RT-qPCR) assays were performed by the SYBR Premix Ex Taq II Kit (TliRnaseH Plus, TaKaRa Dalian Biotechnology Co., Ltd. Dalian, China) on ABI Stepone Real-Time PCR system in MicroAmp fast optical 48-well plates (Applied Biosystems). The expression of the Streptomyces hrdB gene, thought to encode a constitutively expressed vegetative sigma factor, was monitored as a control. A forward primer ('5′-AAG GCG ACG ACG GCG AAC AAG-3′') and a reverse primer ('5′-TCG GCG TTG AGC AGA GGG AC-3′') were used for hrdB (272 bp). The primers used for

Table 1. Crystal parameters, data collection and structure refinement.

|                      | apo-SCO4226 | Ni-SCO4226 |
|----------------------|-------------|------------|
| **Data collection**  |             |            |
| Space group          | P2$_1$      | P2$_1$     |
| Unit cell (Å)        | 29.57, 66.98, 34.27 | 28.33, 67.14, 35.09 |
| Unit cell (°)        | 90.00, 95.02, 90.00 | 90.00, 94.98, 90.00 |
| Resolution range (Å) | 34.14–1.30 (1.32–1.30)* | 28.23–2.03 (2.14–2.04) |
| Unique reflections   | 30,687 (1,081) | 8,364 (1,088) |
| Completeness (%)     | 93.9 (66.6) | 98.3 (88.6) |
| <I/2 <I>             | 19.70 (3.34) | 19.7 (8.1) |
| R$_{merge}^{b}$ (%)  | 10.4 (31.7) | 4.8 (16.1) |
| Average redundancy   | 5.0 (4.1)   | 3.6 (3.5)  |
| **Structure refinement** |         |            |
| Resolution range (Å) | 34.14–1.30 | 28.23–2.04 |
| R-factor/R-free* (%) | 15.2/16.9  | 16.9/21.8  |
| Number of protein atoms | 1276     | 1263       |
| Number of water atoms | 158      | 86         |
| RMSD* bond lengths (Å) | 0.007    | 0.010      |
| RMSD bond angles (°)  | 1.073     | 1.164      |
| Mean B factors (Å$^2$) | 9.95     | 15.78      |
| Ramachandran plot° (residues,%) | 97.47 | 98.10 |
| Most favored (%)     | 97.47      |            |
| Additional allowed (%) | 2.53    | 1.90       |
| Outliers (%)         | 0          | 0          |
| PDB entry            | 40I3       | 40I6       |

*The values in parentheses refer to statistics in the highest bin.

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**Notes:**

1. $R_{merge} = \frac{\sum_{i} \sum_{hkl} |I_i(hkl) - \langle I(hkl) \rangle|^2}{\sum_{i} \sum_{hkl} |I_i(hkl)|^2}$, where $I_i(hkl)$ is the intensity of an observation and $\langle I(hkl) \rangle$ is the mean value for its unique reflection; Summations are over all reflections.

2. $R_{factor} = \frac{\sum_{hkl} |F_{o}(h) - F_{c}(h)|^2}{\sum_{hkl} |F_{o}(h)|^2}$, where $F_o$ and $F_c$ are the observed and calculated structure-factor amplitudes, respectively.

3. R-free was calculated with 5% of the data excluded from the refinement.

4. Root-mean square-deviation from ideal values.

5. Categories were defined by Molprobity.

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RT-PCR analysis were designed as a forward primer (5'-CAC GGG ATC ACC TCG GAC CA-3') and a reverse primer (5'-CGA CAG GGG GAC TTC GTG GA-3') for SCO4226 (210 bp). Each RT-qPCR experiment was carried out from at least three independent RNA biological samples and at least three independent experiments were done from each RNA sample.

**Results and Discussion**

**Overall Structure**

The structures of SCO4226 in both apo- and nickel-bound forms (termed apo- and Ni-SCO4226, respectively) have been solved at 1.30 and 2.04 Å resolution, respectively (PDB IDs: 4OI3 and 4OI4). The two structures are quite similar to each other with a root-mean-square deviation (RMSD) of 0.14 Å for apo-SCO4226, and 0.29 Å for Ni-SCO4226. The two subunits of the dimer are almost identical, with an RMSD of 0.14 Å for apo-SCO4226, and 0.29 Å for Ni-SCO4226. Notably, the Hah1 metallochaperone protein is implicated in copper delivery to the Menkes/Wilson disease proteins. Similar to SCO4226, Hah1 also adopts a ferredoxin-like fold, and belongs to a family of metal binding domains with a conserved MT/HCXXC motif [44]. However, SCO4226 lacks a conserved MT/HCXXC motif, indicating that it should adopt a different metal-binding pattern.

**Nickel coordination pattern**

In the dimeric structure of Ni-SCO4226, four nickel ions and one citrate molecule were identified in the electron density map. These four nickel ions are all surface exposed on the dimer interface with varying occupancies from 0.82 to 0.98. Our ITC data confirmed that four nickel ions are bound in one SCO4226 dimer with the $K_d$ value of 10 μM (Figure S1). All the four nickel ions are placed in the dimer interface. Ni-1 and Ni-2 interact with residues within one dimer whereas Ni-3 and Ni-4 are coordinated by residues from two adjacent dimers by symmetric operation. Specifically, Ni-1 is hexa-coordinated by His76-N=O1, Asp43'-O=O2, Ser46'-O=Oy, and the O4 and O7 and O6 atoms of the citrate molecule (Figure 2A). Ni-2 is penta-coordinated by His23-N=O2, Ala82'-O=O2, and three water molecules (Figure 2B). Ni-3 of an octahedral geometry is hexa-coordinated by His3'-N=O2 and Glu38'-O=O2 of one dimer, His12'-N=O2 of the adjacent dimer, and three water molecules (residues from subunit A of the adjacent dimer were labeled with a double prime) (Figure 2C). Ni-4 coordination is formed by His20'-N=O2 of one dimer, Glu74'-O=O1, Glu74'-O=O2 of the adjacent dimer and three water molecules (Figure 2D). The distances for all coordinate bonds are well within the expected range (2.06-2.46 Å). Sequence analysis revealed that only nickel-binding residues His23, Asp43, Ser46, Glu74 and His76 are exclusively conserved in Streptomyces, among which, Asp43, Ser46, and His76 constitute the complete Ni-1 coordination (Figure 2E). This suggests that the Ni-1 binding site might be conserved whereas others should be plastic.

**In vitro aggregation of SCO4226 upon addition of excess nickel**

As the absorbance at 340 nm ($A_{340\text{nm}}$) could be used to indicate the turbidity of protein solution, the high level of which usually reflects the protein aggregation [45]. Thus we applied the change of $A_{340\text{nm}}$ to explore the behavior of SCO4226 in solution upon binding to Ni$^{2+}$. Without the addition of Ni$^{2+}$, the $A_{340\text{nm}}$ values are approximately zero, which indicated that the SCO4226 protein solution would not aggregate in the absence of Ni$^{2+}$ (Figure 3A). However, the $A_{340\text{nm}}$ values gradually increased upon the addition of Ni$^{2+}$ at varying concentrations (Figure 3A). Moreover, the $A_{340\text{nm}}$ values increased and gradually reached a plateau, resulted from the addition of 1 mM Ni$^{2+}$ to the SCO4226 protein solution of varying concentrations (Figure 3B). Accompanied with the increase of the concentration of either SCO4226 or Ni$^{2+}$, the $A_{340\text{nm}}$ values sharply increased, which indicated the formation of more aggregates (Figure 3A-3B). These results suggested that the excess Ni$^{2+}$ would trigger the aggregation of SCO4226. Notably, the addition of 5 mM EDTA to the
SCO4226 solution in the presence of nickel led to an exponentially decrease of the A340nm value (Figure 3C), demonstrating that Ni^{2+} binding is reversible. However, nickel pre-treated with EDTA will not trigger the aggregation of SCO4226 any more (Figure 3C). It suggested that SCO4226 could be restored once the excess Ni^{2+} ions are depleted.

Moreover, we used DLS experiments to detect the size of SCO4226 protein in solution at a concentration of 50 μM. The initial radius of SCO4226 molecule is approximately 1.2 nm. Upon the addition of 1 mM Ni^{2+}, the SCO4226 molecules aggregated immediately and reached a lower plateau with an average particle radius of 40.0 nm (Figure 3D, plateau a). After incubation for 15 min, SCO4226 molecules kept on accumulating to form larger particles and finally stopped at an upper plateau with a maximum radius of 80.0 nm (Figure 3D, plateau b). These results further demonstrated that the nickel-triggered aggregation of SCO4226 is a dynamic process.

The expression of SCO4226 gene is up-regulated by nickel

To check if the in vivo expression of SCO4226 in S. coelicolor A3(2) strain after 48 hr incubation. The expression levels of SCO4226 gene were assessed by RT-qPCR assays. The addition of 100 μM Ni^{2+} increased the expression levels of SCO4226 gene to about 1.2 folds, as compared to the control without the addition of any metal ions. Accompanied with the increase of Ni^{2+} concentration to 200 and 300 μM, the expression levels of SCO4226 gene were gradually increased to 2 and 3 folds, respectively (Figure 4). In contrast, the addition of Mn^{2+}, Cu^{2+}, and Co^{2+} did not change the expression levels of SCO4226. The results indicated that the expression of SCO4226 gene is specifically up-regulated by the addition of Ni^{2+}, but not other divalent ions such as Cu^{2+}, Mn^{2+} or Co^{2+}.

SCO4226 represents a novel nickel binding protein

The overall structure of SCO4226 reveals a ferredoxin-like fold with four nickel ions coordinated at the dimer interface. Each nickel ion has oxygen-containing ligands like water molecules and at least one histidine ligand (Figure 2), which is consistent with the notion that histidine is a common nickel ligand [46]. Although the crystal was soaked in a buffer of NiSO_{4} as high as 0.8 M, the four nickel-binding sites have an occupancy from 0.82 to 0.98. In fact, SCO4226 possesses a rather low affinity towards nickel, for all nickel-binding sites are solvent-exposed and utilize a few
Figure 2. The coordination patterns of (A) Ni-1, (B) Ni-2, (C) Ni-3 and (D) Ni-4. The Ni$^{2+}$ ions are shown as spheres and colored the same as Figure 1B. Black dotted lines donate the coordinate bonds. The coordinate residues and the citrate ion are shown in sticks. (E) Multiple sequence alignment of SCO4226 and homologs in Streptomyces. The secondary structural elements of Streptomyces coelicolor SCO4226 were labeled on the top. The nickel coordinate residues were labeled with different blue marks [Ni-1 (▲), Ni-2 (●), Ni-3 (●), Ni-4 (▼)]. Colors are chosen according to rules of ESPript (http://espript.ibcp.fr/ESPript/ESPript?): A blue frame represents a similarity across groups; a red character indicates similarity in a group;
coordinate residues. However, despite lacking a conserved metal-binding motif, the abundance of metal-binding residues (eight glutamates, six aspartates and nine histidines) enables SCO4226 to bind multiple nickel ions. Similar cases have been also found in *E. coli* NikRs. The excess nickel binds to a total of 22 possible low-affinity nickel sites on the NikR tetramer. These sites are all on the surface of NikR, and most of them can be described as preferring octahedral geometry, utilizing one to three protein ligands (typically histidine) and at least two water molecules [47]. Despite the similarity of the multiple nickel-binding sites between *E. coli* NikR and SCO4226, further investigation are still needed for elucidating the relevance of nickel binding to the physiological function of SCO4226.

Our data showed that SCO4226 reversibly binds multiple nickel ions with low affinity. Similar cases have also been found in several proteins with multiple metal-binding sites. For instance, *H. pylori* histidine-rich protein Hpn plays a pivotal role in Ni$^{2+}$ homoeostasis, binding or transport [48]. *B. japonicum* HypB with a histidine-rich region can bind multiple nickel ions and is thought to be involved in nickel storage [14,15]. In addition, *A.

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**Figure 3. The aggregation of SCO4226 triggered by nickel.** (A) UV/visible absorbance spectra ($A_{340nm}$) of 50 μM SCO4226 protein solution with the addition of 0, 0.1, 0.2, 0.4 mM Ni$^{2+}$ respectively in the buffer of 0.1 M Tris-HCl, pH 7.5, 100 mM NaCl at 28°C. (B) The $A_{340nm}$ spectra of SCO4226 (15, 25 and 50 μM) in the buffer of 0.1 M Tris-HCl, pH 7.5, 100 mM NaCl upon the addition of 1 mM NiSO$_4$, 3H$_2$O at 28°C. (C) The $A_{340nm}$ spectra of Ni-SCO4226 protein solution with the addition of 5 mM EDTA. The $A_{340nm}$ spectra of SCO4226 protein solution with 1 mM Ni$^{2+}$ that has been pre-treated with 5 mM EDTA. (D) DLS detection of the particle radius of 50 μM SCO4226 protein solution with the addition of 1 mM Ni$^{2+}$. The lower and upper plateaus are labeled with a and b, respectively.

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xylosoxidans NreB with a histidine-rich C-terminus is specifically induced by nickel and confers nickel resistance and reduces nickel accumulation upon heterologous expression in E. coli [18]. Notably, the metal-binding capabilities of these proteins are comparable to that of SCO4226 towards Ni$^{2+}$. We thus propose that SCO4226 might also be involved in cellular nickel homeostasis, detoxification, and/or nickel utilization in specialized cytoplasmic compartments. On the other hand, the increase of cytoplasmic Ni$^{2+}$ concentration triggers the aggregation of SCO4226, and in turn decreases its intracellular concentration. Thus, the up-expression of SCO4226 gene might be a physiological response to guarantee the constant level of functioning SCO4226. Altogether, we proposed that SCO4226 is capable of sequestering excess nickel for fine-tuning the nickel homeostasis. However, the bona fide molecular and cellular function of SCO4226 remains to be investigated.

Supporting Information

Figure S1 ITC titration data for the binding of apo-SCO4226 with nickel. The assays were performed at 28°C in the buffer of 20 mM Tris-HCl, pH 7.5. Raw titration data represent the thermal effect of 40μL injections of Ni$^{2+}$ (500 μM) onto the protein solution (20 μM). The continuous lines represent the best fit of the integrated data, obtained by a non-linear least squares procedure. The calculated number of binding sites and dissociation constant are indicated. (TIF)

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Author Contributions

Conceived and designed the experiments: ML YLJ CZZ. Performed the experiments: ML SW HJ. Analyzed the data: ML YLJ. Contributed reagents/materials/analysis tools: RGZ MJV. Wrote the paper: ML YLJ YC CZZ.

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Figure 4. RT-qPCR analysis of the expression of SCO4226 gene in Streptomyces coelicolor A3(2) strain. The strains were grown for 48 hr on YEME medium with different divalent ions (Ni$^{2+}$, Cu$^{2+}$, Mn$^{2+}$ and Co$^{2+}$) at three concentrations (100, 200 and 300 μM). The expression level of SCO4226 gene without the addition of any metal ions was used as a control. Data are presented as the means ± standard deviations from three independent assays. One-way ANOVA with a post hoc Dunnett test is used for the comparison of statistical significance. The P values of <0.05, 0.01 and 0.001 are indicated with *, ** and *** respectively.

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