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Backbone resonance assignments of the homotetrameric (48 kD) copper sensor CsoR from *Geobacillus thermodenitrificans* in the apo- and Cu(I)-bound states: Insights into copper-mediated allostery

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Abstract

Prokaryotes are highly susceptible to exogenous copper and employ metalloregulatory proteins to control the intracellular concentration. CsoR (copper-sensitive operon repressor) is one such protein that represses transcription of a Cu(I)-effluxing ATPase in its apo form. Cu(I)-binding leads to transcriptional derepression and cellular copper resistance. Herein, we present substantially complete backbone (H^N , N, C', C, C) resonance assignments of tetrameric (48 kD) *Geobacillus thermodenitrificans* (Gt) CsoR in its apo- and Cu(I)-saturated states. These data provide the first spectroscopic evidence that Cu(I)-binding induces an interruption in the long 2 helix of CsoR.

Keywords

metalloregulatory protein; allosteric regulation; CsoR; copper sensor; *Geobacillus thermodenitrificans*; thermophile

Biological Context

Severe bacterial infections such as those caused by multidrug-resistant *Staphylococcus aureus* (MRSA) are becoming more prevalent in hospitals and our current antibiotic regimens are becoming less effective at treatment. In humans, copper is an essential biomineral and functions as a cofactor in selected enzymes, but becomes toxic at high, unregulated concentrations. The toxicity of copper derives from the catalytic production of free radicals via Fenton chemistry and/or disruption and/or replacement of other metal cofactors resulting in pleomorphic metabolic defects, including inhibition of amino acid biosynthesis (Macomber and Imlay 2009). Mechanisms of copper homeostasis and resistance are genetically encoded in all organisms and occur through coordinated copper transport and efflux which collectively minimize deleterious cellular reactions of unregulated Cu(I). In contrast to eukaryotes, non-photosynthetic prokaryotes appear to lack a metabolic requirement for cytoplasmic copper; as a result, the bacterial response to copper toxicity is more of a defense mechanism (Dupont et al. 2011).

Metalloregulatory proteins control the expression of genes necessary to remove exogenous copper from the cell. CsoR (Liu et al. 2007), a metalloregulatory protein, found in a subset of Gram-positive organisms binds operator DNA known as *cso* boxes, which are upstream

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of the genes required for copper resistance. It has reduced affinity for operator DNA in the presence of copper and the subsequent dissociation results in the up-regulation of copper resistance proteins, including the Cu(I)-effluxing ATPase (Liu et al. 2007). CsoR was originally discovered in *Mycobacterium tuberculosis* (*Mtb*) and structurally characterized in the Cu(I)-bound state as a dimer of dimers helical bundle architecture (Liu et al. 2007). Recently, a metal-free apo-structure of CsoR from *Streptomyces lividians* was reported and shown to adopt of dimer-of-dimers structure, with each protomer containing three helical segments, as in *Mtb* CsoR (Dwarakanath et al. 2012). There exists no atomic-level structural description of a conformational change that must occur in a single CsoR in response to Cu(I) coordination that drives the dissociation from corresponding operator DNA.

Previous NMR investigations of CsoR from *B. subtilis* and *M. tuberculosis* have been hampered by very broad resonances indicative of high molecular weight complexes (data not shown). In this note, we employ a thermophilic CsoR from *Geobacillus thermodenitrificans* (*Gt*) to take advantage of faster correlation times obtainable at higher temperatures, thereby making multidimensional experiments feasible on these high molecular weight tetramers (45-50 kD). Here, we present nearly complete backbone ($^1\text{H}^N$, ^1N , C' , C , C) resonance assignments of tetrameric (48 kD) *Gt* CsoR in its apo- and Cu(I)-saturated states, the analysis of which provides unambiguous evidence for a structural transition in the tetramer linked to Cu(I)-binding and negative allosteric regulation of DNA operator binding.

Methods and experiments

Protein expression and purification

The gene encoding the putative *Gt* CsoR (locus tag GTNG_1533) was PCR amplified from *Geobacillus thermodenitrificans* NG80-2 genomic DNA and subcloned into pET15b (Novagen) between the *Nco*I and *Bam*HI sites to drive the expression of T2A *Gt* CsoR, taken as wild-type. The plasmid was transformed into Rosetta BL21(DE3) expression cells using ampicillin and chloramphenicol for selection. A series of starting cultures were utilized to inoculate a 70% D₂O M9 culture supplemented with D-Glucose-¹³C6, C-D7 and ¹⁵N NH₄Cl. The isotopically enriched media was placed at 37 °C shaking at 200 rpm and induced at an O.D. of 0.55 to a final IPTG concentration of 1.0 mM. The recombinant expression was continued for 3 h at 37 °C, afterwards the cells were collected by centrifugation for 20 min at 4000 rpm. The cell pellet was lysed in 100 mL of 10 mM MES, 120 mM NaCl, 5 mM TCEP, 5 mM EDTA by sonication for 9 min (10s on, 50s off) at 65% amplitude. The lysate was then placed in a 65 °C water bath for 30 min and subsequently spun at 9000 rpm for 25 min at 4 °C. Polyethyleneamine was added to the supernatant at a final concentration of 0.015% (v/v) and stirred at 4° for 30 min. After clarification of the supernatant the sample was precipitated with the addition of 20% and 80% NH₄SO₄, stirring for 30 min at 4 °C, with centrifugation at 9000 rpm for 25 min at 4 °C between steps. The pellet from the 80% NH₄SO₄ cut was dissolved with lysis buffer and dialyzed overnight at 4 °C in low salt buffer, 10 mM MES, 50 mM NaCl, 5 mM TCEP, 3 mM EDTA pH 6.0. The supernatant of the dialysis was purified over a 100 min gradient to 100% high salt buffer (10 mM MES, 1 M NaCl, 5 mM TCEP, 3mM EDTA pH 6.0) using a 6 mL SP column. CsoR-containing fractions were identified by SDS-PAGE and pooled for further purification by gel filtration (G75 Superdex) in 10 mM MES, 120 mM NaCl, 5 mM TCEP and 5 mM EDTA pH 6.0. Sample purity was assessed by SDS-PAGE and ESI-MS, and the CsoR fractions absent of impurities or bound Cu(I) were pooled and concentrated to generate the apo CsoR sample. Any fractions that were perceived to have bound Cu(I), indicative of a low 280 nm/260 nm ratio, were pooled for in vitro metallation with Cu(I). The samples were reduced, placed in an anaerobic chamber, buffer exchanged and incubated with one molar equivalent of Cu(I) from an anaerobically prepared CuCl stock. The protein concentration

was measured by determining the concentration of free thiols and the Cu(I) concentration by atomic absorption spectroscopy. The observed molecular weight by ESI-MS was found to be 11926 D (11925 D with the N-terminal Met processed) for unlabeled CsoR. Gel filtration chromatography (G75 Superdex) is consistent with a tetrameric assembly state as are multiple sedimentation velocity (Optima XL-1 analytical ultracentrifuge) runs (10 mM MES, 200 mM NaCl, 5 mM TCEP pH 6.0) at 20 °C (55,000 rpm, monitored at 230 nm, 20 μ M protomer) and 40 °C (60,000 rpm, monitored at 280 nm; 0.16–0.37 mM) which reveal a model-independent $s_{20,w}=3.4 (\pm 0.1)$ S at both temperatures, a value consistent with another tetrameric (≈ 46 kD) *B. subtilis* CsoR in the apo-state (Ma et al. 2009).

NMR spectroscopy

Samples for NMR spectroscopy were prepared at 0.8 mM and 0.6 mM for apo and Cu(I) CsoR, respectively. The sample buffer was 10 mM MES, 120 mM NaCl, 5 mM TCEP, 5 mM EDTA, 20 mM Arginine, 20 mM Glutamate, pH 6.0. All NMR experiments were performed on a Varian DDR 800 MHz spectrometer fitted with a cryogenic probe system at 322 K in the METACyt Biomolecular NMR laboratory at Indiana University. The spectra were referenced to external DSS and NaN_3 was used as a preservative. NMR data processing and analysis were performed using NMRPipe (Delaglio et al. 1995) and SPARKY (Goddard and Kneller). Sequential backbone resonance assignments were obtained using standard triple-resonance NMR spectroscopy: HNCA, HN(CO)CA, HNCACB, HN(COCA)CB, HNCO, and HN(CA)CO (Grzesiek and Bax 1992). All of the 2D & 3D pulse sequences used were acquired using TROSY-based spectral selection and suppression of side chain carboxamide couplings.

Assignments and data deposition

The ^1H , ^{15}N TROSY spectra of apo and Cu(I)-loaded CsoR are shown in Figs. 1 and 2, respectively. A suite of ^{13}C -based multidimensional experiments were used to obtain substantially complete residue-specific ^1H , N, C', C and C assignments obtained in both metal-free apo- and Cu(I)-bound conformational states. At least two backbone nuclei were specifically assigned for over 95% of the non-Pro residues (101 expected ^1H - ^{15}N crosspeaks from 105 residues), with the extreme N-terminal residues 2–4 not assigned in either state. Both spectra are of comparable quality and the linewidths are suggestive of no substantial change in quaternary structure when CsoR binds Cu(I), as previously found for *B. subtilis* CsoR (Ma et al. 2009). However, the chemical shift dispersion of the metallated CsoR is detectably greater than that of apo-CsoR (compare Figs. 1 and 2), and the fact that the spectra are easily distinguished from one another is consistent with a significant conformational change upon metal coordination.

A TALOS+ analysis of apo and Cu(I)-loaded *Gt* CsoR was used to determine backbone and dihedral angles (Fig. 3A and 3B) (Shen et al. 2009). Apo *Gt* CsoR consists of three uninterrupted helices as was recently shown crystallographically in *Streptomyces lividans* apo-CsoR (Dwarakanath et al. 2012) (1: E21–E45; 2: C50–R85; 3: E90–Q102). Although Cu(I)-loaded *Gt* CsoR consists of essentially the same three helices, there is a clear interruption or kink introduced into the 2 helix between the presumptive Cu(I) ligands H75 and C79, likely required to accommodate Cu(I) coordination (1: K20–E45; 2a: C50–A76; 2b: C79–R85; 3: G89–F103). The 2 helix was shown to terminate two residues following the His ligand (H61) in Cu(I)-bound *Mtb* CsoR (Liu et al. 2007). This change in secondary structure is likely coincident with a significant change in tertiary or quaternary structural packing given the large upfield shifts of A76 and A81 when apo-CsoR binds Cu(I). The large downfield shift of I16 upon Cu(I) binding (Fig. 2) may be reporting on local folding in this region of the molecule which sits immediately above the Cu(I)-binding site; the crystal structure of *T. thermophilus* apo-CsoR (Sakamoto et al., 2010) suggests some α -strand

structure in this region that might be stabilized by Cu(I) binding. Backbone (H^N , N, C', C, C) resonance assignments for both apo- and Cu(I)-bound *Gt* CsoR have been deposited in the BioMagResBank database (<http://www.bmrb.wisc.edu>) under the accession numbers 18472 and 18470 for apo- and Cu(I)-CsoR, respectively.

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Fig. 1. ^1H - ^{15}N TROSY spectrum of the apo *Gt* CsoR tetramer. Residue numbers are labeled on the crosspeaks. Crosspeaks from the middle of the spectrum are shown in the inserted box, *left*.

Fig. 2. ^1H - ^{15}N TROSY spectrum of the Cu(I)-bound *Gt* CsoR tetramer. Residue numbers are labeled on the crosspeaks. Crosspeaks from the middle of the spectrum are shown in the inserted box, *left*.

Fig. 3. TALOS+ derived backbone torsion angles and secondary ¹³C chemical shifts of apo-CsoR (A) and Cu(I)-CsoR (B). ¹³C shifts calculated by subtraction of published random coil values from the experimental ¹³C chemical shifts. Filled circles, phi angles; open circles, psi angles; filled diamonds, secondary ¹³C chemical shifts. A secondary structure schematic based on TALOS+ analysis is shown at the top panels A and B for the apo- and Cu(I)-loaded CsoR, respectively.