Cobalt-dependent Transcriptional Switching by a Dual-effector MerR-like Protein Regulates a Cobalt-exporting Variant CPx-type ATPase*

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CoaR associates with and confers cobalt-dependent activation of the coaT operator-promoter. A CoaR mutant (Ser-Asn-Ser) in a carboxyl-terminal Cys-His-Cys motif bound the coaT operator-promoter but did not activate expression in response to cobalt, implicating thiolate and/or imidazole ligands at these residues in an allosteric cobalt binding site. Deletion of 1 or 2 nucleotides from between near consensus, but with aberrant (20 base pairs) spacing, −10 and −35 elements enhanced expression from the coaT operator-promoter but abolished activation by cobalt-CoaR. It is inferred that cobalt effects a transition in CoaR that underwinds the coaT operator-promoter to realign promoter elements. In the absence of cobalt, CoaR represses expression (~50%). CoaR is a fusion of ancestral MerR (mercury-responsive transcriptional activator)- and precorrin isomerase (enzyme of vitamin B₁₂ biosynthesis)-related sequences. Expression from the coaT operator-promoter was enhanced in a partial mutant of cbiE (encoding an enzyme preceding precorrin isomerase in B₁₂ biosynthesis), revealing that this pathway “inhibits” coaT expression. Disruption of coaT reduced cobalt tolerance and increased cytoplasmic 57Co accumulation. coaT-mediated restoration of cobalt tolerance has been used as a selectable marker.

MerR from Tn501 binds to a single site within the mer operator-promoter, and upon binding mercury positively regulates transcription of the mercury resistance operon (1, 2). In the absence of mercury, MerR represses transcription (~2-fold). Several lines of evidence support a model in which mercury-MerR activates transcription by realigning abnormally spaced consensus RNA polymerase recognition sequences via underwinding the mer operator-promoter (3, 4). Within the fully sequenced genome of the cyanobacterium Synechocystis PCC 6803 (5) is an ORF, slr0794, herein designated coaR, encoding a predicted protein with some sequence similarity to MerR.

The amino-terminal one-third of CoaR, which aligns with MerR, is followed by a polypeptide with sequence similarity to precorrin isomerase (see Fig. 1), a methyl transferase involved in the synthesis of the cobalt-containing corrin ring of vitamin B₁₂ (6). Unlike Synechocystis PCC 6803, many organisms do not contain the genes for vitamin B₁₂ biosynthesis, and such organisms have no requirement for cobalt (7). Precorrin isomerase from Pseudomonas denitrificans is known to bind avidly to its product, hydrogenobyrinic acid, which consequently co-purifies with the enzyme (8), suggesting that a domain of CoaR interacts with hydrogenobyrinic acid.

Divergently transcribed from coaR is an ORF, slr0797, designated coaT, encoding a putative P-type ATPase (Fig. 1). CoaT has some sequence features of P₁- (9) or CPx-type ATPases (10) but lacks an amino-terminal metal binding motif and, most significantly, contains a deduced intramembranous Ser-Pro-Cys motif rather than the characteristic Cys-Pro-Cys/His/Ser (CPx). Known CPx-type ATPases transport larger metal ions and include the cadmium transporter CadA, the yeast copper transporter CCC2, the human copper transporters MNK and WND, the bacterial copper transporters CtaA, PacS, CoaP, and CopB (reviewed in Ref. 11), and the zinc transporter ZiaA from Synechocystis PCC 6803 (12) and ZntA from Escherichia coli (13, 14). At present, it is not possible to predict which metal ion is transported in which direction, import or export, merely from the sequence of a CPx-type ATPase, but the divergent organization of coaR and coaT encourages the prediction that the product of the former regulates the latter.

Here we describe experiments that confirm that CoaR does bind to and activate expression from the coaT operator-promoter. The activating effector is shown to be cobalt, and CoaT is shown to confer cobalt resistance and exclusion. Following site-directed mutagenesis, it was revealed that a carboxyl-terminal Cys-His-Cys motif in CoaR is part of the cobalt-sensing site. A partial mutant in the vitamin B₁₂ biosynthetic pathway at a step preceding precorrin isomerase was generated. Enhanced expression from the coaT operator-promoter in this mutant indicates that this pathway inhibits coaT transcription and that CoaR responds to both activating and inhibitory effectors to attune cobalt export with fluctuations in cellular demand as well as with changing cobalt levels.

EXPERIMENTAL PROCEDURES

Bacterial Strains and DNA Manipulation—Synechocystis PCC 6803 was grown in liquid BG-11 medium (15) or on medium C plates with supplement A₅ (16) using previously described conditions (17). Cells were transformed to antibiotic resistance essentially as described by Hagemann and Zuther (18). E. coli strains JM101 or SURE (Strategene) were grown in Luria-Bertani medium (19). Standard DNA manipulations were performed as described by Sambrook et al. (19).

Construction of Plasmids Containing coa-lacZ Fusions—Synechocystis PCC 6803 genomic DNA, isolated as described previously (17), was used as a template for PCR with primers 1 (5′-GAACCTGGGCACTA-AAGCAAAATGAG-3′) and 2 (5′-GAGAAATTCTGGATTTTTACCT-TCTCAGGACC-3′). The amplification product (1.2 kb) containing coaR and the coaT operator-promoter was ligated into the HI/SalI site of pβSK’ (Strategene) to create pJRJC1.1, then subcloned into the BamHI/SalI site of pLACPB2 (20) to create pLACOA.

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1 The abbreviations used are: ORF, open reading frame; bp, base pairs; kb, kilobase pair(s); PCR, polymerase chain reaction.
A derivative of pLACOA was generated in which codon 10 of coaR was converted from GAA to GAA or GAG to CAG. Primer 5'-CCCTCTTGGATGGAATACGCGTTTATCAGG-3' and its reverse complement were used for Quik Change (Stratagene) mutagenesis with pJRJC1.1 as template, creating pJRJC1.2, and the coa sequences were then subcloned into the BamHI/SalI site of pLACPB2 to create pLACOA-OCH.

Two derivatives of pLACOA and pLACOA-OCH were generated with 1 or 2 nucleotides (61 or 62, see Fig. 7A) removed from the coaR operator-promoter. pJRJC1.1 and pJRJC1.2 were used as templates for Quik Change with primer 5'-CTTCTTAGCCGTCATACGATGTTG-3' and its reverse complement for the 61 deletion, or 5'-GATCATGACTCATGAGCTGAGGCT-3' and its reverse complement for the 62 deletion, and the coa sequences were subcloned into the BamHI/SalI site of pLACPB2.

A further derivative of pLACOA was generated in which codons 363 to 365 of coaR, encoding Cys-His-Cys, were converted to encode Ser-Ser. Primer 5'-CATTTGTGGAAGGATCCCGTATCGTTAATG-3' and its reverse complement were used for Quik Change with primer 5'-GTCATCCTGCGATCCCGTTAATC-3' and its reverse complement for the 363 deletion, and the coa sequences were subcloned into the BamHI/SalI site of pLACPB2.

Integration of a coa-lacZ Fusion, or lacZ Alone, into the Synechocystis PCC 6803 Genome—Plasmid pSCCM2 facilitates the integration of translational fusions to lacZ into the Synechocystis PCC 6803 genome (within ORF slr0168) (21). The coaR fragment from pLACOA to generate pJRNR1.1, containing the entire lacZ coding region and Shine-Dalgarno motif. The coa sequences from pJRJC1.1 were subcloned into the PstI/SalI site of pJRNR1.1 to create a transcriptional fusion to lacZ. The resulting plasmid, pJRNR2.1, was used to transform Synechocystis PCC 6803 to kanamycin resistance, generating strain JRNR2.1. JRNR2.1 showed no difference in cobalt tolerance to wild type. As a control, pGEM-T containing lacZ alone, was ligated to pGEM-T before subcloning into the BamHI/SalI site of pLACPB2.

β-Galactosidase Assays—Synechocystis PCC 6803 cultures (final A800 of 0.18 to 0.35) were exposed (20 h) to a range of metal ions under standard growth conditions except where stated otherwise. Overnight cultures of E. coli were diluted 100-fold in fresh medium supplemented with a range of metal ions and grown to an A600 of 0.2 to 0.5. Assays (22) were carried out in triplicate and performed on at least three separate occasions (nine analyses).

Insertional Inactivation of coaR—Synechocystis PCC 6803 genomic DNA was used as a template for PCR with primers 1 and 5'-GAAGGAT-CTCCTGCAAGGCGTTAATC-3' and the amplification product (3.3 kb), containing coaR and coaT, was ligated to pGEM-T (Promega) to create a PCR product that was then ligated into the PstI/SalI site of pTKR2 containing the cat gene to generate pNRJR1.1, containing the entire lacZ coding region and Shine-Dalgarno motif. The coa sequences from pNRJR1.1 were subcloned into the PstI/SalI site of pNRJR1.2 containing the coa operator-promoter as probe. This fragment was released from pSK+ containing the PCR product generated using primers 2 and 5'-GAAGGATCCTTCTTTTATCGTCATC-3' with pJRJC1.1 as template.

RESULTS

Transcription from the coaT Operator-promoter in Synechocystis PCC 6803 Is Maximally Induced by Cobalt—To identify which, if any, metal ions repress or induce transcription from the coaT operator-promoter, 1.2 kb from upstream of coaT (including the coaT operator-promoter and coaR) was fused to a promoterless lacZ gene to generate pJRNR1.2. The transcriptional fusion in plasmid pJRNR1.2 is flanked by sequences from Synechocystis PCC 6803, which facilitated integration by homologous recombination into a remote chromosomal site to generate strain JRNR2.1. After exposure to biologically significant concentrations of various metal ions, maximum induction of β-galactosidase activity was observed.
with elevated cobalt (Fig. 2). A greater than 10-fold reduction in β-galactosidase activity was observed when cells were cultured in modified BG-11 medium devoid of micronutrient (0.15 μM) cobalt, and the consequent response to cobalt was enhanced (Fig. 2B). No induction of β-galactosidase activity was detected using control cells containing lacZ alone (Fig. 2B).

Mutants of Synechocystis PCC 6803 with a Disrupted coaT Gene Have Reduced Tolerance to Cobalt and Increased Accumulation of 57Co in the Cytoplasm—The observation that elevated cobalt enhances transcription from the coaT operator-promoter suggests that coaT may export, and confer resistance to, cobalt. Mutants, Synechocystis PCC 6803(coaT), with disrupted coaT were generated by integration of plasmid pNCOAT, which contains coaT interrupted by a kanamycin resistance gene. Growth of Synechocystis PCC 6803(coaT) and wild type was tested in multiple liquid cultures supplemented with a range of levels of cobalt, cadmium, copper, mercury, nickel, silver, and zinc to determine maximum permissive concentrations (data not shown). Only resistance to cobalt appeared to be reduced in Synechocystis PCC 6803(coaT). Subsequently, growth was examined as a function of time in response to selected concentrations of cobalt and three metals, which are known to be transported by Cpx-type ATPases (Fig. 3A). Again, only resistance to cobalt was reduced. Restoration of cobalt tolerance was also used as a selectable marker to identify mutants of Synechocystis PCC 6803(coaT) in which coaT had reintegrated into the chromosome by homologous recombination. The genotypes of Synechocystis PCC 6803(coaT) and the mutant with reintegrated coaT were confirmed by Southern analysis; the band of lower Mr, represents hybridization to coaR on a smaller fragment, due to the disruption of coaT introducing an additional restriction site (Fig. 3B). Fig. 3C shows the phenotypes of Synechocystis PCC 6803(coaT), wild type and cells with coaT reintroduced into the chromosome, on agar plates.

Synechocystis PCC 6803(coaT) and wild type cells were exposed for 1 h to 1 Kb of 57Co in medium containing 2 μM cobalt. More 57Co was located in the cytoplasm of Synechocystis PCC 6803(coaT) compared with wild type cells (Table I), with equivalent observations being made on two further occasions (data not shown). The disruption of coaT impairs the exclusion of cobalt from the cytoplasm.

CoaR Binds to the coa Operator-promoter—A single complex formed between the coa operator-promoter and extracts from Synechocystis PCC 6803 (Fig. 4). Fig. 5D confirms that a single complex is also formed between the coa operator-promoter and total protein from E. coli cells containing pLACOA, whereas, most importantly, this complex is absent when protein is used from cells containing pLACOA-och (pLACOA containing a stop codon within the coaR ORF). The complex remains stable in reactions containing 0.1 μg μl−1 of poly(dI-dC)poly(dI-dC) competitor DNA (Figs. 4 and 5B). This represents a 1 × 10−5-fold excess of nonspecific competitor DNA to coa probe DNA.
cells containing this construct showed equivalent retardation of the coa operator-promoter as extracts containing nonmutant CoaR (Fig. 5B), confirming that the mutant C363S/H364N/C365S protein is synthesized and can bind to DNA. In the absence of added cobalt, β-galactosidase activity in cells containing mutant CoaR was less than in cells containing pLACOA-OCH (data not shown) and similar to that observed in cells containing pLACOA (Fig. 6), confirming that the mutant C363S/H364N/C365S protein reduces basal expression from the coaT operator-promoter.

Deletions within the coaT Operator-Promoter Enhance Transcription—Known proteins that share sequence similarity to MerR from Tn501 include mercury sensors from other sources (28), the redox sensor SoxR (29), the thiostrepton sensor TipA from Bradyrhizobium japonicum (31), and NolA from Bacillus subtilis (32). These proteins are known, or predicted, to associate with promoters in which consensus −10 and −35 sequences are separated by 19 or 20 bp rather than 16 to 18 bp. The removal of nucleotides from between such elements revealed that suboptimal spacing is essential for normal regulation of mer transcription, with nucleotide deletions leading to constitutive enhanced expression (35). By analogy, 20 bp separate consensus −10 and −35 sequences in the coaT operator-promoter region (Fig. 7A). A degenerate (1 bp mismatch in 13) hyphenated (6 bp) inverted repeat (13-6-13) (Fig. 7A) in this region contains candidate nucleotides for CoaR binding. To test the importance of suboptimal spacing for regulation of transcription from the coaT operator-promoter, variants of constructs pLACOA and pLACOA-OCH were created in which 10 and 35 sequences in the coaT operator-promoter were deleted.

Disruption of cbiE Enhances Transcription from the coaT Operator-Promoter—To test the proposal that interaction be-
between the precorrin isomerase-like domain of CoaR and intermediates in the vitamin B$_{12}$ biosynthetic pathway mediates expression from the coaT operator-promoter. β-galactosidase activity was examined in a mutant of strain JRNR1.2 in which the cbiE gene was insertional inactivated on a proportion of chromosomes. β-Galactosidase activity was activated in the cbiE mutant compared with JRNR1.2 (Fig. 8A), revealing that the vitamin B$_{12}$ pathway mediates repression of transcription from the coaT operator-promoter.

**DISCUSSION**

Cobalt-transporting CPx-type ATPases have not previously been described. Several lines of evidence indicate that CoaT exports this metal ion. Cobalt is the most potent inducer of transcription from the coaT operator-promoter (Figs. 2 and 5A), insertional inactivation of coaT reduces tolerance to cobalt (Fig. 3A), restoration of cobalt tolerance by coaT (Fig. 5B) supports direct interaction with CoaT. Deformation of the carboxyl terminus. In low cobalt, CoaR associates with and represses transcription from the coaT operator-promoter (B). In elevated concentrations of cobalt, CoaR mediates repression of transcription from the coaT operator-promoter.

![Diagram](image)

**Fig. 7. Deletions within the coaT operator-promoter.** Panel A, the coaR and coaT genes corresponding to ORFs slr0794 and slr0797 in the fully sequenced genome of *Synechocystis* PCC 6803 are shown (shaded rectangles). An expanded 40-bp region of the coaT operator-promoter is marked with arrows to indicate a degenerate 13-6-13 hypenated inverted repeat. The near consensus (...).

**Fig. 8. Expression from the coaT operator-promoter in a cbiE mutant of *Synechocystis* PCC 6803 and the proposed mechanism of action of CoaR.** Panel A, strain JRNR1.2 (filled bars) and a mutant of JRNR1.2 with cbiE disrupted on a proportion of chromosomes (shaded bars) were grown with no metal supplement or with added Co$^{2+}$ (1 μM) for ~20 h immediately before assay. Panels B to E, the proposed mechanism of action of CoaR in *Synechocystis* PCC 6803. CoaR is shown as two circles, representing the MerR (M) and precorrin isomerase (P)-like domains, with the Cys-His-Cys motif (CHC) at the carboxyl terminus. In low cobalt, CoaR associates with and represses transcription from the coaT operator-promoter (B). In elevated concentrations of cobalt, CoaR mediates repression of transcription from the coaT operator-promoter.

![Diagram](image)
strate limited. In *P. denitrificans* it is known that hydrogenobyrinic acid precedes the step of cobalt insertion into the corrin ring (6) and is predicted to accumulate when there is insufficient cobalt for vitamin B<sub>12</sub> biosynthesis. An inhibition of CoaT production when hydrogenobyrinic acid accumulates will restrict cobalt export when there is cellular demand (Fig. 8D). Thus, via responses to two effectors, (i) cobalt (positive effector) and (ii) intermediates in the vitamin B<sub>12</sub> pathway (negative effector), CoaR integrates cobalt homeostasis with metabolism. Enzyme recruitment (39) is exemplified by the evolution of the latter response. It is predicted that binding of hydrogenobyrinic acid to the precorrin isomerase domain of CoaR prevents cobalt-mediated conformational change required for activation, possibly occluding the cobalt binding site. Adjacent to the coa divergon in *Synechocystis* PCC 6803 is a deduced operon, starting with ORF slr0793, with similarity to *cnr* and *czc* operons, both of which mediate export of metal ions, including cobalt, across the inner and outer membranes (40). Does transport by CoaT facilitate storage of excess cytoplasmic cobalt in the periplasm while the adjacent genes mediate export across the outer membrane upon saturation of periplasmic stores (Fig. 8E)?

It is now apparent that CoaR senses cobalt (Fig. 5) and some of the residues involved in cobalt sensing have been identified (Fig. 6). During the course of this work, a MerR-like protein from *E. coli* has been shown to activate transcription from the zntA operator-promoter in response to zinc (41), and a similar activity suggested for a homologue from *Proteus mirabilis* (42). It will be intriguing to determine how/ if responses of ZntR are modified coincident with fluctuating requirements for zinc. Clearly there is a subfamily of MerR-like proteins that switch transcription in response to metal ions, mercury, cobalt, and zinc sensors having now been identified. It is probable that there are further members specific for other metals, which await discovery. It is also now apparent that CoaT is a cobalt-transporting variant CPx-type ATPase, adding to the catalogue of resistances (cadmium, copper, zinc, and lead) known to be mediated by these proteins. The next challenge will be to understand metal-specificity.

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