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Cysteine-102 is positioned in the metal binding activation site of the *Corynebacterium diphtheriae* regulatory element DtxR

(gene regulation/diphtheria *tox* repressor/iron binding regulatory element)

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ABSTRACT **DNA sequence analysis of *dtxR* has shown that the M_r 25,316 regulatory protein contains a single cysteine residue at position 102. DtxR readily forms inactive disulfide-linked dimers. We have used saturation site-directed mutagenesis of the cysteine codon (TGC) at position 102 in order to determine the role of this residue in metal ion binding. We show that the insertion of amino acids other than cysteine or aspartic acid into this position abolishes DtxR function both *in vitro* and in recombinant *Escherichia coli* DH5α:λRS45*toxPO/lacZ*. Only those mutant alleles in which the TGC codon for Cys-102 was replaced by either TGT (Cys) or GCA (Asp) were found to direct the expression of active forms of DtxR that regulate the expression of β-galactosidase from the *toxPO/lacZ* transcriptional fusion.**

The structural gene encoding the iron-dependent diphtheria *tox* regulatory element DtxR has been recently cloned from genomic libraries of nontoxigenic *Corynebacterium diphtheriae* (1, 2). DtxR is a M_r 25,613 protein that, in the presence of divalent heavy metal ions, has been shown by gel mobility-shift assay to bind to the diphtheria *tox* regulatory region (3, 4). The binding of DtxR to the *tox* operator requires a 9-bp interrupted palindromic sequence. Moreover, DtxR binding to the *tox* promoter/operator, *toxPO*, probe could be specifically blocked by the addition of either unlabeled probe or anti-DtxR (3). We (5) have recently demonstrated by DNase I protection assays that DtxR may be activated by Fe^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}. In the presence of one of these transition metal ions, DtxR binds to the *tox* operator and protects a 33-nt sequence on the coding strand. The protected region overlaps with a 27-nt segment on the noncoding strand that contains the 9-bp interrupted palindromic sequence.

DNA sequence analysis has shown that DtxR contains a single Cys residue at position 102 (1, 2). Since purified DtxR readily forms disulfide-linked dimers, the sulfhydryl group of Cys-102 appears to be exposed on the surface of this protein. In dimeric form, DtxR is inactive and will not bind to the *toxPO* probe even in the presence of divalent heavy metal ions (4).

To determine whether Cys-102 is in the metal binding activation site of DtxR, we have used saturation site-directed mutagenesis of the TGC codon. We demonstrate that the insertion of all amino acids other than Cys or Asp into position 102 of DtxR results in the loss of regulatory activity. We demonstrate that the mutant DtxR(C102D) is also activated by heavy metal ions and specifically binds to the diphtheria *tox* operator. The differential expression of β-galactosidase in *Escherichia coli* strains that carry a *toxPO/lacZ* transcriptional fusion under the control of either *dtxR* or *dtxR*-102D strongly suggests that the apparent metal binding affinity of DtxR(C102D) is less than that of the wild-type regulatory element.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Medium. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria broth (LB; 10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract per liter). As noted, LB broth or LB agar (15 g of agar per liter of broth) was supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (40 μg/ml). 2X-YT medium contains 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter.

Nucleic Acids and Site-Directed Mutagenesis. Double-stranded plasmid DNA was prepared by using the Magic miniprep system (Promega). Single-stranded DNA was prepared by polyethylene glycol (PEG) precipitation, phenol extraction, and ethanol precipitation. Restriction endonucleases, T4 polynucleotide kinase, and the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories) were used according to the manufacturer's specifications. Restriction endonuclease fragments were separated by electrophoresis on 1% agarose gels or 4% low melting temperature agarose gels in TBE (89 mM Tris/89 mM boric acid/1 mM EDTA, pH 8.3). Appropriate restriction fragments were excised from the gel, extracted with phenol, and ethanol precipitated before use.

Saturation mutagenesis was performed by standard techniques for oligonucleotide-directed mutagenesis (8). The DNA fragment containing *dtxR* was subcloned into the polylinker of phagemid pBluescript II KS+ (Stratagene), generating the phagemid pXT102C. Single-stranded template DNA, containing the sense strand of *dtxR*, was prepared in *E. coli* JM101 by using VCSM13 helper phage (Stratagene). An antisense oligonucleotide with the sequence 5′-CGTGTTCCCAGCGNNNGGCTTCATCGTGAAC-3′, with randomized bases at the position corresponding to the codon for Cys-102, was used for site-directed mutagenesis [N denotes the use of an equimolar mixture of isobutyldeoxyguanosine, benzoyldeoxyadenosine, benzoyldeoxycytidine, and thymidine phosphoramidites in the synthesis reaction mixture (Applied Biosystems)]. A T7-Gen *in vitro* mutagenesis kit (United States Biochemical) was used in the mutagenesis procedure. Following second-strand DNA synthesis and ligation, the unmethylated template DNA was nicked by *Sau*3AI and subsequently digested with exonuclease III. The DNA was then transformed into the SDM strain of *E. coli*. DNA sequencing was performed by the dideoxynucleotide chain-termination method of Sanger *et al.* (9) as modified by Kraft *et al.* (10) using Sequenase (United States Biochemical).

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Abbreviations: X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; DTT, dithiothreitol.

Table 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Genotype	Source or ref.
E. coli		
JM101	*supE, thi*, Δ(*lac-proAB*), [F'*traD36, proAB, lacI*^{q}, *lacZ*ΔM15]	Ref. 6
DH5α	F$^-$(Φ80d *lacZ*ΔM15)Δ(*lacZYA-argF*) U169 *recA1, endA1, hsdR17* (rk$^-$, mk$^+$)*supE44, thiI, gyrA, relA1*	BRL
SDM	*hsdR17, mcrAB, recA1, supE44, Tet*^{r}, Δ(*lac-proAB*), [F'*traD36, proAB*$^+$, *lacI*^{q}ZΔM15]	USB
DH5α:λRS45*toxPO/lacZ*	As DH5α but lysogenic for RS45(*toxPO/lacZYA, kan*^{r})	Ref. 1
HMS174(DE3)pDR-1		Ref. 3
Plasmids		
M13*dtxR*T7	*amp*^{r} *dtxR*$^+$	Ref. 3
pBluescript II KS+	*amp*^{r}	USB
pXT102C	As pBluescript but *dtxR*$^+$	This study
pXT102D	As pBluescript but *dtxR*$^+$	This study
pXT102X*	As pBluescript but *dtxR*	This study
pRS551*toxPO/lacZ*	*amp*^{r}, *kan*^{r}	Ref. 1
pRS551*toxPO/lacZ-dtxR*†	*amp*^{r}	Ref. 7
pRS551*toxPO/lacZ-dtxR*-102D	*amp*^{r}	This study

BRL, Bethesda Research Laboratories; USB, United States Biochemical.
*X denotes all amino acid substitutions except for cysteine and aspartic acid.
†Previously described as pRS551toxPO-C7 (7).

Expression and Purification of DtxR. Expression of *dtxR* from the T7 promoter and purification of DtxR from crude extracts of recombinant *E. coli* were performed as described (3). *E. coli* HMS174(DE3)pDR-1 was grown at 37°C in LB ampicillin medium. Expression of *dtxR* was induced by the addition of isopropyl β-D-thiogalactoside to the growth medium to a final concentration of 0.4 mM. Two hours after induction, bacteria were harvested by centrifugation, resuspended in 10 mM Tris·HCl (pH 7.5), and lysed by sonication. The lysate was centrifuged at 25,000 × *g* for 20 min at 4°C to remove whole bacteria and debris. The supernatant fluid was then applied to a DE-53 (Whatman) anion-exchange column, which was equilibrated with 10 mM Tris·HCl (pH 7.5). The column was washed with application buffer, and DtxR was then eluted with a linear gradient of 40–300 mM NaCl in the same buffer. Fractions containing DtxR were collected, pooled, and dialyzed against 10 mM Tris·HCl, pH 7.5/10 mM dithiothreitol (DTT) buffer before use.

Gel Electrophoresis and Immunoblot Analysis. SDS/PAGE was performed according to the method of Laemmli (11) using 12% polyacrylamide gels in the presence or absence of 0.1 M DTT. Proteins were stained with Coomassie brilliant blue or electrophoretically transferred to poly(vinylidene difluoride) membranes for immunoblot analysis. Immunoblots were probed with a 1:3000 dilution of anti-DtxR antiserum followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Promega) as described (3). Immunoreactive proteins were detected by the addition of nitroblue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate substrate using the ProtoBlot AP system according to the directions of the manufacturer (Promega).

Gel Electrophoresis Mobility-Shift Assay. Gel electrophoresis mobility-shift assays were conducted as described (3). Briefly, the binding of DtxR to the diphtheria *toxPO* probe was carried out in a 16-μl reaction mixture containing 20 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 125 mM $MnCl_2$, 10% (vol/vol) glycerol, 1 μg of poly(dI-dC), 5 μg of bovine serum albumin, purified DtxR, and 3–5 fmol of ^{32}P-labeled *toxPO* probe. DTT was omitted from the reaction mixture in assays performed under nonreducing conditions.

The *toxPO* probe was excised from plasmid pRS551*toxPO/lacZ* by digestion with *Eco*RI and *Bam*HI and labeled with [^{32}P]ATP by filling with Klenow fragment according to standard procedures (12). After the labeled probe was added, the reaction mixture was incubated for 15 min at 25°C. Eight microliters of the reaction mixture was then applied to a 6% polyacrylamide gel in 40 mM Bistris, pH 7.5/125 μM $MnCl_2$/2.5% glycerol buffer and electrophoresed in the same buffer without glycerol at a constant voltage (200 V). After electrophoresis, the gels were dried and analyzed by autoradiography.

β-Galactosidase Activity Measurements. Plasmids that encoded *dtxR* alleles were transformed into *E. coli* DH5α:λRS45*toxPO/lacZ* and the cells were plated on LB plates containing X-Gal, ampicillin, and kanamycin. For quantitative determination of *lacZ* expression, cells were grown in LB medium overnight. β-Galactosidase activities were measured in bacterial extracts after treatment with lysis mixture [toluene/0.2 M $MnSO_4$/10% SDS/2-mercaptoethanol (1:1:1:5)] as described by Miller (13) and modified by Putnam and Koch (14). Results are reported as the means of three independent assays.

RESULTS

As shown in Fig. 1*A* (lane 3), DtxR purified from extracts of recombinant *E. coli* readily forms dimers under oxidizing conditions. In contrast to the monomeric form, DtxR in the dimeric state has a markedly reduced ability to associate with the diphtheria *tox* operator even in the presence of elevated concentrations of heavy metal ions (Fig. 1*B*, lane 3). After

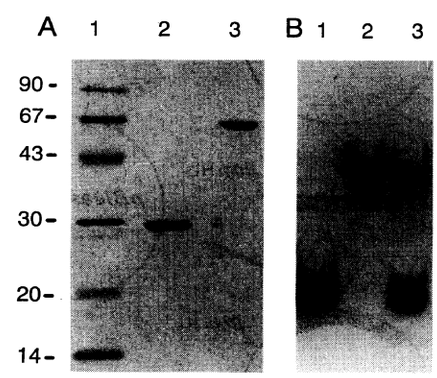

FIG. 1. (*A*) SDS/PAGE analysis of purified DtxR under reduced and oxidized conditions. Lanes: 1, molecular weight standards (M_r × 10^{-3}); 2, reduced DtxR; 3, oxidized DtxR. (*B*) Gel mobility-shift assay of reduced and oxidized DtxR binding to ^{32}P-labeled *toxPO* probe. Lanes: 1, *toxPO* probe; 2, *toxPO* probe and reduced DtxR; 3, *toxPO* probe and oxidized DtxR.

reduction with DTT and conversion of disulfide-linked DtxR to the monomeric form, the regulatory protein becomes fully activated in the presence of heavy metal ions and specifically binds to the *tox* operator (Fig. 1, lanes 2). Based on gel mobility-shift experiments with serial dilutions of monomeric and dimeric forms of DtxR, it is evident that the reduced form binds the *toxPO* probe ≈100 times more avidly than the oxidized form (data not shown). Furthermore, densitometric analysis of Coomassie blue-stained SDS/polyacrylamide gels of reduced and oxidized DtxR indicates that samples of the dimeric form contain ≈1% monomer. Based on these observations, we conclude that the dimeric form of DtxR is inactive and unable to bind to the operator probe.

Since a free sulfhydryl moiety in Cys-102 appeared to be necessary for the activation of DtxR, we have performed site-directed saturation mutagenesis of this residue in order to explore its role in the heavy metal ion activation process. To facilitate the isolation of mutants by phenotype selection as well as their subsequent DNA sequence analysis, the structural gene encoding DtxR was recloned into a phagemid. Coliphage M13*dtxR*T7 was digested with *Bam*HI and *Eco*RI and the 1.5-kb fragment that encodes *dtxR* was recloned into the *Bam*HI and *Eco*RI sites of the phagemid pBluescript II KS+ to form pXT102C (Fig. 2; Table 1). After ligation and transformation of *E. coli* JM101, a single colony was selected, and minilysate DNA was then prepared and analyzed by restriction endonuclease digestion to ensure that *dtxR* was recloned in single copy in the correct orientation.

E. coli JM101(pXT102C) was then grown in 2X-YT ampicillin medium and infected with helper phage in order to isolate single-stranded DNA that carries the sense strand of *dtxR*. Site-directed saturation mutagenesis was performed by hybridizing a 31-mer oligonucleotide, in which the nucleotides corresponding to codon 102 were randomized, to the template DNA. After extension of the primer with T7 DNA polymerase and treatment with DNA ligase to form double-stranded replicons, the unmethylated parental DNA was nicked with *Sau*3AI and digested with exonuclease III. *E. coli* SDM was then transformed and plated on ampicillin plates. Single colonies were picked and grown in LB ampicillin medium and midlogarithmic-phase cultures were infected with helper phage to facilitate the preparation of single-stranded DNA for sequence analysis. After sequence analysis, phagemid DNA was used to transform *E. coli* DH5α:λRS45*toxPO/lacZ*. Since β-galactosidase is constitutively expressed in this strain, the absence of functional DtxR activity is detected by a blue colony phenotype on X-Gal-containing medium.

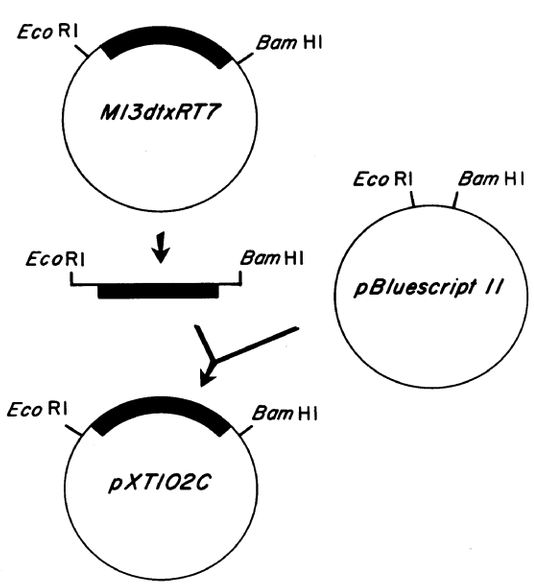

FIG. 2. Plasmid constructions used in this study.

We isolated mutant *dtxR* alleles in which Cys-102 is replaced with each of the 20 amino acids. Analysis of transformants with a white colony phenotype gave rise to two mutant *dtxR* alleles. In the first mutant, the TGC codon for Cys-102 was replaced with the alternative TGT codon for cysteine, thereby giving rise to a wild-type DtxR. In addition, we isolated transformants with a white colony phenotype in which the mutant *dtxR* alleles encoded aspartic acid at position 102.

DNA sequence analysis of the *dtxR* allele from >150 single colonies, which subsequently had a blue phenotype on X-Gal medium, gave rise to a family of *dtxR* mutants in which Cys-102 was replaced with each of the other 18 amino acids. Since a possible explanation for the blue phenotype of *E. coli* DH5α:λRS45*toxPO/lacZ*, which carried these *dtxR* alleles, was proteolytic degradation of the mutant DtxR, we analyzed crude cell-free extracts of strains carrying each plasmid by immunoblot using anti-DtxR antiserum. As shown in Fig. 3, DtxR expressed from each of the mutant alleles was not degraded and was found to have an electrophoretic mobility corresponding to that of the wild-type regulatory element. It should be noted that in the case of DtxR(C102D) and DtxR(C102E) the mutant regulatory proteins have an electrophoretic mobility in SDS/polyacrylamide gels that was slightly less than that of the wild type corresponding to an M_r of 28,500.

Since only low levels of β-galactosidase are expressed from the single genomic copy of the *toxPO/lacZ* transcriptional fusion, we recloned the *dtxR*-102D allele into the reporter plasmid pRS551*toxPO/lacZ*. In this instance, the copy number of the regulatory element and the reporter gene were equivalent in recombinant *E. coli* DH5α. We then compared the levels of β-galactosidase produced in strains of DH5α, which carried the empty vector, the vector with wild-type *dtxR*, and the vector with the *dtxR*-102D allele in the presence and absence of the chelator 2,2′-dipyridyl. As shown in Table 2, the expression of β-galactosidase in *E. coli*(pRS551*toxPO/lacZ*) was constitutive. The addition of 2,2′-dipyridyl to concentrations >100 μM was found to inhibit both the growth and expression of β-galactosidase in *E. coli* strains. As anticipated from earlier studies (1), the expression of β-galactosidase from the *toxPO/lacZ* transcriptional fusion in strains that carried the wild-type *dtxR* allele was regulated and required the addition of 2,2′-dipyridyl to 200 μM to induce expression of the reporter gene. It is most interesting to note, however, that the expression of β-galactosidase in strains of *E. coli* that carried the *dtxR*-102D allele was also found to be sensitive to the concentration of Fe^{2+} in the growth medium. The addition of Fe^{2+} to 100 μM was found to inhibit the expression of β-galactosidase relative to that produced in LB medium alone. Furthermore, the addition of increasing concentrations of 2,2′-dipyridyl to the growth medium resulted in increased levels of *lacZ* expression.

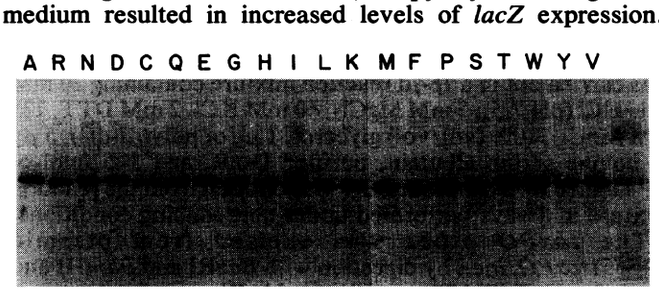

FIG. 3. Immunoblot analysis of crude extracts of recombinant *E. coli* that carry wild-type and mutant *dtxR* alleles. Single-letter code indicates amino acid substitution at position 102 of DtxR.

Table 2. Expression of β-galactosidase from the *toxPO/lacZ* transcriptional fusion in *E. coli* strains following growth in the presence and absence of 2,2′-dipyridyl

Addition	Concentration, μM	pRS551 derivative		
		toxPO/lacZ-dtxR	*toxPO/lacZ-dtxR*-102D	*toxPO/lacZ*
Fe^{2+}	100	39 ± 5	196 ± 16	2916 ± 133
None		21 ± 3	408 ± 51	2862 ± 142
2,2′-Dipyridyl	100	17 ± 9	1074 ± 350	2631 ± 80
2,2′-Dipyridyl	200	526 ± 113	1650 ± 71	1943 ± 33
2,2′-Dipyridyl	300	847 ± 28	1021 ± 68	1190 ± 228

These results strongly suggest that the mutant DtxR(C102D), like the wild-type DtxR, is able to function as an iron-dependent regulatory element and control expression from the *tox* promoter/operator.

To rigorously demonstrate that DtxR(C102D) was able to specifically bind to the *tox* operator, we performed a series of gel mobility-shift experiments using a ^{32}P-labeled diphtheria *tox* operator probe. We have previously shown that binding of the wild-type DtxR to the *tox* operator requires the presence of divalent heavy metal ions and the 9-bp interrupted palindromic sequence (3). As shown in Fig. 4, the addition of crude extracts of *E. coli*(pXT102D) to the gel mobility-shift reaction mixture results in the specific interaction between the mutant regulatory element and the *tox* operator. The addition of either excess unlabeled probe or anti-DtxR antiserum to the reaction mixture was found to block the interaction between DtxR(C102D) and the labeled probe (lanes 2 and 3).

DISCUSSION

The aim of this study was to determine whether Cys-102 was essential for the regulatory activity of DtxR. We performed saturation site-directed mutagenesis at amino acid position 102, and mutant *dtxR* alleles carrying each of the 20 amino acids at this position were isolated and characterized. Each of the cloned *dtxR* alleles was transformed into *E. coli* DH5α:λRS45*toxPO/lacZ*, a reporter strain that carries a single genomic copy of a transcriptional fusion between the diphtheria *tox* regulatory region and *lacZ*. DtxR has been shown to repress *lacZ* expression in this strain and give rise to white colony phenotype on X-Gal-containing medium (1).

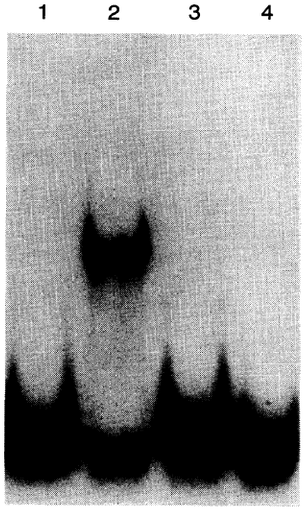

FIG. 4. Gel mobility-shift assay of DtxR(C102D) binding to the ^{32}P-labeled *toxPO* probe in the presence and absence of inhibitors. Lanes: 1, ^{32}P-labeled *toxPO* probe alone; 2, ^{32}P-labeled *toxPO* probe plus DtxR(C102D); 3, ^{32}P-labeled *toxPO* probe, DtxR(C102D), plus 200 fmol of unlabeled probe; 4, ^{32}P-labeled *toxPO* probe, DtxR(C102D), plus 1:160 dilution of anti-DtxR antiserum.

From all of the *dtxR* alleles studied, only the wild type and two mutants were found to encode an active *tox* regulatory element. The two mutant *dtxR* alleles were found to carry either the alternative codon for cysteine (TGT) or the codon for aspartic acid at position 102.

It is well known that the introduction of a mutation(s) at certain positions in a protein may increase susceptibility to intracellular proteolysis (15). To rule out the possibility that degradation of mutant DtxR gave rise to the blue colony phenotype in the reporter strain, we analyzed crude extracts of *E. coli* that carried the wild-type and mutant *dtxR* alleles by immunoblot using anti-DtxR antiserum as described (3). We have shown that when expressed all mutant DtxR had electrophoretic mobilities similar to that of the wild type ($M_r \approx 28,000$). These results demonstrate that the insertion of amino acids other than Cys and Asp into position 102 abolished DtxR function.

Gene expression from the heterologous diphtheria *tox* promoter in recombinant strains of *E. coli* is relatively weak. Since the lysogen DH5α:λRS45*toxPO/lacZ* only carries a single copy of *toxPO/lacZ*, strains that carry either *dtxR* or *dtxR*-102D on multicopy plasmids were found to repress β-galactosidase expression even in low iron medium. To demonstrate rigorously that DtxR(C102D)-mediated repression of *lacZ* was iron dependent, we cloned the *dtxR*-102D allele into pRS551*toxPO/lacZ*. In this instance, the bacterial host strain carries an equal copy number of repressor and reporter genes. The results obtained from quantitative measurement of β-galactosidase produced in strains that carried either *dtxR* or the *dtxR*-102D mutant indicate that both DtxR and DtxR(C102D) function as iron-dependent regulatory elements. Since the growth of recombinant *E. coli* strain is inhibited in the presence of elevated concentrations of 2,2′-dipyridyl, the addition of 2,2′-dipyridyl to concentrations >100 μM resulted in a decrease in the level of β-galactosidase expressed. Nonetheless, β-galactosidase activities in both *E. coli*(pRS551*toxPO/lacZ-dtxR*-102D) and *E. coli*(pRS551*toxPO/lacZ-dtxR*) increased with increasing concentrations of 2,2′-dipyridyl. It is of interest to note that the addition of equivalent concentrations of 2,2′-dipyridyl to the medium resulted in the expression of higher levels of β-galactosidase in strains that carried the *dtxR*-102D allele. DtxR(C102D) was unable to completely repress *lacZ* expression even in medium supplemented with 100 μM Fe^{2+}. Even though the regulatory activity of DtxR(C102D) was not as sensitive to the concentration of iron as DtxR, it was able to control β-galactosidase expression from the *toxPO/lacZ* transcriptional fusion in an iron-dependent manner. In addition, we have demonstrated by gel mobility shift that the interaction between DtxR(C102D) and the *tox* operator is both specific and dependent on the presence of heavy metal ions in the reaction mixture.

The fact that only one amino acid substitution is allowed for Cys-102 suggests that this residue plays an important role in DtxR-mediated regulatory activity. Both Cys and Asp have been shown to serve as metal binding residues in many metalloproteins (16). Based on either x-ray crystal structure or NMR spectroscopy, the sulfhydryl group of Cys has been

found to be a coordination site in alcohol dehydrogenase (17) and in the transcription factor TFIIIA-like zinc finger protein (18), whereas the carboxyl group of Asp has been shown to bind metal in alkaline phosphatase (19).

His and Glu residues are also found to be metal-coordination sites in many metalloproteins; however, neither of these amino acids was able to substitute Cys-102 in DtxR. The bulky side chain of His and the additional carbon atom of Glu may not meet the geometric requirements necessary for DtxR activation. This explanation may also explain why DtxR(C102D) is less avid with respect to metal binding than wild-type DtxR. As yet, we have not determined whether the Asp substitution at position 102 results in either a decrease in the metal binding affinity or a decrease in DNA binding affinity.

DtxR has been shown to regulate the diphtheria *tox* operon in a manner analogous to Fur in *E. coli*, although the two regulatory proteins have different DNA target sequences. Coy and Neilands (20) have shown that the C-terminal region of Fur is sufficient for binding to metal ions and Cys residues may directly or indirectly interact with metals. In addition, His residues in Fur may also be involved in metal binding (21). DtxR has not been shown to contain significant homology to any known metal binding motif. While we have shown that Cys-102 plays a critical role in heavy metal activation of DtxR, we cannot rule out the possibility that other amino acids are also involved in metal ion binding. A detailed understanding of the interactions between DtxR and the heavy metal ions that activate this protein must await determination of the x-ray crystal structure.

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