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Characterization of Mutations That Inactivate the Diphtheria Toxin Repressor Gene (*dtxR*)

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The diphtheria toxin repressor (DtxR) is an iron-dependent regulator of diphtheria toxin production and iron uptake in *Corynebacterium diphtheriae*. It is activated in vitro by divalent metal ions including Fe^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}. We characterized 20 different mutations in *dtxR* induced by bisulfite mutagenesis, 18 of which caused single-amino-acid substitutions in DtxR and two of which were chain-terminating mutations. Six of the amino acid replacements were clustered between residues 39 and 52 in a predicted helix-turn-helix motif that exhibits homology with several other repressors and is identified as the putative DNA-binding domain of DtxR. Three substitutions occurred within a predicted alpha-helical region with the sequence His-98–X_3–Cys-102–X_3–His-106 that resembles metal-binding motifs in several other proteins and is identified as the putative metal-binding site of DtxR. Several purified variants of DtxR with decreased repressor activity failed to bind in gel retardation assays to DNA fragments that contained the *tox* operator. A quantitative assay for binding of DtxR to $^{63}Ni^{2+}$ was also developed. Scatchard analysis revealed that DtxR has a single class of high-affinity $^{63}Ni^{2+}$-binding sites with a K_d of 2.11×10^{-6} M and a maximum binding capacity of approximately 1.2 atoms of Ni^{2+} per DtxR monomer. The P39L, T40I, T44I, and R47H variants of DtxR exhibited normal to slightly decreased $^{63}Ni^{2+}$-binding activity, but H106Y, which has an amino acid substitution in the presumed metal-binding domain, exhibited markedly decreased $^{63}Ni^{2+}$-binding activity.

The diphtheria toxin repressor (DtxR) is a negative regulator that controls the expression of diphtheria toxin and the high-affinity iron uptake system of *Corynebacterium diphtheriae* in response to the environmental iron concentration (20, 21, 30, 37). Under high-iron conditions, the production of diphtheria toxin is repressed, and optimal production of diphtheria toxin occurs under low-iron conditions. The structural gene for diphtheria toxin, *tox*, is present on the genome of temperate corynebacteriophages, such as phage β (20, 42). Expression of the *tox* gene is regulated by DtxR at the level of transcription (4, 9, 12, 15, 19).

Genetic and biochemical analysis of DtxR was facilitated by cloning and sequencing the *dtxR* gene and by purifying the DtxR protein (5, 30–32, 34, 38, 39). DtxR contains 226 amino acid residues and has a predicted molecular mass of 25,316 Da. DtxR is an iron-dependent, sequence-specific, DNA-binding protein. Gel retardation and DNase I protection experiments demonstrated that it binds to an approximately 30-bp DNA sequence that contains a region of dyad symmetry and which overlaps the −10 sequence of the *tox* promoter (4, 9, 31, 32, 34, 38, 39). In addition to Fe^{2+}, other first-row transition cations, including Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, also activate the DNA-binding activity of DtxR (33, 39). Hydroxyl radical protection experiments revealed that DtxR binds to the deoxyribose-phosphate backbone of the *tox* operator and other DtxR-regulated operators of *C. diphtheriae* in a twofold symmetrical manner about the dyad axis (32, 33). These findings provide strong but indirect evidence that DtxR binds to its cognate operators as a dimer or multimer, similar to the manner by which several other well-characterized repressors, including Cro, LacR, TrpR, and MerR, bind to their respective operators (6, 10, 14, 17, 22). DtxR, therefore, has at least three distinct activities: (i) the ability to bind DNA and inhibit transcription from the *tox* promoter and other DtxR-regulated promoters; (ii) the ability to bind ferrous iron and other related divalent metal ions; and (iii) the ability to form dimers or multimers.

The DtxR protein from *C. diphtheriae* is functionally similar to the Fur protein from *Escherichia coli*. Fur is also an iron-dependent repressor, and it regulates numerous genes in *E. coli* (2, 8, 29). Computer searches for homologous sequences at the nucleotide and amino acid levels revealed that the *dtxR* and *fur* genes as well as their protein products have low levels of homology (2, 5, 31). Nevertheless, DtxR and Fur have different DNA-binding specificities, and they interact with different sets of operators (5, 31–34, 38, 39).

In this study, we used molecular genetic methods to assess the structural basis for the distinct functions of the DtxR protein discussed above. We subjected the *dtxR* gene to random mutagenesis in vitro, and we isolated and characterized a set of mutant *dtxR* alleles that determined single-amino-acid substitutions or chain termination in the DtxR polypeptide. We identified putative DNA-binding and metal ion-binding domains in the amino-terminal half of DtxR within which amino acid substitutions caused decreased repressor activity. We also purified several representative variants of DtxR with alterations in the putative DNA-binding or metal-binding motif and tested their site-specific DNA-binding activity and their metal-binding activity in vitro.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains DH5α, XL-1 Blue, and MC1009 (Bethesda Research Laboratories, Gaith-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source[a]
E. coli		
DH5α	F^- *supE44* Δ*lacU169* (φ80 *lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	BRL
MC1009	F^- Δ(*lacIPOZY*) *chi-74* Δ(*ara-leu*)*7697 galK galU recA rpsL* λ	BRL
XL-1 Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac* F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(Tet^r)]	BRL
Plasmids		
pMS298	pBluescriptKS carrying 1.4-kb *Pvu*II insert containing the *dtxR*$^+$ allele (Amp^r)	30
pSKdtxR	0.8-kb *Nco*I-*Nar*I insert from pMS298 carrying the *dtxR*$^+$ allele (without its native promoter) under *lacZ* promoter control in pBluescriptSK vector (Amp^r)	This study
pSKlac	pBluescriptSK vector with *lacZ* deletion (*Pvu*II-*Pvu*II) (Amp^r)	This study
pCMZ100	*tox-lacZ* translational fusion in *E. coli-C. diphtheriae* shuttle vector pCM2.6 (Cm^r)	31
pDSK29	5-kb fragment carrying *dtxR*$^+$ allele in RSF1010-derived vector (low copy number) (Kan^r)	31

[a] BRL, Bethesda Research Laboratories.

ersburg, Md.) were used for all experiments. Stock cultures were stored at −70°C in 20% glycerol. The strains and plasmids used in this study are listed in Table 1.

Reagents, media, and growth conditions. *E. coli* strains were routinely cultured in Luria broth (LB) medium or on LB agar medium. LB medium contains about 30 μM iron and is considered to be a high-iron medium. The following supplements were added as needed: ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), kanamycin (50 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml). LB medium was made low iron by the addition of an iron chelator, ethelenediamine-di-*o*-hydroxyphenyl acetic acid (EDDA), at 500 μg/ml, and LB agar medium was made iron deficient by adding EDDA at 40 μg/ml. EDDA was deferrated by the method of Rogers (24) prior to use as an iron chelator.

Bisulfite mutagenesis. Bisulfite mutagenesis was performed by the method of Pine and Huang (23). Briefly, gapped duplex molecules (containing gaps of approximately 250 to 300 bp) were formed by annealing 2-μg amounts of selected, double-stranded DNA fragments from plasmid pSKdtxR with 1 μg of single-stranded, full-length, genomic DNA from pSKdtxR. The single-stranded DNA was isolated from *E. coli* XL-1 Blue(pSKdtxR) by established procedures (16). Reaction conditions were determined empirically (23) with the intent of introducing approximately one C-to-T transition per *dtxR* gene. In each case, 750 ng of gapped duplex DNA was treated for 10 min at 37°C with 3 M sodium bisulfite and 2 mM hydroquinone. Reaction mixtures were transferred to dialysis tubing and dialyzed at 4°C against 5 mM sodium phosphate to terminate the reaction. Gaps on the mutagenized, gapped duplex DNA were filled by using the Klenow fragment of DNA polymerase and T4 ligase, and the completely double-stranded pSKdtxR plasmids were transformed into *E. coli* DH5α (pCMZ100) (7).

Identification of *dtxR* mutants. Mutagenized pSKdtxR plasmids were transformed into *E. coli* DH5α(pCMZ100). The transformants were grown on LB agar medium containing X-Gal and the antibiotics ampicillin and chloramphenicol to maintain positive selection for both plasmids. A mixture of blue and white colonies appeared on this medium after 24 h at 37°C. Blue colonies were defective in DtxR repressor activity, with increasing color indicating progressively greater impairment of repressor function. Colonial phenotypes were designated as follows: −, white; ±, trace blue; +, light blue; ++, medium blue; and +++, dark blue. The nucleotide sequences of inactivated *dtxR* alleles were determined, and *dtxR* alleles in several white colonies were also sequenced to identify phenotypically DtxR$^+$ strains with silent mutations in the *dtxR* gene. Dominant negative *dtxR* mutations were identified by transforming each of the mutant pSKdtxR plasmids into *E. coli* DH5α(pCMZ100, pDSK29) and screening for blue colonies on LB agar medium containing X-Gal and the antibiotics ampicillin, chloramphenicol, and kanamycin to maintain positive selection for all three plasmids. Blue colonies were scored as described above for the intensity of the blue color, and + or ++ indicated that the mutant *dtxR* allele blocked expression of the wild-type repressor activity encoded by the *dtxR*$^+$ allele of plasmid pDSK29.

DNA sequencing and analysis. Double-stranded DNA for sequencing was isolated from the appropriate *E. coli* DH5α clones, and for each clone the sequence of the segment of the *dtxR* gene that had been subjected to mutagenesis was determined by the dideoxy chain termination method of Sanger et al. (27). Dideoxy chain termination reactions were done with T7 polymerase (Sequenase 2.0; United States Biochemical, Cleveland, Ohio) by using the following oligonucleotide primers: MCS-1, 5′-ACAAAAGCTGGAGCTCCAC-3′; MW-1, 5′[326]-CTCATAACGTGTTCCCAGC-3′[308]; MCS-2, 5′[158]-TTGTCGTTGTCGCCTCAGA-3′[176]; MW-2, 5′[555]-AGCATCGAGGAGCTGTGTA-3′[537]; MCS-3, 5′[410]-AACTCGGCGTAGGCAATTC-3′[428]; MW-3, 5′-ATACGACTCACTATAGGGC-3′. (The numbers in brackets for MW-1, MCS-2, MW-2, and MCS-3 refer to nucleotide positions within the published DNA sequence of the *dtxR* gene [5].) Primers MCS-1 and MW-3 were located within the multicloning site of plasmid pBluescript SK. Reaction products were resolved on 6% polyacrylamide-urea gels (16). The deduced amino acid sequence of DtxR was analyzed by using the PROTEIN ANALYSIS program (GCG Sequence Analysis Software Package, University of Wisconsin Biotechnology Center, Madison).

Assay of β-galactosidase. Because the *tox-lacZ* translational fusion gene in pCMZ100 is negatively regulated by the DtxR repressor, intracellular levels of β-galactosidase were inversely proportional to DtxR repressor activity. β-Galactosidase activity of *E. coli* strains carrying the various plasmids was determined as described by Miller (18).

Western blot (immunoblot) analysis of DtxR proteins in bacterial extracts. *E. coli* strains with pSKdtxR plasmids harboring wild type or mutant *dtxR* alleles were grown overnight with aeration at 37°C in LB medium containing ampicillin. Bacteria from 5 ml of culture were harvested by centrifugation at 5,000 × *g* for 10 min, and subsequent procedures were performed at 4°C. Bacteria were resuspended in 1.5 ml of sonication buffer (10 mM sodium phosphate buffer [pH 7.0], 50

TABLE 2. Characterization of mutant *dtxR* alleles

Strain or plasmid	Codon change in *dtxR*	Functional assay[a]		Dominance of *dtxR* allele[b]
		Phenotype	β-Gal activity (U)	
Mutants of pSKdtxR[c]				
T67I	ACA→ATA	−	0.3 ± 0.3	−
T24I	ACC→ATC	−	0.3 ± 0.2	−
E19K	GAA→AAA	±	0.8 ± 0.3	−
T44I	ACC→ATT	±	0.8 ± 0.2	±
T7I	ACC→ATC	±	1.3 ± 0.4	−
R47H	CGT→CAT	+	2.0 ± 0.1	+
A72V	GCG→GTG	+	2.6 ± 0.1	±
R13C	CGT→TGT	+	2.7 ± 0.5	−
R84H	CGC→CAC	++	4.8 ± 0.6	±
D88N	GAT→AAT	++	5.7 ± 1.6	±
R77H	CGT→CAT	++	7.1 ± 0.8	+
W104[d]	TGG→TAG	+++	9.5 ± 0.1	+
T40I	ACA→ATA	+++	11.7 ± 1.1	+
A147V	GCC→GTC	+++	11.9 ± 0.9	−
H106Y	CAC→TAC	+++	12.0 ± 0.3	+
E100K	GAA→AAA	+++	12.6 ± 1.9	++
P39L	CCT→CTT	+++	14.1 ± 1.2	+
Q36	CAA→TAA	+++	17.1 ± 1.8	−
G52E	GGA→GAA	+++	17.7 ± 1.9	+
A46V	GCC→GTC	+++	28.8 ± 1.9	−
Control plasmids				
pSKdtxR		−	0.2 ± 0.2	−
pDSK29		−	0.3 ± 0.2	ND[e]
pSKlac		+++	51.9 ± 4.1	ND

[a] Functional assays for repressor included colonial phenotype and quantitative measurement of β-galactosidase (β-gal) activity. For colonial phenotype designations, see Materials and Methods. Increasing colonial color or enzyme activity indicates progressively greater impairment of repressor function. The control value for β-galactosidase activity in DH5α(pCMZ100) without a second or third plasmid was 86.0 ± 7.1 U.

[b] Increasing colonial color indicates progressively greater inhibition of wild-type DtxR.

[c] Strain designations reflect the amino acid substitution or premature chain termination that occurs in the DtxR protein encoded by the *dtxR* allele in the mutant pSKdtxR plasmid.

[d] *E. coli* DH5α contains a *supE* allele that permits production of some W104Q as well as the truncated protein W104.

[e] ND, not determined.

mM NaCl, 0.02% NaN_3) and disrupted by sonication. Insoluble debris was removed by centrifugation at 25,000 × *g* for 20 min. Ten-microliter samples of the crude cell extracts were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE). The proteins were transferred to nitrocellulose and then incubated either with polyclonal rabbit antiserum against a DtxR-MalE fusion protein (34) or with mouse monoclonal antibody 8G5 against DtxR prepared in our laboratory by E. M. Twiddy (41a). The immobilized rabbit antibodies or mouse antibodies were then treated with an enzyme-labelled second antibody of appropriate specificity, and the blots were developed by using a chromogenic substrate as described previously (11).

DtxR protein purification. The DtxR variants P39L, T40I, T44I, A46V, and H106Y were purified from bacterial extracts by Ni^{2+}-nitrilotriacetic acid (NTA)-agarose chromatography (Qiagen Inc., Chatsworth, Calif.) as described previously (32). A small sample of the purified R47H variant from *C. diphtheriae* C7(β)hm723 was available from a previous study (32), and purified wild-type DtxR was prepared by Suping Zhang in our laboratory. The purified proteins, which contained small amounts of contaminants other than DtxR, were treated with 10 mM 2-mercaptoethanol for 15 min at 25°C to reduce disulfide bonds, dialyzed at 4°C for 4 h to remove most of the 2-mercaptoethanol, and stored frozen at −70°C.

In vitro $^{63}Ni^{2+}$-binding assay. $^{63}Ni^{2+}$ (900 Ci/mol) was purchased from Amersham Life Science, Arlington Heights, Ill. Various amounts of $^{63}Ni^{2+}$ were incubated in 250-μl reaction mixtures with wild-type or mutant DtxR protein at the concentrations indicated in the text in buffer (10 mM sodium phosphate [pH 7.0], 50 mM NaCl, 1 mM 2-mercaptoethanol) for 15 min at 25°C. Aliquots (200 μl) were filtered through Immobilon transfer membranes (Millipore Corp., Bedford, Mass.) in a Millipore filtration apparatus. Total $^{63}Ni^{2+}$ was determined by counting samples of the reaction mixtures in a Beckman model LS-7500 scintillation counter. The amounts of $^{63}Ni^{2+}$ bound to the protein were determined by counting the filter disks, and the values for these amounts were corrected by subtracting the amounts of $^{63}Ni^{2+}$ bound nonspecifically to disks in samples that contained no DtxR. The statistical significance of observed differences in binding of $^{63}Ni^{2+}$ by wild-type DtxR and mutant DtxR proteins was evaluated by *t* tests.

RESULTS

Phenotypes of strains with diminished DtxR activity. *E. coli* DH5α containing the reporter plasmid pCMZ100, which carries a *tox-lacZ* gene fusion under transcriptional control of the *tox* promoter-operator region, formed blue (Lac^+) colonies on LB agar (high-iron conditions) containing X-Gal. When the wild-type *dtxR* gene, present on the multicopy plasmid pSK dtxR, was transformed into DH5α(pCMZ100), transcription from the *tox* promoter was repressed under these high-iron conditions, and white colonies (Lac^-) were formed. Plasmids pSKdtxR(+) and pSKdtxR(−), derived from the pBluescript

SK(+) and SK(−) phagemid vectors, respectively, were used to generate single-stranded DNA templates for each strand of the *dtxR* gene. These single-stranded DNA templates were used to construct gapped duplex DNA molecules that were mutagenized with sodium bisulfite and transformed into *E. coli* DH5α(pCMZ100). The transformants carrying the mutagenized plasmids were then tested for the production of β-galactosidase to determine the phenotypes associated with their *dtxR* alleles.

To restrict the target for mutagenesis and facilitate sequencing of the mutant *dtxR* alleles obtained, three sets of gapped duplex molecules were prepared. The 678-bp *dtxR* gene was divided into three regions, with the single-stranded segments of the gapped duplex molecules corresponding to an N-terminal region (−30 to 280 bp; *Xba*I-*Xba*I), a central region (220 to 470 bp; *Pst*I-*Sph*I), and a C-terminal region (470 to 700 bp, *Sph*I-*Sal*I). Each region on each strand was subjected to bisulfite mutagenesis, and more than 1,000 transformants mutagenized within each of the three regions of the *dtxR* gene were screened. Among transformants that had been subjected to mutagenesis in the segment that encoded the N-terminal region, the central region, and the C-terminal region of DtxR, blue colonies represented 55, 51, and 21%, respectively, of the total colonies. Three sets of clones, each consisting of 30 blue colonies and 4 white colonies representing a different mutagenized region of the *dtxR* gene, were selected randomly and purified by single-colony isolations, and the nucleotide sequences of the *dtxR* alleles in the 102 selected clones were determined. Strains were excluded from further analysis if they contained mutations in more than one codon or if they represented independent isolates of *dtxR* alleles already identified. In this manner, 20 mutant *dtxR* alleles with alterations in single codons were identified, two of which resulted in premature termination. Characterization of these 20 mutant *dtxR* alleles is summarized in Table 2.

DtxR variants with single-amino-acid substitutions were designated by the one-letter code for the wild-type amino acid, its number in the sequence of the DtxR polypeptide, and the one-letter code for the amino acid at that position in the mutant polypeptide (e.g., T67I for the variant with isoleucine replacing threonine at residue number 67). T67I and T24I were phenotypically indistinguishable from wild-type DtxR. They are encoded by *dtxR* alleles with silent mutations identified among the strains that retained the ability to form white colonies on LB agar plates containing X-Gal. All variants of DtxR that exhibited decreased repressor activity were from strains that formed blue colonies on LB agar plates with X-Gal. Rank ordering of the strains based on quantitative assays for β-galactosidase activity correlated well with rank ordering based qualitatively on intensity of the blue color of the colonies. All of the mutants may retain at least trace levels of repressor activity since the β-galactosidase activity observed in the presence of the negative control plasmid pSKlac$^-$ (51.9 U) was greater than that observed with any of the *dtxR* mutants.

Dominance of *dtxR* alleles. Mutant forms of repressor that lack activity and also interfere with activity of the wild-type repressor protein have been obtained for several *E. coli* repressors (13, 41). This dominant negative phenotype could result either from formation of nonfunctional heterodimers of the mutant and wild-type repressor polypeptides or, less probably, from competition between homodimers of the nonfunctional mutant polypeptides and wild-type DtxR. We therefore tested the nonsense and missense mutants of *dtxR* to determine whether any expressed a dominant negative phenotype (Table 2).

To optimize the sensitivity of these tests, we used plasmid pDSK29, a low-copy-number plasmid that contains the wild-type *dtxR* allele, to repress the *tox-lacZ* reporter gene of pCMZ100. Each mutant pSKdtxR plasmid was transformed into DH5α(pCMZ100, pDSK29), and the transformants were screened on LB agar containing X-Gal. Positive selection with antibiotics was used to ensure maintenance of all three plasmids, and the presence of all three plasmid DNAs in the transformants was verified by agarose electrophoresis (data not shown). Since the mutant *dtxR* genes were present on the high-copy-number plasmid pSKdtxR, the transformants were expected to express the mutant DtxR polypeptides in large excess over the wild-type DtxR polypeptides. Decreased repressor activity of a transformant containing the mutant and wild-type *dtxR* alleles was reflected by an increase in expression of β-galactosidase and indicated that the defective DtxR repressor protein interfered with the activity of the wild-type DtxR. Twelve of the 16 *dtxR* missense mutants with deficient repressor activity expressed the dominant negative phenotype in this assay system.

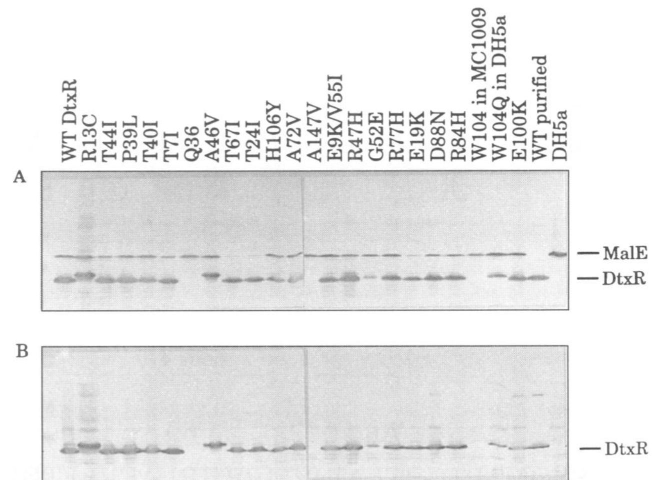

FIG. 1. Western blot analysis of mutant forms of DtxR. Extracts of *E. coli* DH5α containing pSKdtxR plasmids with wild-type or mutant *dtxR* alleles were subjected to SDS–10% PAGE. The plasmid encoding the W104 variant of DtxR was also tested in the suppressor-negative *E. coli* strain MC1009. The separated proteins were transferred to nitrocellulose membranes, and the DtxR proteins on the membrane were allowed to react with polyclonal rabbit antibody against DtxR-MalE fusion protein (A) or a monoclonal mouse anti-DtxR antibody (B). The immobilized antibodies were then allowed to react with an enzyme-labelled secondary antibody, and the blots were developed by using a chromogenic substrate for the enzyme activity (peroxidase).

Detection of mutant DtxR proteins by Western blotting analysis. The intracellular level of each of the mutant repressor proteins was analyzed by Western blotting analysis (Fig. 1). Most of the defective DtxR proteins were immunoreactive, were produced at levels comparable to that of wild-type DtxR, and had the same mobility in SDS-PAGE as the wild-type DtxR did. R13C, A46V, and W104Q had slightly slower electrophoretic mobilities. No immunoreactivity was detected for A147V or the truncated polypeptide Q36 or W104, and G52E gave a weak reaction. These results indicated that most of the mutant repressors were present in *E. coli* in amounts comparable to that of wild-type DtxR. With the possible exception of G52E and A147V, the mutant phenotypes of the DtxR variants with single-amino-acid substitutions were unlikely to result from decreased synthesis or accelerated degradation in comparison with wild-type DtxR.

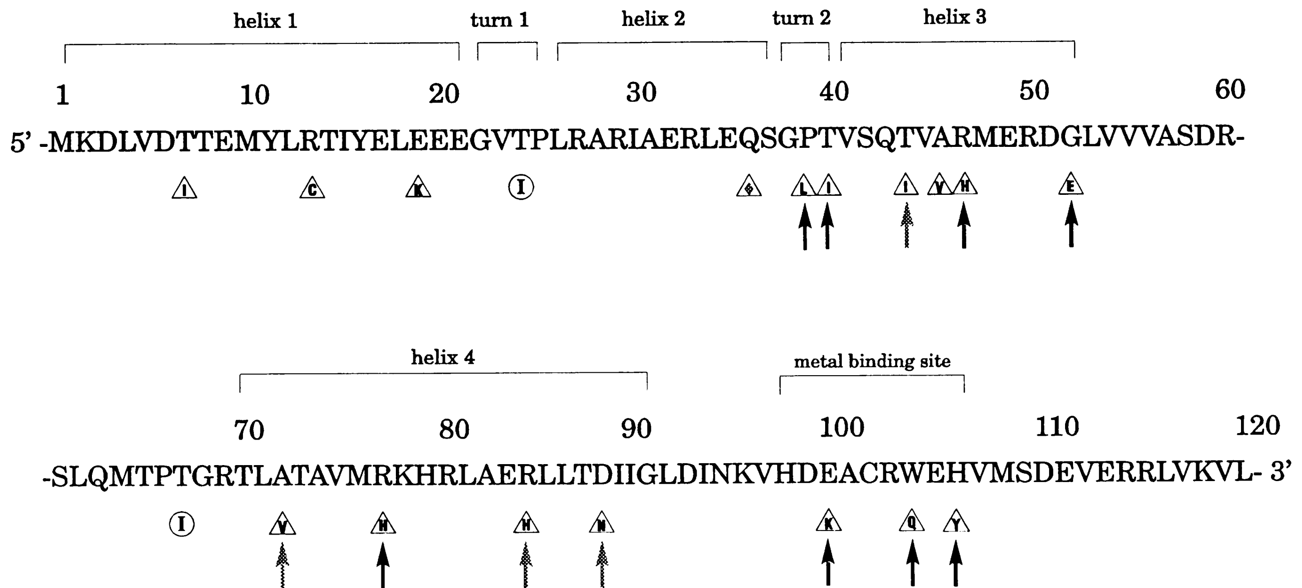

FIG. 2. Amino acid sequence of the amino-terminal half of wild-type DtxR (1 to 120 amino acids) and positions of amino acid substitutions in mutant forms of DtxR. Predicted secondary structures and functional domains are bracketed. Symbols: △, an amino acid substitution that resulted in defective repressor activity; ○, a substitution that had no effect on repressor activity. The letters within the triangles and circles indicate the amino acid residues substituted for the wild-type residues. A diamond within a triangle indicates a chain-terminating mutant. Arrows indicate the mutants with dominant negative phenotypes. Solid and shaded arrows represent strong and weak interference, respectively, with the repressor activity of wild-type DtxR.

Distribution of amino acid substitutions in DtxR. Seventeen of the 18 single-amino-acid substitutions that resulted in decreased repressor activity were located within the amino-terminal half of DtxR (Fig. 2).

Computer analysis revealed a predicted helix-turn-helix-turn-helix secondary structure at the amino terminus (residues 1 to 52). Nine of the repressor-deficient variants have substitutions within these 52 residues. The helix-turn-helix motif between residues 28 and 52 was found to be similar to DNA-binding motifs in several other well-characterized proteins (Fig. 3) (6, 10, 28). Six of the amino acid substitutions in DtxR are clustered in the segment between residues 39 and 52, including five that caused dominant negative phenotypes and four that resulted in quantitatively severe deficits in repressor function (+++ colonial phenotypes and a β-galactosidase activity of >10 U). In contrast, the amino acid substitutions located within the first 25 residues at the amino terminus of DtxR caused only moderate to negligible decreases in repres-

		helix										turn					recognition helix								
DtxR	28-	A	R	I	A	E	R	L	E	Q	S	G	P	T	V	S	.	Q	T	V	A	R	M	E	R
LacR	4-	V	T	L	Y	D	V	A	E	Y	A	G	.	.	V	S	Y	Q	T	V	S	R	V	V	N
λ Cro	14-	F	G	Q	T	K	T	A	K	D	L	G	.	.	V	Y	Q	S	A	I	N	K	A	I	H
434 Cro	16-	M	T	Q	T	E	L	A	T	K	A	G	.	.	V	K	Q	Q	S	I	Q	L	I	E	A
P22 Cro	11	G	T	Q	R	A	V	A	K	A	L	G	.	.	I	S	D	A	A	V	S	Q	W	K	E
λ Repressor	31-	L	S	Q	E	S	V	A	D	K	M	G	.	.	M	G	Q	S	G	V	G	A	L	F	N
434 Repressor	16-	L	N	Q	A	E	L	A	Q	K	V	G	.	.	T	T	Q	Q	S	I	E	Q	L	E	N
P22 Repressor	19-	I	R	Q	A	A	L	G	K	M	V	G	.	.	V	S	N	V	A	I	S	Q	W	E	R
λ cII	24-	L	G	T	E	K	T	A	E	A	V	G	.	.	V	D	K	S	Q	I	S	R	W	K	R
CAP	167-	I	T	R	Q	E	I	G	Q	I	V	G	.	.	C	S	R	E	T	V	G	R	I	L	K
trp Repressor	66-	M	S	Q	R	E	L	K	N	E	L	G	.	.	A	G	I	A	T	I	T	R	G	S	N

FIG. 3. Comparison of residues 28 through 50 of DtxR with the helix-turn-helix motifs of several other DNA-binding proteins. The sequences shown are from references 4, 6, and 10. The most highly conserved residues are boxed. Vertical bars between the DtxR and LacR sequences indicate identical residues. The predicted helix 3 of DtxR (see Fig. 2) appears to correspond with the DNA recognition helixes of the other repressor proteins. Underlined residues in DtxR and the other repressors indicate the positions at which amino acid substitutions abolished or diminished repressor activity.

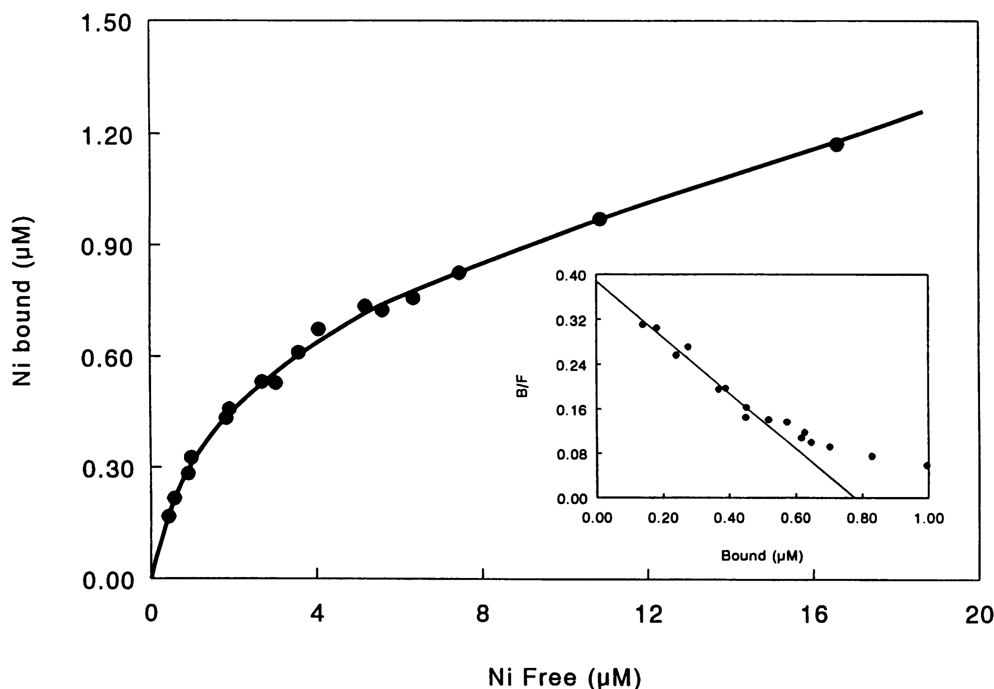

FIG. 4. Binding of $^{63}Ni^{2+}$ by wild-type DtxR. Samples containing DtxR at 100 μg/ml (equivalent to monomer at 3.9 μM) were incubated with $^{63}Ni^{2+}$ at molar ratios of Ni^{2+} to DtxR of from 0.5 to 30. Bound (B) and free (F) $^{63}Ni^{2+}$ were then separated by filtering the samples through Immobilon membranes as described in Materials and Methods. The figure shows the saturation curve for binding of $^{63}Ni^{2+}$ by DtxR, and the insert shows the Scatchard transformation of the binding data. Each point represents the mean of four independent determinations. The standard deviations did not exceed 10% of the mean values.

sor activity. The region between residues 39 and 52 is an excellent candidate for the domain that recognizes and interacts with DtxR-regulated operators.

The His-98–XXX–Cys-102–XXX–His-106 sequence has a predicted helical secondary structure and similarity with metal-binding motifs found in several other proteins (1). Three of the DtxR variants, all with severe deficits in repressor function, have amino acid substitutions within this motif. The region between residues 98 and 106 is, therefore, an excellent candidate for the metal-binding domain involved in activation of DtxR by Fe^{2+} or other divalent metal ions.

Four DtxR variants with moderate deficiencies in repressor activity were located in a sequence between the presumed DNA-binding and metal-binding sites that contains a predicted long helix (residues 70 to 90). The role of this region in the function of DtxR is currently unknown.

Amino acid substitutions associated with the dominant negative phenotype were clustered in all three regions of DtxR described above, between residues 39 and 52, 70 and 90, and 98 and 106.

Analysis of purified mutant DtxR proteins. It was previously demonstrated that wild-type DtxR and the R47H variant of DtxR bind to Ni^{2+}-NTA-agarose resin and can be purified in a single step by chromatography on this resin (32). Each DtxR variant that was detected by Western blotting (Fig. 1) was also tested and shown to be capable of binding to Ni^{2+}-NTA-agarose resin (data not shown). Wild-type DtxR, the variants P39L, T40I, T44I, and A46V with substitutions in the presumed DNA-binding motif, and H106Y with an amino acid substitution in the presumed metal-binding motif were selected for further study and purified by Ni^{2+}-NTA-agarose chromatography as described in published methods (32). A sample of R47H purified previously (32) was also available for analysis.

In the presence of 150 μM Co^{2+}, wild-type DtxR binds in gel retardation assays to DNA fragments that contain the *tox* promoter-operator region (32). Under identical conditions, the purified proteins P39L, T40I, T44I, A46V, R47H, and H106Y did not bind in gel retardation assays to the *tox* promoter-operator region (data not shown). Amino acid substitutions either in the presumed DNA-binding domain or metal-binding domain of DtxR can, therefore, abolish or markedly diminish the sequence-specific DNA-binding activity of DtxR.

Certain divalent cations, i.e., Fe^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, can function as corepressors for DtxR in vitro (32, 34, 38, 39). Binding of divalent cations by the DtxR aporepressor is presumed to cause an allosteric change in the conformation of DtxR, which enables the activated repressor to bind to the *tox* operator. We used $^{63}Ni^{2+}$ as a convenient and suitable isotope for the development of a quantitative assay for the metal-binding activity of DtxR, based on a published method for Hg^{2+}-binding activity of the MerR protein (35, 36). The saturation binding curve for Ni^{2+} binding by wild-type DtxR and a Scatchard transformation of the binding data are shown in Fig. 4. The results were consistent with a single class of high-affinity Ni^{2+}-binding sites plus additional binding sites for Ni^{2+} with lower affinity. The equilibrium dissociation constant (K_d) for the high-affinity site was 2.11×10^{-6} M. Maximum binding at the high-affinity site was estimated to be 1.24 mol of Ni^{2+} per mol of DtxR monomer, consistent with one high-affinity Ni^{2}-binding site per monomer.

The Ni^{2+}-binding activities of purified wild-type DtxR, P39L, T40I, T44I, R47H, and H106Y were compared at

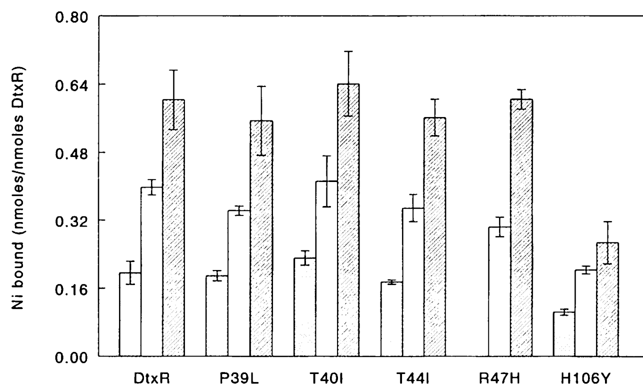

FIG. 5. Binding of $^{63}Ni^{2+}$ by wild-type and mutant forms of DtxR. Samples containing wild-type or mutant DtxR protein at 85 μg/ml (equivalent to monomer at 3.3 μM) were incubated with $^{63}Ni^{2+}$ at 0.78 (▒), 1.96 (□), or 4.19 (▨) μM and then processed by filtration through Immobilon membranes as described in Materials and Methods. Each value represents the mean of four independent experiments, and error bars indicate ±1 standard deviation.

concentrations of Ni^{2+} corresponding approximately to 20, 40, and 60% calculated occupancy of the Ni^{2+}-binding sites of wild-type DtxR (Fig. 5). A46V was not available in sufficient quantity to include in this experiment. At the higher and lower Ni^{2+} concentrations, the Ni^{2+}-binding activities of P39L, T40I, T44I, and R47H, which have substitutions within the presumed DNA-binding region, were not significantly different from that of wild-type DtxR. At the intermediate Ni^{2+} concentration, the P39L and R47H variants showed a slight but statistically significant ($P < 0.05$) decrease in Ni^{2+} binding. In contrast, at each concentration of Ni^{2+} tested, the H106Y variant of DtxR, with an amino acid substitution in the presumed metal-binding domain, exhibited a much greater and statistically significant decrease in Ni^{2+} binding to about 50% of the level observed with wild-type DtxR. These results provide direct evidence that H106Y exhibits a quantitatively greater abnormality in metal-binding activity than P39L, T40I, T44I, or R47H and are consistent with the model that His-106 is part of the metal-binding site of DtxR.

DISCUSSION

DtxR is an iron-dependent repressor of *C. diphtheriae* that differs in DNA-binding specificity from the Fur protein of *E. coli* (2, 32, 34, 38, 39). The structural basis for the biological activity of DtxR has not yet been defined in detail. Toward this end, we introduced random mutations into the 5′, middle, and 3′ segments of the *dtxR* gene by in vitro bisulfite mutagenesis and characterized a representative set of clones with decreased DtxR activity.

Clones with diminished repressor activity represented a greater proportion of the mutagenized pool when bisulfite treatment was targeted to the 5′ segment or middle segment of *dtxR*. Among 102 mutant alleles sequenced, including 34 that had been mutagenized in each third of the *dtxR* gene, we identified 18 with a single missense mutation, 2 with a chain-terminating mutation, and 82 with multiple mutations or duplications of mutations already identified. Strikingly, all but one of the single-amino-acid substitutions that impaired repressor activity were in the amino-terminal half of DtxR (Fig. 2). Boyd et al. (3) reported that the wild-type DtxRs from *C. diphtheriae* 1030 and C7 differ by six amino acid residues, all of which are located in the carboxyl-terminal third of the molecule. Taken together, these findings indicate that the amino-terminal half of DtxR is particularly important for its biological function.

The single-amino-acid substitutions that severely diminished or abolished repressor activity (giving +, ++, or +++ phenotypes [Table 2]) were not randomly distributed in the amino-terminal half of DtxR. Instead, they were clustered in three distinct regions that appear to be important for different functions of DtxR.

Six mutant proteins (P39L, T40I, T44I, A46V, R47H, and G52E) had amino acid substitutions between Pro-39 and Gly-52 in the distal part of a predicted helix-turn-helix motif (Fig. 2). This sequence exhibits homology with the DNA recognition region of the LacR repressor and, to a lesser extent, with several other well-characterized repressors (Fig. 3) (6, 10, 14, 22). We concluded that the region from Pro-39 to Gly-52 in DtxR is likely to be involved in recognition of the *tox* operator (4, 15, 32, 39) and other DtxR-regulated operators (33) of *C. diphtheriae.*

A high-affinity chelating site for a divalent cation can be formed from as few as two properly positioned metal-coordinating ligands in a protein. Histidines or cysteines are important metal-coordinating ligands at neutral pH, and two histidines or cysteines separated by three intervening residues in an α-helix constitute a potential metal-binding motif (1, 25, 26). The His-98–XXX–Cys-102–XXX–His-106 sequence in DtxR fits this pattern, and previous reports implicated Cys-102, the only cysteine residue, in the metal-binding activity of DtxR (32, 40). Three mutant proteins (E100K, W104Q, and H106Y) had amino acid substitutions within this sequence. We concluded that the sequence from His-98 to His-106 is the probable binding site for divalent cations that activate DtxR.

A third cluster of substitutions (including A72V, R77H, R84H, and D88N) was within a predicted α-helix from residues Thr-70 to Gly-90 (Fig. 2). This sequence is located between the presumed DNA recognition region and the presumed metal-binding site, and we speculate that it is involved in conformational changes induced by metal binding that activate or expose the DNA recognition domain of DtxR.

Hydroxyl radical footprinting studies demonstrated that binding of activated DtxR to its cognate operators involves protein-DNA interactions that occur symmetrically with respect to the dyad axis of the operator sequence (32, 33), suggesting that the active form of DtxR may be a dimer or multimer. Dominant negative phenotypes are often observed with mutant forms of polypeptides from oligomeric proteins that lose biological activity but retain the ability to form nonfunctional oligomers. As shown in Fig. 2, most of the mutant forms of DtxR with single-amino-acid substitutions expressed a dominant negative phenotype. Additional studies are needed to provide direct evidence for formation of functional dimers or oligomers by wild-type DtxR and to define the structural requirements for the assembly of such complexes.

We purified several variants of DtxR with single-amino-acid substitutions in the putative DNA recognition or metal-binding domain and tested them directly for DNA-binding and metal-binding activities. All lacked sequence-specific DNA-binding activity in a gel shift assay. This finding demonstrated that their binding activity for the *tox* operator is significantly less than that of wild-type DtxR, but more quantitative studies will be required to demonstrate whether these variant DtxR proteins have any residual DNA-binding activity (32). The H106Y variant was found to exhibit much less Ni^{2+}-binding activity than wild-type DtxR or the P39L, T40I, T44I, and R47H variants (Fig. 5). This finding supports the involvement of

His-106 in the metal-binding site of DtxR. In a previous study (32), we demonstrated that R47H exhibits DNA-binding activity in a DNase I footprinting assay but only at higher concentrations of metal ions than are needed for the DNA-binding activity of wild-type DtxR, and we concluded that R47H was likely to have decreased binding activity for divalent cations. The direct demonstration in the present study that binding of Ni^{2+} by R47H and that by wild-type DtxR are similar (Fig. 5) necessitates a modification of that conclusion and suggests that activation of repressor activity is less tightly coupled to binding of divalent cations for R47H than it is for wild-type DtxR. The substitution of histidine for arginine at position 47 could potentially function by destabilizing the active conformation of DtxR, by creating a new site for the binding of a divalent metal ion that is not linked to activation of DtxR, or by other mechanisms. Additional genetic and crystallographic studies will be needed to define all of the ligands in DtxR that coordinate with the physiological activator Fe^{2+} or other bound divalent metal ions and to explain fully the molecular basis for the activation of DtxR.

In summary, the studies presented here identify several distinct regions in the amino-terminal half of DtxR that are required for repressor activity. On the basis of the phenotypes of our *dtxR* mutations, the properties of several purified DtxR variants, and similarities in amino acid sequence between DtxR and other repressors or divalent metal ion-binding proteins, motifs of DtxR that are presumed to be associated with operator recognition and binding of divalent metal-ion activators have been identified.

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