Transition metal ion activation of DNA binding by the diphtheria tox repressor requires the formation of stable homodimers

(Xin Tao, Hui Yan Zeng, and John R. Murphy*)

ABSTRACT The diphtheria tox repressor (DtxR) is a transition metal ion-dependent regulatory element that controls the expression of diphtheria toxin and several genes involved in the synthesis of siderophores in Corynebacterium diphtheriae. In the presence of transition metal ions apo-DtxR becomes activated and specifically binds to its target DNA sequences. We demonstrate by glutaraldehyde cross-linking that monomeric apo-DtxR is in weak equilibrium with a dimeric form and that upon addition of activating metal ions to the reaction mixture a dimeric complex is stabilized. Addition of the DNA-binding-defective mutant apo-DtxR(Δ1-47) to apo-DtxR in the absence of transition metal ions inhibits conversion of the apo-repressor to its activated DNA-binding form. We also show that the binding of Ni2+ to both apo-DtxR and apo-DtxR(Δ1-47) is cooperative and that upon ion binding there is a conformational change in the environment of the indole ring moiety of Trp-104. For the wild-type repressor the consequences of this conformational change include a shift in equilibrium toward dimer formation and activation of target DNA binding by the repressor. We conclude that the formation of DtxR homodimers is mediated through a protein–protein interaction domain that is also activated on metal ion binding.

The Corynebacterium diphtheriae dtxR gene encodes a Mr 24,316 transition metal ion-activated repressor (1-3). Although dtxR was initially shown to regulate expression of the diphtheria tox structural gene (1, 3), it is now known that the diphtheria tox repressor (DtxR) also controls the expression of C. diphtheriae genes involved in siderophore synthesis (4, 5). In the presence of divalent heavy metal ions, DtxR becomes activated and specifically binds to its target DNA sequences (3). The minimal essential nucleotide sequence required for DtxR binding has been recently determined by both in vitro genetic methods (6) and the cloning and sequence analysis of two DtxR-responsive promoters from C. diphtheriae (5).

Although dtxR has been cloned and DtxR has been produced and purified from recombinant Escherichia coli in high yield (1-3, 7), little is known of the process by which apo-DtxR becomes activated by transition metal ions. In the present communication, we describe the genetic construction and properties of an N-terminal deletion mutant of DtxR, DtxR(Δ1-47), and demonstrate by gel mobility-shift analysis that apo-DtxR(Δ1-47) is an inhibitor of the conversion of apo-DtxR to its activated DNA-binding form. In addition, we have used glutaraldehyde cross-linking and show that monomeric apo-DtxR is in weak equilibrium with a dimeric form. Upon the addition of activating metal ions, there is a shift in the equilibrium, and the dimeric complex is stabilized. Finally, on the basis of these results a working model of the process by which apo-DtxR becomes activated by iron is presented.

MATERIALS AND METHODS

Bacterial Strains, Plasmid, and Medium. The bacterial strains and plasmids used 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). LB broth or LB agar was supplemented with ampicillin (100 μg/ml) and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (40 μg/ml) as described.

Nuclear Acids. Double-stranded plasmid DNA was prepared by using the Magic miniprep system (Promega). Restriction endonucleases, T4 polynucleotide kinase, and Klenow fragment of DNA polymerase (Bethesda Research Laboratories, New England Biolabs) were used according to the manufacturer's specifications. Restriction endonuclease fragments were separated by electrophoresis in 1% agarose or 4% low-melting-temperature agarose gels in TBE (89 mM Tris/89 mM boric acid/1 mM EDTA, pH 8.3).

Expression and Purification of DtxR and DtxR(Δ1-47). Expression of dtxR and dtxR(Δ1-47) was directed from a T7 promoter on pDR-1 (3) and pDR(Δ1-47), respectively. DtxR was purified from crude extracts of recombinant E. coli as described (8). Plasmid pDR(Δ1-47) was transformed into E. coli HMS174(DE3), and expression from the T7 promoter was induced by addition of isopropyl β-D-thiogalactoside (IPTG). Two hours after induction, bacteria were harvested by centrifugation, resuspended in 10 mM Tris-HCl buffer (pH 7.5), and lysed by passage through a French pressure cell (14,000 psi; 1 psi = 6.9 kPa). The lysate was centrifuged at 25,000 × g for 20 min at 4°C, and the pellet was resuspended in 10 mM Tris-HCl/0.5 M NaCl, pH 7.5 buffer. The suspension was mixed for 5 min; the pellet was washed three times and then resuspended in 10 mM Tris-HCl/0.5 M NaCl, pH 7.5/0.5% deoxycholic acid. The resulting inclusion body suspension was then solubilized by sonication. DtxR(Δ1-47) was purified by immunoadfinity chromatography on an anti-DtxR affinity column that was equilibrated with 20 mM phosphate buffer, pH 7.5 (9). The column was washed with 1 M NaCl in the same buffer followed by a wash with 10 mM phosphate buffer, pH 7.5. DtxR(Δ1-47) was eluted with 1% acetic acid solution. One-milliliter fractions were collected and immediately neutralized by the addition of 1 M NaHCO3. All procedures were done at 4°C. Fractons containing DtxR(Δ1-47) were identified by SDS/PAGE and immunoblot.

Gel Electrophoresis and Immunoblot Analysis. SDS/PAGE was done according to the method of Laemmli (10) using 12% polyacrylamide gels in the presence or absence of 0.1 M dithiothreitol. Proteins were stained with Coomassie blue or electrophoretically transferred to poly(vinylidene difluoride) membranes for immunoblot analysis. Immunoblots were

Abbreviations: toxPO, tox promoter/operator; DtxR, diphtheria tox repressor.

*To whom reprint requests should be addressed at: Department of Medicine, Boston University Medical Center Hospital, 88 East Newton Street, Boston, MA 02118.

†Deceased, March 21, 1995.
probed with a 1:3000 dilution of anti-DtxR serum 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-indoly phosphate substrate using the Protoblot alkaline phosphatase system according to the directions of the manufacturer (Promega).

**Gel Mobility-Shift Assay.** The gel electrophoresis mobility-shift assay used in this study was similar to that described (3). DtxR, DtxR(A1-47), and Mn²⁺ were added to the reaction mixture in different order before analysis by gel mobility-shift electrophoresis. Apo-DtxR and apo-DtxR(A1-47) were mixed, Mn²⁺ was added, and after 8-min incubation at room temperature a 3²P-labeled tox promoter/operator (toxPO) probe (3) was added to the reaction mixture. As described, apo-DtxR was activated with Mn²⁺, apo-DtxR(A1-47) was added, and after 8-min incubation the 3²P-labeled toxPO probe was added. Finally, apo-DtxR(A1-47) was activated with Mn²⁺, apo-DtxR was then added, and after 8-min incubation the 3²P-labeled toxPO probe was added. In each instance, 12 µl of the reaction mixture was applied to a polyacrylamide gel, and the binding of activated DtxR to the labeled probe was measured by gel mobility-shift analysis (3).

**Chemical Cross-Linking.** Apo-DtxR was dialyzed against 20 mM Hepes buffer, pH 7.5/10 mM dithiothreitol. The protein was then diluted to 2 µg/ml with reaction buffer containing 20 mM Hepes (pH 7.5), 5 mM MgCl₂, 20 mM NaCl, 20 mM KCl, and 2 mM dithiothreitol. DtxR samples were then incubated at room temperature in the presence or absence of 125 µM Mn²⁺ for 10 min, and glutaraldehyde was added to a final concentration of 0.2%. Cross-linking reactions were allowed to proceed for 1 min and stopped by adding 10 vol of SDS/PAGE loading buffer and warming to 90°C for 3 min before analysis by SDS/PAGE. After electrophoresis the glutaraldehyde cross-linked products were visualized by immunoblot analysis using anti-DtxR antiserum as described (3).

**Fluorescence Spectroscopy.** The intrinsic fluorescence emission of apo-DtxR was measured in a 1-cm quartz cuvette using a model LS-5B luminescence spectrometer (Perkin–Elmer). The excitation wavelength was 280 nm, and the emission wavelength was 330 nm. The final concentration of DtxR was 1.0 µM. Transition metal ion solutions were prepared as 1 M stock solutions by dissolving the salts in 0.1 M HCl. Immediately before use stock solutions were diluted with milli-Q water (Millipore) to a concentration of 0.6 mM. Dithiothreitol was removed from apo-DtxR preparations by either centrifugation using G-50 Sephadex Quick Spin columns (Boehringer Mannheim) or dialysis under nitrogen immediately before use. The intrinsic tryptophan fluorescence was measured for 5 sec at room temperature (25°C). The data obtained were corrected for increased volume upon addition of metal ion to the sample. The largest correction factor used was <1%.

**RESULTS**

**Expression and Purification of DtxR(A1-47).** We have previously described the construction of plasmid pDR-1 in which an Nde I site was introduced at the dtxR translational initiation site (3). The dtxR allele from the iron-insensitive C7hm723 strain of C. diphtheriae carries a point mutation resulting in both the introduction of a unique Nde I restrictionendonuclease site in the dtxR(hm723) allele and the Arg-47 → His substitution (11, 12). We therefore digested the dtxR(hm723) gene with Nde I and BamHI and recloned the truncated gene into the Nde I and BamHI sites of pET11c to form pDR(A1-47), which encodes DtxR(A1-47). Preliminary studies of the x-ray crystal structure of apo-DtxR show that amino acids 27–50 form a helix–turn–helix DNA-binding motif (N. Schiering, X.T., J.R.M., and D. Ringe, unpublished work). Therefore, we anticipated that DtxR(A1-47) would be inactive. In fact, DtxR(A1-47) was unable to regulate the expression of β-galactosidase from the toxPO/lacZ transcriptional fusion in pRSS513−PO/lacZ-dtxR(A1-47) (data not shown).

Because a large portion of the helix–turn–helix motif was deleted in DtxR(A1-47), it was of interest to express, purify, and characterize this repressor mutant. The DtxR(A1-47) gene in pDR(A1-47) is under the control of a T7 promoter, and translation of DtxR(A1-47) is initiated at Met-48. E. coli HMS174(DE3) was transformed with pDR(A1-47), and expression of DtxR(A1-47) was induced by addition of isopropyl β-D-thiogalactoside to the medium. Analysis of the high-speed supernatant fluid and inclusion-body pellet of whole-cell lysates by SDS/PAGE and immunoblot revealed that DtxR(A1-47) has a Mr of 23,000 and partitioned in both the soluble and insoluble fractions. Further, by immunoblot analysis DtxR(A1-47) was partially degraded in the soluble fraction, whereas only the Mr 23,000 form of the mutant repressor was found in the inclusion-body pellet (data not shown). After extensive washing to remove associated proteins, inclusion bodies were resuspended in Tris-HCl buffer/0.5% deoxycholic acid and solubilized by sonication; DtxR(A1-47) was then purified to apparent homogeneity by immunoaffinity chromatography on a Sepharose anti-DtxR matrix (Fig. 1). Apo-DtxR(A1-47) Inhibits DNA Binding by DtxR. We used a 3²P-labeled toxPO probe (3) and gel mobility-shift experiments to study quantitatively the behavior of apo-DtxR(A1-47) in the presence and absence of activating divalent metal ions. Fig. 2B (lane 7) shows that DtxR(A1-47) fails to bind to the labeled probe. However, because the preliminary x-ray crystallographic analysis of apo-DtxR showed that there were two molecules of the repressor associated with each other in the asymmetric unit of the unit cell (8), we anticipated that the addition of apo-DtxR(A1-47) to apo-DtxR might result in the formation of heterodimers that fail to bind to the toxPO probe.

**Table 1. Bacterial strains and plasmids used**

<table>
<thead>
<tr>
<th>Strain and plasmid</th>
<th>Genotype</th>
<th>Ref. or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli HMS174(DE3)</td>
<td>F⁺ recA K₁₂− mK₁₂− nfr (DE3)</td>
<td>Novagen</td>
</tr>
<tr>
<td>Plasmid pET11c</td>
<td>amp²</td>
<td>Novagen</td>
</tr>
<tr>
<td>pDR-1</td>
<td>amp²</td>
<td>Ref. 3</td>
</tr>
<tr>
<td>pDR(A1-47)</td>
<td>amp²</td>
<td>This study</td>
</tr>
<tr>
<td>pRSS513 toxPO/ lacZ-dtxR(A1-47)</td>
<td>amp²</td>
<td>This study</td>
</tr>
</tbody>
</table>
To test this hypothesis, serial dilutions of wild-type apo-DtxR were mixed with 40 ng of DtxR(Δ1-47) and incubated at room temperature for 1 min; then Mn²⁺ was added to a final concentration of 125 μM to activate the DNA-binding activity of the repressor. The 32P-labeled toxPO probe was then added, and the reaction mixture was assayed by gel mobility-shift electrophoresis. As shown in Fig. 2A and B, the addition of apo-DtxR(Δ1-47) to apo-DtxR before the addition of Mn²⁺ ions reduced DtxR DNA-binding activity by 75–85%. Because only a single mobility-shifted band was observed, we conclude that the addition of apo-DtxR(Δ1-47) to the reaction mixture directly inhibits activated DtxR binding to the toxPO probe. These results also suggest that apo-DtxR forms a stable dimer upon metal ion activation.

If the binding of metal ions by DtxR resulted in the stabilization of a dimeric complex, the addition of apo-DtxR(Δ1-47) to the reaction mixture after addition of Mn²⁺ ions should not inhibit conversion of apo-DtxR to its activated form. In fact, this was found to be the case. When either Mn²⁺ was added to apo-DtxR before addition of apo-DtxR(Δ1-47) or apo-DtxR(Δ1-47) was mixed with Mn²⁺ before addition of apo-DtxR to the reaction mixture, we were unable to detect any inhibition in the DNA-binding activity of DtxR (Fig. 3A and B).

Monomeric apo-DtxR Is in Weak Equilibrium with a Homodimer Even in the Absence of Transition Metal Ions. Because the results of the gel mobility-shift experiments with mixtures of apo-DtxR(Δ1-47) and apo-DtxR suggested that Mn²⁺ ions stabilized at least a dimeric form of DtxR, we performed a series of glutaraldehyde cross-linking experiments with and without metal ions. In these experiments, glutaraldehyde was added to a final concentration of 1.0% to apo-DtxR with or without 125 μM Mn²⁺ under reducing conditions. Cross-linking reactions were allowed to proceed for 1 min and then stopped by addition of SDS/PAGE loading buffer. The reaction mixture was then electrophoresed on SDS/polyacrylamide gels and analyzed by immunoblot using polyclonal anti-DtxR antibody. As shown in Fig. 4 (lane 2), in the absence of Mn²⁺, the addition of glutaraldehyde to apo-DtxR results in the formation of low levels of dimeric DtxR with a Mₐ of ~63,000. In contrast, after activation with Mn²⁺, the level of dimeric cross-linked DtxR was significantly increased (Fig. 4, lane 3). Addition of glutaraldehyde to either apo-DtxR or metal ion-activated DtxR does not result in the formation of trimeric, tetrameric, or higher-order aggregates that would be indicative of nonspecific cross-linking.

Allosteric Activation of DtxR by Ni²⁺. DtxR contains a single tryptophan residue at position 104 that is located in the metal ion activation domain of the repressor. We measured the quenching of intrinsic tryptophan fluorescence of apo-DtxR after the addition of various concentrations of Ni²⁺ in an attempt to measure changes in the environment of the indole ring moiety of Trp-104 upon metal ion binding. In Fig. 5, the quenching of intrinsic fluorescence is plotted against the final Ni²⁺ concentration. At Ni²⁺ concentrations of <0.5 μM there was little, if any, quenching of intrinsic fluorescence; however, addition of Ni²⁺ to concentrations of 0.5–1.5 μM resulted in marked quenching that became saturated at 5 μM Ni²⁺. The apparent affinity of Ni²⁺ binding, as estimated from half-saturation of quenching of intrinsic tryptophan fluorescence, is 9 × 10⁻⁷ M. Importantly, the plot of quenching of intrinsic fluorescence is sigmoidal, thereby indicating that metal ion binding by apo-DtxR is cooperative. These results further demonstrate that upon metal ion binding, DtxR is in at least a dimeric form. In a similar fashion, we measured the quench-
ing of intrinsic tryptophan fluorescence of apo-DtxR (Δ1–47) and have found that the apparent Kd of Ni2+ binding to the metal ion activation domain is 1.2 × 10−6 M (data not shown).

It has been reported (13) that each DtxR molecule has a single metal ion-binding site with a Kd of 2.11 × 10−6 M. We have independently confirmed these results by equilibrium dialysis under nitrogen and have found by this method that the apparent Kd for Ni2+ is 9 × 10−7 M (data not shown).

**DISCUSSION**

Recent x-ray crystal structure analysis of apo-DtxR has revealed that amino acid residues 27–50 form a helix–turn–helix motif that is characteristic of DNA-binding proteins (14; N. Schiering, X.T., J.R.M., and D. Ringe, unpublished work). We constructed an N-terminal in-frame deletion mutant, DtxR (Δ1–47), in which a large portion of the helix–turn–helix motif was removed. We have found that DtxR (Δ1–47) is unable to regulate the expression of a lacZ reporter gene that is under the control of the diphtheria toxPO (data not shown) and unable to bind to a 32P-labeled toxPO probe in a gel mobility-shift assay in vitro. Nonetheless, the addition of apo-DtxR (Δ1–47) to wild-type apo-DtxR blocked the DNA-binding activity of DtxR probe provided that apo-DtxR (Δ1–47) was added to apo-DtxR before the addition of activating metal ions to the reaction mixture. Because of the well-known instability of Fe2+ under aerobic conditions, we have used either Mn2+ or Ni2+ ions to activate DtxR as described (9).

Previous experiments had shown that the Arg-47 → His mutation carried by the dtxR (hm723) allele is sufficient to almost completely abolish the regulation of tox and siderophore gene expression in lysogenic toxigenic strains of C. diphtheriae and recombinant E. coli (11, 12). In the case of recombinant E. coli that carries the dtxR (hm723) allele on a multicopy plasmid, the expression of β-galactosidase from a toxPO/lacZ transcriptional fusion has been shown to be only weakly regulated by iron (12). It is likely that the Arg-47 → His mutation, which occurs in the helix–turn–helix DNA-binding motif of DtxR, either alters the conformation of the DNA-binding domain or His-47 is unable to hydrogen bond with a nucleotide base in the recognition sequence, thereby resulting in a decrease in affinity between the mutant repressor and its target sequence(s).

In the present study, we demonstrate that the deletion of amino acids 1–47 of DtxR results in a complete loss of DNA-binding activity. However, because only apo-DtxR (Δ1–47) is able to inhibit the formation of functional DtxR, both the metal ion activation domain and the protein–protein interaction domain appear to be functional in the mutant repressor.

Cross-linking experiments suggest that monomeric apo-DtxR is in weak equilibrium with a dimeric form, even in the absence of activating heavy metal ions. However, the possibility of trace contamination with divalent heavy metal ions, which would result in low levels of dimeric DtxR, cannot be ruled out. To avoid nonspecific protein–protein interaction that may occur at high protein concentrations, these experiments were conducted with low concentrations (8 × 10−8 M) of DtxR and analysis by immunoblot with anti-DtxR antibody. The addition of activating heavy metal ions to the reaction mixture results in a marked increase in the concentration of glutaraldehyde cross-linked dimers of DtxR. These results suggest that upon metal ion binding by the repressor, there is a shift in the equilibrium between monomeric and dimeric forms and a stabilization of the dimeric complex.

Because Trp-104 is positioned in the metal ion activation domain of DtxR (13), we have used the quenching of intrinsic tryptophan fluorescence as a measure of changes in the environment of this residue upon addition of activating metal ions to apo-DtxR. The results of these experiments clearly demonstrate that the binding of Ni2+ by apo-DtxR is cooperative. Further, the apparent Kd of Ni2+ binding is 9 × 10−7 M. In addition, we have demonstrated by quenching of intrinsic tryptophan fluorescence that the apparent Kd of Ni2+ binding by apo-DtxR (Δ1–47) is 1.2 × 10−6 M. These results agree well with those of Wang et al. (15), who recently demonstrated by saturation binding experiments that DtxR has a single class of Ni2+-binding sites with an apparent Kd of 2.11 × 10−6 M. We have independently confirmed that each molecule of DtxR has a single binding site for an activating metal ion by equilibrium dialysis under nitrogen and have also measured an apparent Kd of 9 × 10−7 M by this method (data not shown).

The results reported here add further evidence that DtxR binds to its target DNA sequence through its helix–turn–helix motif. As is well known, the x-ray crystallographic structures of a number of regulatory proteins have been determined at high resolution. Among these regulatory elements, E. coli CAP (16), Trp repressor (17, 18), 434 repressor (19, 20), and λ Cro repressor (21) are examples that recognize their respective DNA targets through a helix–turn–helix motif. A common feature is that these regulatory elements bind their respective targets as a dimer with each monomer bind in the complex recognizing half of their respective palindromic operator sequence. It is tempting to speculate that apo-DtxR (Δ1–47) inhibits the
conversion of apo-DtxR to its activated state by the formation of a DtxR/DtxR(Δ1–47) heterodimer. As yet, we are not able to determine directly whether DtxR binds to its target sequence(s) in a dimeric or higher-order complex.

It is striking that the addition of activating metal ions to apo-DtxR results in a shift in the equilibrium between monomeric and dimeric forms. In the presence of transition metal ions, once the dimeric complex is formed we have not been able to detect subunit exchange with either DtxR or DtxR(Δ1–47). It is clear from the increased quenching of tryptophan fluorescence upon Ni²⁺ addition that the environment of the indolyl ring of Trp-104 changes dramatically during conversion of apo-DtxR to its activated DNA-binding form.

On the basis of the results described here, we propose a working model of the process by which apo-DtxR becomes activated in the presence of iron (Fig. 6). Apo-DtxR is composed of at least the following three functional domains: (i) the helix-turn-helix DNA-binding motif (B), (ii) a transition metal ion activation domain (A), and (iii) a protein–protein interaction domain (PPI). In the absence of activating metal ions, monomeric apo-DtxR is in weak equilibrium with a dimeric form of the repressor. Because of the possibility of trace contamination with divalent heavy metal ions, we cannot rule out the possibility that monomeric apo-DtxR forms a dimer upon metal ion binding. Nonetheless, binding of the first metal ion to one of the monomers induces a conformational change in the activation domain that results in a partial stabilization of the dimeric complex through increased protein–protein interactions. These changes facilitate binding of the second metal ion to the complex and directly lead to a further strengthening of the protein–protein interactions between the two monomers. Importantly, the conformational changes induced by metal ion binding orient the helix-turn-helix motif of each monomer in the complex so that they can bind to their target DNA sequence(s) with high affinity. In this fashion, the conversion of apo-DtxR to its activated form in the presence of either iron or other activating transition metal ions forms the basis of an extremely sensitive genetic switch in C. diphtheriae: a switch that governs the expression of genes involved in both siderophore synthesis and diphtheria toxin production.

We thank Dr. Thomas Laue for helpful suggestions, and Drs. Johanna vanderSpek and Dagmar Ringe for their critical reading of the manuscript. This work was supported by Public Health Service Grant AI-21628 from the National Institute of Allergy and Infectious Diseases.