Functional Studies of the Mycobacterium tuberculosis Iron-dependent Regulator*

Received for publication, July 1, 2004, and in revised form, September 27, 2004 Published, JBC Papers in Press, September 29, 2004, DOI 10.1074/jbc.M407385200


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The iron-dependent regulator (IdeR) protein in Mycobacterium tuberculosis, and its better characterized homologue, the diphtheria toxin repressor (DtxR) from Corynebacterium diphtheriae, are iron-dependent regulatory proteins that control gene expression in response to iron availability in bacteria. IdeR regulates several genes required for iron uptake and storage including those involved in the synthesis of transition metal chelators called siderophores that are linked to the M. tuberculosis virulence. In this study, the metal ion and binding affinities for IdeR binding to an fxbA operator duplex DNA were estimated using fluorescence assays. The Fe²⁺, Co²⁺, and Ni²⁺ affinities of the two metal ion binding sites in IdeR that are involved in the activation of the regulator DNA binding process in vitro were independently estimated. Binding to the two metal ion binding sites is apparently cooperative and the two affinities differ significantly. Occupation of the first metal ion binding site causes dimerization of IdeR, and the metal ion affinity is about 4 μM for Ni²⁺ and much less for Fe²⁺ and Co²⁺. Binding of the second metal ion fully activates IdeR for binding to the fxbA operator. The equilibrium metal ion dissociation constants for IdeR-fxbA operator binding are ~9 μM for Fe²⁺, 13 μM for Ni²⁺, and 23 μM for Co²⁺. Interestingly, the natural IdeR cofactor, Fe²⁺, shows high affinities toward both binding sites. These results provide insight into the possible roles for each metal binding site in IdeR activation.

Iron is an essential nutrient that is used in many critical physiological functions in living organisms. In mammalian hosts, iron is present as Fe³⁺ chelated to transferrin and lactoferrin. The concentration of free Fe³⁺ in human serum is extremely low and studies have reported its concentration to be from 10⁻¹⁰ to 10⁻¹⁵ M (1, 2), which is far below what is needed for normal bacterial growth. Pathogenic bacteria such as Mycobacterium tuberculosis and Corynebacterium diphtheriae have developed an elaborate iron uptake system to overcome the low Fe³⁺ availability. This iron uptake system is tightly regulated because excess iron can cause irreparable oxidative damage to the organism once the Fe³⁺ is reduced to Fe²⁺ within the bacteria (3). The iron-dependent regulator (IdeR)¹ protein from M. tuberculosis and its better characterized homologue, the diphtheria toxin repressor (DtxR) from C. diphtheriae, regulate iron uptake at the transcriptional level. In the presence of reduced Fe²⁺, IdeR binds to the operator region of the genes that code for enzymes crucial for the production of siderophores and other virulence factors. Siderophores are transition metal chelators secreted by the bacteria to scavenge iron. Siderophore production insures the availability of Fe²⁺ needed for bacterial growth, and survival of the pathogen within the host is closely linked to virulence (4–6). IdeR is a key regulator of siderophore production in M. tuberculosis (7, 8). Upon entering the host, M. tuberculosis secretes siderophores and other virulence factors capable of extracting iron from heme and iron transport proteins in the host (9, 10). For example, the fxbA gene encodes a putative formyl-transferase that is crucial for siderophore production in M. smegmatis. The gene is transcribed under low iron conditions when IdeR is deactivated. Under high iron conditions, binding of Fe²⁺ activates IdeR binding to the fxbA operator, which prevents fxbA gene expression. Thus IdeR is critical to the survival and the virulence of M. tuberculosis because it plays a role in the maintenance of iron homeostasis and it regulates expression of virulence factors during infection. Indeed, ideR is an essential gene for M. tuberculosis survival. IdeR cannot be disrupted in M. tuberculosis in the absence of a second functional copy of the gene. Mutation studies have suggested that this essentiality of the ideR gene is related to its regulation of intracellular levels of iron (11).

Crystal structures of IdeR and DtxR have shown that the regulators are homodimeric proteins in their active state. Two IdeR/DtxR homodimers bind to the operator region to form a dimer-dimer DNA binding complex (12–17). A single monomer of IdeR contains three domains and two metal ion binding sites (12, 13). Domain 1 is the DNA binding domain, and it contains a helix-turn-helix motif. Domain 2 is the dimerization domain and it contains metal ion binding sites 1 and 2. Domain 3 folds similarly to the well known Src-SH3 domains (18) even though it has no significant sequence homology to such domains. It is attached to domain 2 via a flexible tether region. Recent NMR studies reported by Wang et al. (19) suggest that this third SH3-like domain in DtxR binds to a proline-rich region of domain 2, residues 125–139, possibly stabilizing the inactive monomer form of IdeR. However, in the crystallographic studies*

* This work was supported by National Institutes of Health Grants CA65656 (to W. G. J. H) and AI14107 (to R. K. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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The abbreviations used are: IdeR, iron-dependent regulator; BSA, bovine serum albumin; DtxR, diphtheria toxin repressor; DLS, dynamic light scattering; SH, Src homology domain .

¹ The abbreviations used are: IdeR, iron-dependent regulator; BSA, bovine serum albumin; DtxR, diphtheria toxin repressor; DLS, dynamic light scattering; SH, Src homology domain .
ies (Pohl et al., Ref. 15) of the fully activated DtxR dimer in complex with DNA, as well as in the activated IdeR structure (Feese et al., Ref. 13), and in the complex of IdeR with DNA (17), the SH3-like domain is found to wedge itself between domains 1 and 2 via multiple protein contacts contributing two of the ligands for the metal ion binding at site 1. It was postulated from these studies that the domain 3 to metal ion site 1 interaction facilitates DNA binding. Similar interactions have also been observed in IdeR (13). The observation of two different binding modes for the third domain probably reflects the high flexibility of the tether region that connects the second and third domain. Even in crystal structures where the third domain is bound to the metal ion site 1, electron density for the tether region is often not or only poorly observed. It is also possible that the third domain interacts with different regions of the protein depending on the iron concentration.

IdeR and DtxR also bind to and are activated by, divalent metal ions other than Fe$^{2+}$ in vitro. Other activating metals include Co$^{2+}$, Ni$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$. Gel mobility shift and DNase protection assays have shown that these metal ions have variable abilities to promote IdeR and DtxR binding to DNA (20–22). HoloDtxR/IdeR binding to different divalent metal ions has also been characterized by crystallographic studies (12, 13, 23, 24). The two metal ion binding sites have distinctly different characteristics. The metal ion at site 1 is pentavalent coordinate with distorted trigonal bipyramidal geometry while the metal ion at site 2 is hexavalent coordinate with octahedral geometry (Fig. 1, A and B). These geometries are common, fairly low energy states for Fe$^{2+}$, the biological IdeR cofactor. In IdeR, the side chains of Met$^{10}$, Cys$^{102}$, Glu$^{105}$, His$^{106}$, and the carbonyl oxygen atom of Cys$^{102}$ coordinate the metal ion in binding site 2 (Fig. 1B). In the early DtxR crystal structures, the repressor was not co-crystallized with operator oligonucleotides, and the electron density for metal ion at binding site 2 was not consistently observed. Electron density for the metal ion in binding site 2 was either absent or far less (~50% less) than the density seen in binding site 1, and Cys$^{102}$ was found to be oxidized to a sulfenyl (RSO$^-$) group, which is probably not its biologically relevant oxidation state. The structures of either the DtxR-DNA operator complex or fully activated IdeR exhibit complete metal ion electron density for both metal ion binding sites, and Cys$^{102}$ is not oxidized. These results suggest that coordination with metal ion and DNA binding might make Cys$^{102}$ less sensitive to oxidation. The coordination of the metal ion at binding site 2 by Sy of Cys$^{102}$ and Sy of Met$^{10}$ also causes the amino acid residues to move by 1.8 and 4.5 Å, respectively, in comparison to the aporepressor. These shifts in position are a possible indication of conformational changes due to metal ion binding and could be significant for the repressor activity.

At metal binding site 1 (Fig. 1A), the side chains of His$^{79}$, Glu$^{83}$, and His$^{98}$ of the dimerization domain bind to the metal ion. Initially, a fourth ligand for the metal ion had been identified as a sulfate anion (18). Although there is a formal possibility that the anion could also be the phosphate, the crystals were obtained in the presence of 2 m sulfate. The sulfate forms extensive hydrogen bonds with multiple contacts to other domains of the protein, i.e., Arg$^{80}$, Asn$^{130}$, and Ser$^{126}$. It also interacts with an ordered water molecule in metal ion binding site 1, which in turn forms a possible communication network between Arg$^{13}$ from domain 1 and Glu$^{105}$ from domain 2. Mutagenesis studies reported by Goranson-Siekieirke et al. (25) have demonstrated that the anion-coordinating residues, Arg$^{80}$, Asn$^{130}$, and Ser$^{126}$, at binding site 1 are essential for the biological activity of the repressor. These observations support the suggestion that the sulfate anion is a mimic of a phosphate anion that would normally be part of site 1. Crystal structures of fully activated, un-oxidized IdeR and DtxR bound to DNA duplexes have been solved. Surprisingly, in several cases, the anion site in these structures is occupied by third domain residues: Glu$^{172}$ and Glu$^{175}$ in IdeR or Glu$^{170}$, Glu$^{172}$, and Glu$^{175}$ are coordinated to the metal ion. Metal ion at site 2 of IdeR is hexavalent coordinate with an octahedral geometry. The side chains of Met$^{11}$, Cys$^{102}$, Glu$^{105}$, His$^{106}$, and an ordered water are coordinated to the metal ion.

Metal ion binding sites 1 and 2 also seem to communicate with each other through Glu$^{105}$ and His$^{79}$ which implies cooperativity between the two sites. This communication might have to do with the observation of different electron density for the metal ion in site 2. Based on the crystal structures of IdeR and DtxR, it has been assumed that site 1 has a higher metal ion affinity than site 2. For example, site 1 was occupied in all
crystal structures, while site 2 was only occupied when IdeR was expressed from the plasmids pMTBKS and pMTBKSC102S, respectively in Escherichia coli DH5a (Invitrogen, Life Technologies, Inc.) as described (12). IdeR was purified from the expression strain by means of collecting cells by centrifugation and lysing in 10 mM sodium phosphate buffer pH 7.0, 50 mM NaCl, the lystate was centrifuged at 12,000 x g for 30 min and the supernatant was filtered through a 0.22-micron filter. The cleared lysate was loaded onto a Ni-nitrilotriacetic acid affinity column. The protein was then eluted by a linear gradient of imidazole from 0 to 100 mM in 10 mM sodium phosphate, pH 7.0. The protein elution was monitored using a UV detector set at 280 nm. The fractions with IdeR that were more than 80% pure were pooled, and applied to an anion exchange column and eluted with a linear gradient of NaCl from 50 mM to 1M in 10 mM Tris-HCl, pH 7.5. The fractions with ideR were confirmed by DNA sequencing to ensure they were not contaminated by the targeted DNA operator. In the presence of excess divalent metal cations, the association constant, and $h$ is the Hill coefficient. All equation parameters were solved individually in 5-MM increments into a buffer containing 2 μM IdeR, 20 mM fbaB duplex, 5 mM MgCl₂, 50 mM NaCl, 20 mM Tris-Cl, 60 μg/ml acetylated BSA, and 10 μg/ml poly(dI-dC) nucleotides at pH 7.0. The antioxidant dithiothreitol (1 mM) was added to the buffer and the stock divalent metal solution when Fe²⁺ was used. All solutions were treated with Chelex 100 and sterile-filtered before use. Steady-state fluorescence anisotropy, r, was calculated as in Equation 1.

$$r = \frac{I_{\perp} - G_{\perp}}{I_{\parallel} + 2G_{\parallel}}$$

where $I_{\perp}$ and $I_{\parallel}$ are the parallel and perpendicular polarized fluorescence intensities measured with the vertically polarized excitation light. G is the monochromatic grating correction factor given by G = $I_{\parallel}/I_{\parallel}$, and $I_{\parallel}$ and $I_{\perp}$ are exact fluorescence intensities polarized with the excitation horizontally polarized. The r value is normalized to the maximum between the Ni²⁺, Co²⁺, and Fe²⁺ experiments.

The DNA binding activity of IdeR was evaluated by measuring changes in the fluorescence anisotropy value of a 5’-end-labeled fbaB operator. In the presence of excess divalent metal cations, the association of IdeR with fbaB operator was described as the binding of two dimers to form a dimer-dimer protein-DNA complex. It was assumed that the initial binding event between the duplex fbaB sequences (FbaB) and an IdeR dimer (IdeR₂) was described by the equilibrium fbaB + IdeR₂ $\rightarrow$ fbaB-IdeR₂, and that the process is cooperative. With limited DNA concentration (i.e., [IdeR₂] < [IdeR₂]₀), the total anisotropy of IdeR/fbaB operator binding reactions were plotted as a function of concentration of the protein dimer, and the data were fit to a cooperative Hill equation with free floating h value (Equation 2) as described by Wilson et al. (32),

$$A = A_{\text{free}} + (A_{\text{bound}} - A_{\text{free}}) \frac{[\text{IdeR}_{2}] / K_h}{1 + [\text{IdeR}_{2}] / K_h}$$

where $A_{\text{free}}$ is the initial anisotropy in the absence of IdeR, $A_{\text{free}}$ is the final anisotropy when fbaB is fully bound, $K_h$ is the equilibrium constant, and h is the Hill coefficient. All equation parameters were solved by nonlinear regression of $A_{\text{total}}$ versus [IdeR₂] data sets using KaleidaGraph version 3.6. A simple non-cooperative 2:1 (IdeR₂/DNA) binding model did not fit the observed data; the fit gave nonsense errors. Intrinsic Fluorescence Emission—IdeR contains two tryptophan residues at positions 94 and 104. The tryptophan residue at position 104 is located at the dimerization interface. It transmits from solvent-exposed to buried upon dimerization, and quenching of the corresponding DtxR tryptophan fluorescence has been used to assay for dimerization (33). The initial concentration of IdeR was 2 μM in a buffer containing 5 mM MgCl₂, 50 mM NaCl, and 20 mM Tris-Cl at pH 7.0. The antioxidant (1 mM) dithiothreitol was added to the buffer and the stock divalent metal solution when Fe²⁺ was used. The intrinsic fluorescence emission of apoIdeR was measured in the SPEX Fluorolog 1681 0.22m Spectrometer without the polarizer after addition of various concentrations of Ni²⁺, Co²⁺, and Fe²⁺ at the excitation wavelength of 280 nm and the emission wavelength of 355 nm. Dynamic Light Scattering (DLS)—Stokes radii and estimated IdeR molecular weights were determined using a Protein Solutions DynaPro...
Molecular Sizing Instrument (Protein Solution Ltd, Bucks, England). The measurements were taken using 1 mg/ml IdeR in a buffer solution containing 5 mM Mg\textsuperscript{2+}, 50 mM NaCl, and 20 mM Tris-HCl at 20 °C. Because DLS is sensitive to as little as 0.01% mass aggregate, the autocorrelation functions were fit to double exponential functions to give two radii. In all cases, the high molecular size species, i.e. aggregates or particulates with molecular masses of > 2 x 10\textsuperscript{4} Da, represented less that 0.05% of the solute mass. Higher order fits did not produce any additional low molecular mass species, and, thus, the solutions were effectively monodisperse.

Data Analysis—Utilizing the anisotropy assay and the tryptophan intrinsic fluorescence of Trp\textsuperscript{104} of the IdeR, the divalent metal ion concentration requirement for the dimerization process and the fxbA operator sequence binding were measured for Ni\textsuperscript{2+}, Co\textsuperscript{2+}, and Fe\textsuperscript{2+}. The cooperative Hill equation was used to fit the normalized curve as shown in Equation 3 (34).

$$A = \frac{[M/K_d]^h}{1+[M/K_d]^h}$$

\(K_d\) is the equilibrium dissociation constant where half of the IdeR is activated and bound to the fxbA operator region, \(M\) is the divalent metal ion concentration, and \(h\) is the Hill coefficient, which is often used as a measurement of cooperativity. All equation parameters were solved by nonlinear regression of \(A\) versus \([M]\) data sets using KaleidaGraph version 3.6. The advantage of using Equations 2 and 3 is that only the properties of the equilibrium curve around the midpoint of the complex formation need to be known. In addition, the Hill coefficient measures the degree of cooperativity for dimer-dimer binding to the fxbA operator and for metal ion binding.

RESULTS

Previously, IdeR binding to operator sites in the presence of divalent metal ion has only been demonstrated by gel mobility shift assays, DNase protection assays, and crystallization studies. Thus, the IdeR operator and metal ion affinities have not been defined. Utilizing fluorescence anisotropy, we were able to determine the binding affinity of holoIdeR for the fxbA operator in the presence of excess metal ion. The increased fluorescence anisotropy of fluorescein-labeled fxbA caused by added IdeR was fit to Equation 2, to give an apparent equilibrium dissociation constant of 0.14 ± 0.02 μM \((n = 3; R^2 = 0.997)\) for the holoIdeR\textsubscript{2} (dimer) binding to fxbA in excess Ni\textsuperscript{2+} (Fig. 2A). The concentration is for holoIdeR\textsubscript{n}, because the model used for the fit is holoIdeR\textsubscript{2} bound by 4 metal ions binding to one single, average operator site. The average Hill coefficient is 3.3. As a control, apoIdeR was also added to labeled fxbA duplex in the absence of divalent metal ion (Fig. 2B). As expected, no change in anisotropy was observed up to an excess of 1 μM IdeR. In addition, the total fluorescence intensity did not change significantly during either titration indicating that the anisotropy signal was not convoluted with changes in fluorescence lifetime or contaminated by scattered excitation light (Fig. 2C).

Previous studies have shown that DtxR is monomeric at low concentrations when devoid of divalent metal ion. At higher protein concentrations or upon addition of metal ion, DtxR dimerization is observed (33). Solutions of IdeR were first examined with DLS to determine the oligomerization state of IdeR in the absence and presence of metal ion. The Stokes radius \(R_s = 2.29 ± 0.13 nm\) obtained from DLS analyses of a dilute IdeR solution (40 μM) free of divalent metal ion gives an estimated mass of 29 ± 1 kDa for a spherical protein. The calculated IdeR monomer has a mass of 25.2 kDa (NP_337286). Addition of 1.0 equivalent of Co\textsuperscript{2+} results in quantitative formation of a single species with \(R_s = 2.94 ± 0.05 nm\), which corresponds to the dimer with an estimated mass of 54 ± 1 kDa. Because the DLS signal-to-noise was poor for IdeR concentrations below 40 μM, fluorescence spectrophotometry was used to estimate the metal ion-dependent dimerization affinity.

![Fig. 2. IdeR binding to the fxbA operator monitored by fluorescence anisotropy. A. IdeR is titrated in 0.025 μM increments into buffer containing 20 μM fluorescein-labeled fxbA duplexes, 5 mM Mg\textsuperscript{2+}, 0.2 mM Ni\textsuperscript{2+}, 50 mM NaCl, 20 mM Tris-HCl, 60 μg/ml acetylated BSA, and 10 μg/ml poly(dI-dC) nucleotides at pH 7.0. The IdeR concentration is corrected for the dilution. B. IdeR-fxbA titration in the absence of divalent metal ion. IdeR was titrated in 0.025 μM increments into buffer containing 20 μM fxbA duplexes, 5 mM Mg\textsuperscript{2+}, 50 mM NaCl, 20 mM Tris-HCl, 60 μg/ml acetylated BSA, and 10 μg/ml poly(dI-dC) nucleotides without divalent metal ion at pH 7.0. C. Total fluorescence intensity during an IdeR-fxbA titration. The change is less than 6%.](image-url)
shown). The $K_d$ for Ni$^{2+}$-dependent IdeR dimerization was found to be $3.8 \pm 0.2 \mu M$ ($n = 3; R^2 = 0.994$) and the stoichiometry is 1:1 (Fig. 3A). This value is comparable to the $K_d$ reported for DtxR dimerization (33). However, the titration curves for Co$^{2+}$ and Fe$^{2+}$ plateau at 1 metal ion/monomer for 2 $\mu M$ IdeR (Fig. 3B). The dimerization $K_d$ values for Co$^{2+}$ and Fe$^{2+}$ are significantly lower than for Ni$^{2+}$. Similar 1:1 stoichiometric titration curves were also observed with 1 and 0.5 $\mu M$ IdeR, and at lower concentrations the intrinsic fluorescence was too low to reliably measure. Thus, the dimerization $K_d$ values for Co$^{2+}$ and Fe$^{2+}$ are less than 0.5 $\mu M$.

Because early crystal structures of the DtxR aporepressor are of homodimers with only metal ion site 1 occupied, it has been inferred that this site is a higher affinity site that regulates dimerization (27–28, 33). However, in most of these structures the Cys$^{102}$ residue at site 2 was oxidized, which compromises this inference. Thus, in order to provide definitive identification of the high affinity metal ion site in IdeR, we mutated the Cys$^{102}$ in site 2 to serine. We hypothesized that this mutation would largely eliminate site 2 metal ion binding while producing little or no effect on site 1 binding. The DLS analyses of 40 $\mu M$ solutions of IdeR-C102S in the absence of metal ion gave a Stokes radius of $R_h = 1.66 \pm 0.11$ nm corresponding to an estimated mass of 19.2 $\pm 2.7$ kDa. As observed for native IdeR, addition of one equivalent of Co$^{2+}$ produced a quantitative shift to a species with $R_h = 2.14 \pm 0.09$ nm corresponding to a dimer with a mass of 33.3 $\pm 1.8$ kDa. Analyses of more dilute solutions necessitated use of fluorescence spectrophotometry. Shown in Fig. 3C are representative dimerization induced fluorescence quenching results for IdeR-C102S titrated with Co$^{2+}$ and Ni$^{2+}$. As observed with native IdeR, 1:1 stoichiometric titration curves that were obtained with Co$^{2+}$ and Ni$^{2+}$ gave a $K_d$ for IdeR dimerization of about 5 $\mu M$.

Fluorescence anisotropy changes measured during divalent metal ion titrations of IdeR in the presence of fluorescein-labeled fxbA duplex were used to measure the Ni$^{2+}$, Co$^{2+}$, and Fe$^{2+}$ apparent $K_d$ values (Fig. 4). The concentration of IdeR used is sufficient to saturate the fxbA operator, and the changes in anisotropy were fit to the cooperative Hill equation (Equation 3) using the model of a single metal ion binding to an average site on the IdeR dimer already bound to DNA. In the absence of IdeR, there were no changes in fluorescence anisotropy throughout the titration (data not shown). The affinity of each divalent metal ion for fxbA binding, Table I, correlates well with the divalent metal ion dependence of DtxR binding to the tox operator region as determined by gel mobility shifts (35). Addition of Ni$^{2+}$ to the IdeR-C102S mutant in the presence of fluorescein-labeled fxbA duplex did not cause corresponding increases in fluorescence anisotropy (Fig. 4B). Addition of Co$^{2+}$ also did not activate the IdeR-C102S mutant (data not shown).

It has been reported that both Zn$^{2+}$ and Cd$^{2+}$ will activate DtxR as measured with gel mobility shift assays (20–22). Although both of these ions would be expected to bind readily to site 1 with the distorted trigonal bipyramidal geometry, binding at site 2 with the octahedral geometry is not favored for these ions. Indeed, in the one DtxR structure with Cd$^{2+}$ in site 2, the geometry is a highly distorted tetrahedral (24). Given the geometric differences between the two sites we hypothesized that Cd$^{2+}$ would enable IdeR dimerization at very low concentrations while exhibiting a much reduced ability to activate DNA binding. As seen with Co$^{2+}$ and Fe$^{2+}$, dimerization of IdeR induced by Cd$^{2+}$ measured by fluorescence quenching gave 1:1 stoichiometric titrations for IdeR solutions of 2–5 $\mu M$ (Fig. 5A). However, the binding of IdeR to fxbA required at least

**Fig. 3.** IdeR dimerization monitored from the intrinsic tryptophan fluorescence emission. A, solution of Ni$^{2+}$ is titrated into a solution containing 2 $\mu M$ IdeR, 5 mM Mg$^{2+}$, 50 mM NaCl, and 20 mM Tris-HCl at pH 7.0. The intrinsic tryptophan fluorescence quenching rate is calculated using $(I_o - I)/I_o$, while $I_o$ is the initial tryptophan intensity, and $I$ is the observed intensity at each point during titration. The metal ion concentration is corrected for the dilution factor at each titration point. B, IdeR dimerization titration for Co$^{2+}$ and Fe$^{2+}$. The curve plateaus at 2 $\mu M$ metal ion concentration, which equals the initial concentration of IdeR. C, Co$^{2+}$ and Ni$^{2+}$ dimerization titrations for the IdeR-C102S mutant at protein concentrations ranging from 2–5 $\mu M$. 

Note: Figures and text are placeholders and need to be replaced by actual content.
a 10-fold higher concentration of Cd$^{2+}$ than did either Ni$^{2+}$ or Fe$^{2+}$ (Fig. 5B).

**DISCUSSION**

IdeR and DtxR bind to their specific DNA operator sequences in a divalent metal ion-dependent manner. Using intrinsic fluorescence of the IdeR tryptophan residue and fluorescence anisotropy of a labeled DNA duplex, we monitored the multiple steps for metal ion activation of IdeR from the initial dimerization step to the DNA binding step. For the IdeR-fxbA operator complex, in the presence of excess divalent metal ion, the apparent $K_D$ of a holoIdeR dimer for a site on the fxbA operator is $0.14 \mu M$. The binding curve of IdeR dimer to fxbA operator is sigmoidal in nature with Hill coefficient of 3.3. The Hill coefficient suggests that the binding of activated holoIdeR$_2$ to the fxbA operator is highly cooperative. The IdeR protein binds to DNA as a homodimer, and each monomer has a DNA binding domain. It is likely that the cooperativity is due to coupling between the two sites within a single homodimer, i.e. when one subunit binds to its recognition site on cognate DNA, the binding of the second subunit to its recognition site is promoted. In addition, because two IdeR dimers bind to one operator region (17), it is also possible that the cooperativity is due in part to coupling between binding of the first and then the second homodimer. Although, there are no contacts between the two homodimers in the crystal structures of IdeR and DtxR, the DNA is significantly distorted from a normal B-form conformation (13, 15–17), which suggests the possibility that binding of the first dimer might induce changes in the DNA conformation that facilitate binding of the second dimer to the promoter.

The cooperative nature of IdeR dimer binding to the fxbA operator sequence is consistent with recent observations in the DtxR system. In contrast, the actual IdeR-fxbA affinity is relatively low in comparison to the DtxR-tox affinity ($K_D = 1 \text{ nM}$) and the estimated IdeR-irg2 binding affinity that is 12–16 times greater than the fxbA affinity (7, 16). From a functional perspective, the significance of these differences is not clear given our incomplete knowledge of the numbers of IdeR-mediated operators and their range of affinities. From a structural perspective, we suggest that the lower IdeR-fxbA affinity is probably due to the presence of a T at the −7 position (Fig. 6).

**TABLE I**

Equilibrium constants for metal ion activation of IdeR-fxbA binding

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>$K_D$</th>
<th>Correlation coefficient ($R^2$)</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni$^{2+}$</td>
<td>13.1 ± 0.1</td>
<td>0.9667</td>
<td>3.6</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>22.6 ± 1.9</td>
<td>0.9969</td>
<td>2.4</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>9.5 ± 1.3</td>
<td>0.9716</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* The average value of at least three titrations ± S.E.
on the complementary strand of an IdeR operator sequence are apparently important for high affinity IdeR-DNA binding (16, 17). The fxbA sequence does not possess the conserved A at the \(-7\) position (Fig. 6).

Earlier studies have suggested that IdeR undergoes either a hinge motion conformational change or perhaps a disordered-to-ordered folding rearrangement upon binding metal ion (13, 36). The role of the SH3-like domain 3 in this transition has been debated. NMR studies suggest that the SH3-like domain could bind to a proline-rich region at residues 125–139 near the metal ion binding site 1 (19). It has been suggested that this binding is functionally similar to the role of its Src-SH3 homologue, which binds to a polyproline region to stabilize the inactive form of the Src protein. However, in one crystal structure of DtxR (15) and in two of IdeR (13, 17), the SH3-like domain provides two side chains as ligands to metal ion binding site 1, and these are two highly conserved residues in domain 3 among different IdeR homologs. In either case, it has been suggested that this binding is functionally similar to the role of its Src-SH3 homologue, which binds to a polyproline region to stabilize the inactive form of the Src protein. However, in one crystal structure of DtxR (15) and in two of IdeR (13, 17), the SH3-like domain provides two side chains as ligands to metal ion binding site 1, and these are two highly conserved residues in domain 3 among different IdeR homologs. In either case, it is possible that this binding is to both sites depending on the occupancy of site 1. The IdeR protein forms a dimer in low concentrations of Ni\(^{2+}\), Co\(^{2+}\), Cd\(^{2+}\), and Fe\(^{2+}\). Of the four, the \(K_d\) in the presence of Ni\(^{2+}\) was the only one that could be determined from changes in intrinsic tryptophan fluorescence. The dimerization \(K_d\) values for the others were estimated to be less than 0.5 \(\mu\)M. The dimerization titration curves plateau at one metal ion per IdeR monomer, which suggests that a single high affinity site is responsible for dimerization. Previous studies of DtxR have theorized that metal ion binding site 1 is the higher affinity site (27, 28). Because Cd\(^{2+}\) would not be expected to bind favorably to the octahedral site 2, the observations that it has a high affinity for dimerization and a very low affinity for DNA binding are consistent with the assignment of site 1 as the high affinity site. More importantly, disruption of the metal ion site 2 in the IdeR-C102S mutant had no effect on dimerization. Based on these results we suggest that the metal ion binding site 1 of IdeR is the high affinity site that is responsible for the dimerization that occurs before DNA binding, which involves binding site 2. The dimerization titration curves were modeled as a single metal ion binding event to one average site on the homodimer. The fit for Ni\(^{2+}\) gave a Hill coefficient of 1.5 suggesting that the two metal ion binding sites 1 from different monomers are coupled. It is possible that the coupling between the high affinity metal ion sites involves the movements of domain 3. The movements of domain 3 from the proline-rich region located within the dimerization domain binding to metal ion site 1 binding could potentially facilitate IdeR dimer formation in a cooperative manner.

It is important to note that once metal ion binding site 1 is occupied, Glu\(^{172}\) and Gln\(^{175}\) from the SH3-like domain will be able to form multiple contacts through hydrogen bonds with residues Glu\(^{20}\), Arg\(^{80}\), Asn\(^{130}\), and Ser\(^{126}\) from domains 1 and 2. All of these are residues that have been shown to be critical for IdeR function even though the metal ion ligand residues at site 1 are not (25). Also, a single amino acid substitution, E175K, at the SH3-like domain near metal binding site 1 has been shown to behave as a super-repressor for DtxR (37). A conformational change that facilitates dimerization while also helping to preform the proper configuration at site 2 needed for metal ion binding has been attributed to site 1 occupation, although the measured Hill coefficient for Ni\(^{2+}\) is not particularly high.
However, the relatively high Hill coefficient for site 2 binding is consistent with coupling of site 2 occupation and the hinge bending of domain 1 that occurs upon DNA binding.

The Ni$^{2+}$, Co$^{2+}$, or Fe$^{2+}$ concentrations required for the specific $fxbA$ operator binding by IdeR are all significantly greater than the concentrations required for IdeR dimerization. This is in agreement with previous studies suggesting that occupation of site 2 is critical for DNA binding (13–15, 24). A recent crystal structure of IdeR has shown that its N terminus undergoes a rearrangement in the presence of Co$^{2+}$, forming a Co$^{2+}$-water-Leu4 carbonyl interaction at site 2 (13). This rearrangement of N-terminal residues is postulated to properly orient and stabilize the DNA binding helices of domain 1 for the DNA operator binding. A critical role for site 2 in DNA binding is also supported by our observation that IdeR C102S does not bind DNA even though it dimerizes with high affinity. Hence, we conclude that metal ion binding site 1 is responsible for the dimerization process of the IdeR homodimer, and the metal ion binding site 2 is mainly responsible for the DNA binding.

All together, these observations support a multiple step activation model from the inactive apoform of IdeR to its fully active metal ion-bound form as a regulator (Fig. 7). In the absence of metal ion, apoIdeR may transit between an “open,” free SH3-like domain, and “closed” conformation, with domain 3 bound to the proline-rich region, according to previous observation of its DtxR homologue (19). Once a transition metal ion is bound to metal binding site 1, an active IdeR dimer is formed and stabilized through the SH3-like domain ligands Glu172 and Gln173. The occupation of metal binding site 1 also contributes to the proper orientation of metal binding site 2.

The two metal binding sites are also dramatically different in metal binding affinities. This observation could explain why single alanine substitutions of DtxR at the high affinity metal ion binding site 1 (i.e. H79A, H83A, H98A) show only minor effect on DtxR DNA binding activity while single alanine substitution mutations at site 2 completely abolish DtxR activity (25, 29). A double alanine substitution at site 1 also abolishes DtxR activity (25). These observations are consistent with our observation that site 1 has far greater metal ion binding affinity than site 2 and, thus, a mutation that diminishes site 1 affinity will not alter the apparent metal ion dependence for DNA binding unless the affinity is decreased to less than that of site 2.

We found that Fe$^{2+}$, the natural cofactor of IdeR, is the optimal metal ion for IdeR function. The Fe$^{2+}$ concentration required for dimerization is very low whereas the concentration for another ion like Ni$^{2+}$ is much higher (Fig. 2, A and B). The Fe$^{2+}$ concentration required for DNA binding is also very low, unlike the concentration required for another ion like Co$^{2+}$ (Fig. 3). Spiering et al. (27) have reported that DtxR, also, is optimized for Fe$^{2+}$. These results suggest that the IdeR metal ion binding sites are selective toward their natural cofactor even though other divalent metal ions can still activate the protein. It will be interesting to see if other proteins in the DtxR family that use different natural cofactors, such as the Mn$^{2+}$ regulator MntR, display similar selectivities for their natural cofactors (38).

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doi: 10.1074/jbc.M407385200 originally published online September 29, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407385200

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