Roles of Active Site Residues and the HUH Motif of the F Plasmid TraI Relaxase*

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Bacterial conjugation, transfer of a single strand of a conjugative plasmid between bacteria, requires sequence-specific single-stranded DNA endonucleases called relaxases or nickases. Relaxases contain an HUH (His-hydrophobe-His) motif, part of a three-His cluster that binds a divalent cation required for the cleavage reaction. Crystal structures of the F plasmid TraI relaxase domain, with and without bound single-stranded DNA, revealed an extensive network of interactions involving HUH and other residues. Here we study the roles of these residues in TraI function. Whereas substitutions for the three His residues alter metal-binding properties of the protein, the same substitution at each position elicits different effects, indicating that the residues contribute asymmetrically to metal binding. Substitutions for a conserved Asp that interacts with one HUH His demonstrate that the Asp modulates metal affinity despite its distance from the metal. The bound metal enhances binding of ssDNA to the protein, consistent with a role for the metal in positioning the scissile phosphate for cleavage. Most substitutions tested caused significantly reduced in vitro cleavage activities and in vivo transfer efficiencies. In summary, the results suggest that the metal-binding His cluster in TraI is a finely tuned structure that achieves a sufficient affinity for metal while avoiding the unfavorable electrostatics that would result from placing an acidic residue near the scissile phosphate of the bound ssDNA.

Bacterial conjugation contributes to diversification of prokaryotic genomes through horizontal transfer of conjugative plasmids between bacteria. During conjugation, a single strand of the plasmid is cleaved in the donor cell and transferred to the recipient bacterium using plasmid-encoded secretion machinery (1). Conjugation may play important roles in evolution of pathogenic Escherichia coli (2) and the formation of biofilms (3). Despite its importance in genetic diversification and the acquisition of new traits, however, the mechanism of bacterial conjugation is still a relatively poorly understood process. Initiation of conjugation requires strand- and site-specific cleavage of the plasmid within the origin of transfer (oriT) at a site termed nic. The relaxase or mobilization (Mob) protein encoded by the plasmid carries out the metal-dependent cleavage reaction. TraI is the relaxase for F plasmid (4, 5). The cleavage reaction is facilitated by accessory proteins that recruit TraI to oriT, perhaps by directly interacting with the relaxase or creating a favorable DNA conformation for the relaxase to bind (6–8). The cleavage reaction results in a long-lived physical link between the plasmid and the relaxase, through a phosphotyrosyl bond bridging the catalytic tyrosine residue and the DNA backbone (9, 10). After transfer of the plasmid to the recipient, possibly as a protein-DNA conjugate (11), the cut plasmid is recircularized, most likely by the relaxase, and second strand synthesis occurs. The process produces a donor and recipient that are both competent for further transfer of the plasmid.

F TraI is a 192-kDa bifunctional protein essential for bacterial conjugation (4). The N-terminal 330 amino acids contain the specific single-stranded DNA (ssDNA)5 recognition and cleavage activities of the protein, whereas the helicase activity is contained in the C-terminal region (12, 13). The relaxase and helicase activities must be linked for efficient plasmid transfer (13). Most relaxases have a His-hydrophobe-His (HUH) motif that, when first identified, was proposed to be a metal-binding site (14). This HUH motif is conserved in plasmid and viral initiation factors (15). F TraI and relaxases from related plasmids, including TraI from R100 and R1, and R388 TrwC, are further defined by an YY-X5–6-YY motif located in the N-terminal portion of the protein. This motif includes the catalytic Tyr and other residues important for relaxase function (12, 16). Recent structures of members of this family of proteins have shown that the HUH motif participates in metal coordination (16–21). The His residues in the HUH motif have also been shown to be necessary for efficient conjugation facilitated by R388 TrwC (22). Beyond this, however, few details about the function of the HUH motif are known.

The physiologically relevant metal for relaxases is unknown, but the proteins are able to utilize a variety of metal ions to catalyze the in vitro ssDNA cleavage reaction (16, 18, 20). These metals include Mg2+, Mn2+, and Zn2+, as well as metals that are less abundant in E. coli, including Ni2+. We previously*

5 The abbreviations used are: ssDNA, single-stranded DNA; ITC, isothermal titration calorimetry.
showed that the TraI relaxase domain (TraI36) is active with Mn$^{2+}$ and Mg$^{2+}$ at micromolar and millimolar concentrations, respectively (18, 20). These concentrations are similar to the total concentrations of these metals in *E. coli* (23). We have attributed electron density in the TraI36 (20) and TraI36: ssDNA (18) structures to a bound Mg$^{2+}$ ion, as have Lujan and colleagues (21) in their recent structure of a similar F TraI relaxase domain. In addition, the R1162 MobA relaxase domain has a bound Mn$^{2+}$ (19), and a 3-His cluster coordinates a Mg$^{2+}$ in one structure of an HNH bacterial colicin (24).

We previously generated a 36-kDa N-terminal TraI fragment (TraI36) that retains the ssDNA binding and cleavage functions of the protein (12). To further explore the nature of the cleavage reaction of TraI and the role of the HUH motif in that reaction, we have characterized TraI36 variants that have substitutions for conserved residues in the active site, including the HUH motif and adjacent residues. We have assessed the ability of the variants to bind ssDNA, Mg$^{2+}$, and Mn$^{2+}$, to cleave single-stranded *oriT* DNA substrates, and to facilitate bacterial conjugation in vivo. In general, TraI tolerated the substitutions poorly, with variants often showing altered affinities for metals and reduced cleavage of ssDNA substrates, and facilitating transfer with reduced efficiencies. The results suggest that the 3-His metal binding cluster and the residues that interact with it represent a refined and subtle solution to the problem of positioning a divalent cation and scissile phosphate in the context of high-affinity, extremely sequence-specific ssDNA recognition.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Protein Purification**—Expression constructs for TraI36 variant proteins were engineered from the pET24a-traI36 plasmid (12) using the QuikChange mutagenesis kit (Stratagene). Mutant plasmids were verified by sequencing. Sequences of mutagenic primers are available on request. Wild-type and variant TraI36 were expressed and purified as described (12).

Vectors for full-length TraI variants were constructed by digesting a variant pET24a-traI36 construct with PvuI and Stul to produce a fragment encoding the N-terminal portion of the protein with the substitution of interest. These fragments were then ligated to a PvuI/Stul fragment created from the pET24a-traI construct that expresses the full-length protein (12). The ligated constructs were checked for size by PCR and the presence of mutations were verified by sequencing.

**DNA Binding Affinity Measurements**—DNA affinities of TraI36 and variant proteins were measured by following increases in fluorescence emission intensity of a 3′‐carboxytetramethyl‐rhodamine‐labeled single‐stranded oligonucleotide (5′‐TTTGGTGGGTGGT^GGTGCCTTT‐3′) upon protein binding. The oligonucleotide was synthesized by Integrated DNA Technologies and purified as described (25). The oligonucleotide was diluted to 4 nM in binding buffer (50 mM Tris, pH 7.5, 0.1 mM EDTA) plus 100 or 200 mM NaCl as indicated. Fluorescence emission intensity was measured in an AVIV automatic titrating fluorometer model ATF-105. Data were fit to a single binding site model using KaleidaGraph (Synergy Software) and the equation, $$(BL_U - BL_L)(2 \times [D_T]) \times ([D_T] + [P_T] + K_D)$$

$$\pm \sqrt{[([D_T] + [P_T] + K_D)^2 - (4 \times [D_T][P_T])]} + BL_L \quad \text{(Eq. 1)}$$

where $BL_U$ is the upper baseline, $BL_L$ is the lower baseline, $D_T$ is the total amount of labeled ssDNA, $P_T$ is the total amount of protein, and $K_D$ is the dissociation constant.

**DNA Cleavage Measurements**—DNA cleavage activity of TraI36 and variants was assessed using a 5′-Cy5-labeled oligonucleotide (5′-Cy5-TTTGGTGGGTGGT^GGTGCCTTT-3′; Integrated DNA Technologies). Oligonucleotide (1 nm) was mixed with 1, 10, 100, or 1000 nm protein in cleavage buffer (100 mM NaCl, 50 mM Tris, pH 7.5, 0.1 mM EDTA) with either 20 mM MgCl$_2$ or 2 mM MnCl$_2$. Samples were incubated for 60 min at 37 °C, reactions were stopped by adding SDS to 0.1%, samples were applied to a 16% urea denaturing polyacrylamide gel (National Diagnostics), and results were visualized using a Typhoon 9410 Imaging system. Bands of cleaved and uncleaved oligonucleotide were quantified using ImageJ (26) and percentage of cleaved product was calculated. One + was awarded for each reaction where >50% of oligonucleotide had been cleaved.

**Isothermal Titration Calorimetry**—Isothermal titration calorimetry to measure affinity for Mn$^{2+}$ was performed using a MicroCal VP-ITC calorimeter. Protein was dialyzed against 25 mM Na-HEPES (pH 7.5), 25 mM NaCl, with 7.5–10 g/liter of Chelex beads in the dialysis buffer. Concentrated protein and metal solutions were degassed immediately before loading into the calorimeter. For the measurement of Mn$^{2+}$ binding, 1 mM MnCl$_2$ was diluted to 1.5 mM in the dialysis buffer and injected into 130–200 μM TraI36. Typically 25–32 7.5-μl injections were performed with stirring at 300 rpm. Experiments were performed at 25 °C. Data analysis was carried out by fitting to a single site model as defined in the Origin software package.

**Quantitative Conjugation**—Quantitative conjugation was performed as described (27), except strain XK1502 F^ΔI was the donor.

**RESULTS AND DISCUSSION**

While long postulated (14), structural data only recently established that the HUH motif coordinates the metal ion in viral initiation proteins and relaxases (17–22). As observed in the F TraI36-ssDNA crystal structure, His$^{159}$, His$^{157}$, and His$^{146}$ comprise the TraI metal-binding site (Fig. 1) (18, 20). The HUH motif includes His$^{157}$, Thr$^{158}$, and His$^{159}$. When TraI36 binds ssDNA, the 5′-bridging and a non-bridging oxygen of the scissile phosphate provide additional ligands. Thus, the bound metal orients the ssDNA substrate and polarizes the scissile phosphate for efficient attack by the nucleophile.

Relaxases also have a conserved acidic residue near the metal-binding site. In *TraI36, Asp$^{95}$ forms hydrogen bonds with both His$^{159}$ and Tyr$^{17}$. To examine the roles of the 3 His and the residues with which they interact, variants with single amino acid substitutions were engineered and characterized. Affinities of wild-type and variant TraI36 proteins for Mn$^{2+}$ were measured by isothermal titration calorimetry (ITC) (Table 1). Affinities of the proteins for an ssDNA *oriT* oligonucleotide...
Asp and Asn for His157 as these residues are often seen coordinating Mg$^{2+}$ in endonuclease structures (28). The H157N variant shows no detectable Mn$^{2+}$ binding, but has near wild-type ssDNA affinity. In contrast, H157D has an affinity for Mn$^{2+}$ that is too tight to measure via ITC, whereas the affinity for ssDNA is reduced by ~100-fold. Similar to the H157A variant, substituting Ala for His$^{157}$ reduces Mn$^{2+}$ binding to undetectable levels (Table 1) and reduces affinity for ssDNA 13-fold (Table 2).

Neither H146A nor H146N detectably bind Mn$^{2+}$, whereas the H146D variant shows a ~30-fold reduced affinity for Mn$^{2+}$ relative to wild type. Affinities of H46D and H146N for ssDNA, however, are near wild type. Variant H146A shows improved binding for ssDNA, with a ~20-fold higher affinity than wild type.

We next examined the functional effects of the substitutions for the 3 His residues, assessing the ability of the proteins to cleave a 22-base 5'-Cy5-labeled F oriT oligonucleotide (Table 3). We tested cleavage activities at 1, 10, 100, and 1000 nM protein in the presence of either MnCl$_2$ (2 mM) or MgCl$_2$ (20 mM). By measuring activity with both metals at these concentrations, we can better assess the activity of the protein than if using a single metal. The affinity of TraI36 for Mn$^{2+}$ is over 4 orders of magnitude greater than its affinity for Mg$^{2+}$ (0.5 μM versus...
The higher affinity of Mn$^{2+}$ is observed for the active site variants. The His146 variants have significantly reduced affinity for MnCl₂, but still show activity in the presence of MnCl₂. H157D has dramatically reduced activity with MgCl₂, even though it has a higher affinity for ssDNA. The reduced H157D activity may reflect its reduced affinity for ssDNA, or may reflect effects from a partial neutralization of the metal ion by the charged Asp residue. While this second possibility is intriguing, if this were the case, we might expect H146D to have less than its wild-type cleavage activity (see below).

The H146A variant cleaved DNA slightly better than wild type with MnCl₂, and slightly worse with MgCl₂. Variants H146D and H146N displayed wild-type cleavage abilities. The high cleavage activity of the His¹⁴⁶ variants with Mg$^{2+}$ compared with the reduced activities of H159A and the His¹⁵⁹ variants, and the different Mn$^{2+}$ affinities of H146D and H157D indicate that the His contribute asymmetrically to metal binding and function, with His¹⁴⁶ being less important than the others to function.

**DNA Affinity in the Presence of Metal**—Some variants showing no metal binding by ITC nevertheless retain in vitro cleavage function, suggesting that the proteins possess significant, if reduced, metal affinity. The interactions between two ssDNA phosphate oxygens and the bound metal in the TraI36:ssDNA crystal structure (18) suggest that the metal can influence the affinity of TraI36 for ssDNA. We could not measure metal binding in the presence of ssDNA directly, so we compared ssDNA affinity in the presence and absence of 1 mM MnCl₂. The higher affinity of Mn$^{2+}$ than Mg$^{2+}$ for TraI36 allowed use of a lower concentration, reducing the detrimental effect of ionic strength on the affinity of TraI36 for ssDNA (29). In initial measurements we used the TraI36 Y16F variant, which has wild-type affinity but drastically reduced ssDNA cleavage activity (Tables 1–3) (25), to minimize the potential effects of cleavage of the oligonucleotide on the assay. Measurements for the Y16F variant were also performed in 200 mM NaCl to better observe any increase in affinity. At 100 mM NaCl, the conditions we normally use, TraI36 affinity for ssDNA is near the upper limit of reliable measurement. Mn$^{2+}$ affinity for TraI36 is unchanged in 25 or 400 mM NaCl (data not shown). With no metal, variant Y16F had a $K_d$ of 11 nM (Table 2). With 1 mM MnCl₂ present, the $K_d$ improved to 0.7 nM (Fig. 3). We previously reported no apparent effect of bound Mg$^{2+}$ on affinity of Y16F for ssDNA when measured in 100 mM NaCl with 5 mM MgCl₂ instead of the 200 mM NaCl and 1 mM MnCl₂ used here (25). The conditions used previously apparently obscured the enhanced affinity due to the bound metal.

We then examined H157A and H159A for an influence of metal on DNA affinity in 100 mM NaCl (Table 2). With no metal present, H157A had a $K_d$ of 4 nM, but had a $K_d$ of 0.8 nM in the presence of 1 mM MnCl₂ (Table 2). Similarly, H159A had a $K_d$ of 8 nM without Mn$^{2+}$ and a $K_d$ of 0.5 nM with Mn$^{2+}$. Thus, we observe an influence of Mn$^{2+}$ on ssDNA binding by these variants despite the variant proteins showing no apparent affinity for Mn$^{2+}$ by ITC.

We also measured the affinity of the H157A/H159A variant for ssDNA in the presence and absence of MnCl₂. The variant had a 78 nM $K_d$ in the absence of metal, but reduced affinity ($K_d = 480$ nM) in the presence of 1 mM MnCl₂. The affinity of H157A/H159A for Mn$^{2+}$ apparently has dropped too low to allow metal binding to be rescued by the presence of bound ssDNA, at least at the Mn$^{2+}$ concentration used. The affinity of H157A/H159A for ssDNA is reduced in the presence of metal, rather than being unaffected, likely due to interactions between Mn$^{2+}$ and ssDNA phosphates that must be disrupted to permit binding. The effect of Mn$^{2+}$ on ssDNA affinity presumably is compensated for in Y16F, H157A, and H159A by the favorable interactions between the metal bound to the HUH site and the oxygens of the scissile phosphate.
TABLE 4
Transfer efficiencies of TraI active site variants

| Protein variant | Transfer efficiency | -Fold reduction from wt |
|-----------------|---------------------|------------------------|
| wt TraI         | 0.97                |                        |
| Y16F            | 0.0105              | 100                    |
| Y17F            | 0.23                | 4                      |
| D81A            | 0.015               | 70                     |
| D81N            | 0.012               | 70                     |
| H146A           | 0.025               | 40                     |
| H146D           | 0.049               | 20                     |
| H157A           | 0.00011             | 10,000                 |
| H157D           | 0.0004              | 2,500                  |

Transfer Efficiency Enabled by His Variants—To assess the ability of TraI variants to promote F plasmid transfer, variants were engineered in full-length TraI and used in transfer assays to complement an F’lac plasmid that has traI replaced with Tn10 tetRA (Table 4). Complementation with TraI variant Y16F reduces transfer efficiency a relatively modest 100-fold, relative to complementation with wild-type TraI. Y17F reduces transfer only 4-fold.

The transfer results from the variants confirm the importance of His146 and His157 residues to function, and emphasize their unequal contributions. The H146A and H146D variants are down 40- and 20-fold in transfer efficiency, respectively, despite demonstrating wild-type or better cleavage abilities in vitro. H157A causes the greatest reduction, with transfer efficiency down 10,000-fold from wild-type. The H157D mutant, despite its enhanced metal binding via ITC, is also deficient in transfer, with efficiency down 2500-fold from wild type.

The 10,000-fold reduced transfer efficiency of H157A, relative to wild type, is comparable with the reduction resulting from the equivalent H161A substitution in TrwC (22). Whereas H157A retains in vitro metal binding and ssDNA cleavage activities, the poor transfer efficiency facilitated by H157A suggests that its affinity for metal has fallen too far, and the in vivo metal concentration is too low, for the protein to bind metal and cleave the plasmid.

Results from the His157 variants may suggest that Mg$^{2+}$ is the metal preferred by TraI in vivo. H157A and H157D poorly complement a traI deletion in transfer assays. Both variants show wild-type in vitro ssDNA cleavage activity with Mn$^{2+}$ but greatly reduced activity with Mg$^{2+}$. The apparent correlation between Mg$^{2+}$ cleavage results and transfer efficiency may indicate that Mg$^{2+}$ is the preferred metal in vivo. These observations, however, might also reflect the significantly greater ratio of metal concentration to K_d for Mn$^{2+}$ versus Mg$^{2+}$ in our in vitro assay.

Whereas TraI better tolerates substitutions for His146 than for His157 or His159, the decreased transfer efficiency for H146A and H146D indicate His146 has a functional role. Substituting Ala at the equivalent TrwC position reduces transfer ~200-fold (22). His146 is conserved among bacterial relaxases, but is replaced with an acidic residue in the viral initiation factors (14). Here, too, the residue is important. A Glu to Cys substitution at the equivalent position in AAV2 Rep dramatically reduces in vitro cleavage (30). The reduced transfer efficiency seen with His146 variants can be attributed to reduced metal binding. The conservation of His at this position in relaxases, whereas Glu and Asp are found in viral initiation proteins, however, remains unexplained.

Y16F, a variant that lacks the catalytic Tyr of TraI, has dramatically reduced in vitro cleavage (Table 3; (12)). Y16F, however, facilitates plasmid transfer with an efficiency reduced only 100-fold from wild-type TraI and 100-fold higher than that facilitated by H157A. Similar results were obtained with comparable TrwC variants (22, 31). Results from extensive mutagenesis of TrwC indicates that there is redundancy in TrwC cleavage activity (31), and our Y16F result suggests the same is true for F TraI. This result is difficult to correlate with the TraI36 structure, which shows Tyr16 perfectly positioned relative to the scissile phosphate for cleavage. There is, however, considerable flexibility in the TraI36 region that includes Tyr16 as judged by the often high B factors in the model and weak electron density in the maps (18, 20), which may allow another amino acid in the region to provide the catalytic nucleophile when Tyr16 is absent.

Asp$^{81}$ Modulates Metal Affinity—In TraI36, Asp$^{81}$ forms hydrogen bonds with both His$^{159}$ and Tyr$^{17}$. Variant D81A binds Mn$^{2+}$ with 150-fold lower affinity than wild type (Table 1), although its affinity for ssDNA remains wild type (18, 20). In contrast, D81N binds Mn$^{2+}$ with 140-fold lower, and ssDNA with 120-fold lower affinity relative to wild type (Table 2).

Substitutions for Asp$^{81}$ generally reduce the cleavage activity of the protein. The D81A variant exhibits minimal activity with MgCl$_2$ but wild-type activity with MnCl$_2$. The D81N variant has a significantly diminished activity with MnCl$_2$, and almost no activity with MgCl$_2$. The lower activity of the D81N variant relative to the D81A variant may result from the lower ssDNA affinity of D81N. Why these two variants have similar affinities for metals but different affinities for ssDNA, however, is unclear.

D81A and D81N variants facilitate plasmid transfer with a 70-fold decrease in efficiency. Substitutions for the equivalent TrwC Asp$^{85}$ residue reduce efficiency ~10,000-fold (22). The E83C variant of AAV2 Rep showed no cleavage of ssDNA substrate with Mg$^{2+}$ and a ~50% reduction in cleavage with Mn$^{2+}$ (30). These substantial functional effects suggest that the acidic residue does more than orient a His to coordinate the metal, and probably modulates the charge of the His on the metal, allowing greater polarization of the scissile phosphate. TraI Asp$^{81}$ may also be required to ensure that the His$^{159}$ Nδ1 is protonated, leaving an unprotonated Ne2 to interact with the metal. If this were the role of Asp$^{81}$, however, we might expect the D81N substitution to have a smaller effect on metal binding and function. The Asn side chain amide is capable of serving as a hydrogen bond acceptor from a protonated His$^{159}$ Nδ1 and a hydrogen bond donor to the Tyr$^{17}$ hydroxyl. We see no obvious structural reason why Asn$^{81}$ would not adopt the conformation that would allow these interactions.

In contrast to results from other proteins, the R1162 MobA variant E74A (MobA Glu$^{74}$ is equivalent to TraI Asp$^{74}$) shows only a minor reduction in transfer (19). In the structures of TraI (Protein Data Bank code 2AOI) (18) and TrwC (PDB code 2CDM) (16) relaxase domains with bound ssDNA, the Asp carboxylate forms a bifurcated hydrogen bond with the Nδ1 of the second HUH His. For TraI36, the shortest NH-O distance is 2.7 Å, whereas for the TrwC relaxase it is 3.0 Å. For MobA (PDB code 2NS6) (19), the shortest NH-O distance between a Glu$^{74}$ and...
carboxyl oxygen and the His^{122} N\delta1 is 4.1 Å. Instead, MobA Glu^{74} forms a hydrogen bond with Lys^{22}. No comparable interaction is present in either TrwC or TraI. The distance of MobA Glu^{74} from His^{122} may limit the effect of Glu^{74} on MobA metal affinity. We speculate that the E74A MobA variant has wild-type metal affinity, explaining the high transfer efficiency E74A facilitates.

In F TraL36, the Asp^{81} carboxylate also forms a hydrogen bond with the hydroxyl of Tyr^{17}, the second tyrosine of the YY-XXX-YY motif that defines this family of bacterial relaxases (15). The role of this Tyr in the cleavage mechanism is unclear. Work described previously (12) and here shows that Y17F has dramatically reduced in vitro activity with both MnCl_{2} and MgCl_{2}, even though the K_{D} of Y17F for Mn^{2+} is near wild type at 0.24 µM (Table 1). Y17F in vivo activity is apparently down, but transfer efficiency is reduced only 4-fold relative to wild type. The Tyr^{17}/Asp^{81} interaction suggests that Tyr^{17} may participate in a charge network that polarizes the scissile phosphate. We cannot, however, dismiss the possibility that a structural rearrangement not suggested by the available structures allows Tyr^{17} to directly participate in cleavage.

His Usage in Relaxase Metal Binding—His is a more neutral metal ligand than Asp or Glu (32). Thus coordination by His leaves a greater positive charge on the metal, allowing greater polarization of the scissile phosphate and making the phosphorus more susceptible to nucleophilic attack by the catalytic Tyr. The reduced transfer efficiencies of the H157D and H146D variants are consistent with reduced relaxase activity due to greater neutralization of the metal, although there are alternative explanations for the reduced activity of the variants. H157D has reduced affinity for ssDNA and H146D has reduced affinity for metal, relative to wild type.

Recently Lujan and colleagues (21) reached a similar conclusion about the functional requirement for maintaining the full charge on the bound metal. Their conclusion was based on results from TraI variants H159Q, which retained wild-type in vitro function, and H159E, which was inactive in vitro. What is not known, however, is how the proximity of acidic side chains at positions 81 and 159 in the latter variant might affect local structure.

Although the HUH motif and 3-His metal coordination are widely conserved in relaxases, the 3 His residues are replaced by the HEN motif in the ColE1 MbeA and some relaxases closely related to MbeA (33). Mutagenesis experiments confirmed that the HEN motif is important for function. Replacement of the HEN motif by 3 His residues yielded a protein that facilitated transfer at a low but detectable level. The reduced function of the variant, although, clearly indicates that the MbeA protein is optimized for use of the HEN motif. The differences between MbeA and the HUH relaxases required to allow efficient use of the HEN motif have not yet been identified.

Conclusions—Although the 3 His residues that coordinate the metal do not contribute equally to TraI relaxase function, the transfer results of the variants indicate that all 3 His residues are important to TraI function. Asp^{81}, which forms a hydrogen bond to His^{159}, is also essential to function. The network of residues seen in relaxase structures that work to bind metals represent a clever solution to problems inherent in the relaxase active site. Within a relatively small volume, the enzyme must bring together the scissile phosphate, the Tyr hydroxyl, the metal, and the residues that bind the metal. The combination of the 3 His and the interacting Asp allows a sufficiently high affinity for metal without compromising affinity for ssDNA by placing an acidic amino acid near a ssDNA phosphate. Furthermore, the use of 3 His residues allows for binding of the metal while minimizing the neutralization of the charge of the metal, which may be necessary for optimal cleavage activity.

TraI is able to successfully employ the 3-His motif to bind metal because the protein uses a single metal ion in its cleavage mechanism. Enzymes that use a two-metal reaction mechanism frequently employ acidic residues to coordinate metal ions, probably because partial neutralization is required to permit the close spacing of the ions. We do not yet understand, however, why other HUH proteins, such as ColE1 MbeA and AAV-5 Rep, presumably use a single metal in their cleavage reaction but have an acidic residue participating in metal coordination.

Beyond the initial cleavage of the plasmid, TraI further needs to unwind, assist in transfer of and ligate the conjugative plasmid. More genetic, biochemical, and structural studies will be necessary to elucidate these activities.

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