Homing in on the Role of Transition Metals in the HNH Motif of Colicin Endonucleases*

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Ansgar J. Pommer‡, Ulrike C. Kühlmann‡, Alan Cooper§, Andrew M. Hemmings‡¶, Geoffrey R. Moore¶, Richard James‡, and Colin Kleanthous‡¶

From the ‡School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, the §Department of Chemistry, University of Glasgow, Glasgow G12 8QQ, and the ¶School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom

The cytotoxic domain of the bacteriocin colicin E9 (the E9 DNase) is a nonspecific endonuclease that must traverse two membranes to reach its cellular target, bacterial DNA. Recent structural studies revealed that the active site of colicin DNases encompasses the HNH motif found in homing endonucleases, and bound within this motif a single transition metal ion (either Zn$^{2+}$ or Ni$^{2+}$) of which is unknown. In the present work we find that neither Zn$^{2+}$ nor Ni$^{2+}$ is required for DNase activity, which instead requires Mg$^{2+}$ ions, but binding transition metals to the E9 DNase causes subtle changes to both secondary and tertiary structure. Spectroscopic, proteolytic, and calorimetric data show that, accompanying the binding of 1 eq of Zn$^{2+}$, Ni$^{2+}$, or Co$^{2+}$, the thermodynamic stability of the domain increased substantially, and that the equilibrium dissociation constant for Zn$^{2+}$ was less than or equal to nanomolar, while that for Co$^{2+}$ and Ni$^{2+}$ was micromolar. Our data demonstrate that the transition metal is not essential for colicin DNase activity but rather serves a structural role. We speculate that the HNH motif has been adapted for use by endonuclease colicins because of its involvement in DNA recognition and because removal of the bound metal ion destabilizes the DNase domain, a likely prerequisite for its translocation across bacterial membranes.

Colicins are a formidable weapon in the armory of a bacterium as it competes for nutrients with other bacteria, exemplified by the first-order kinetics of colicin-mediated cell death, which imply that a single toxin molecule is sufficient to kill a cell (1). Colicins have been intensively studied since the late 1950s (2), with much of this work revolving around how these folded proteins are able to traverse the membrane barriers of a bacterium (reviewed in Ref. 3). This process is distinct from that of mitochondrial import in eukaryotic cells since colicins do not possess signal sequences but rather are dependent on the activities of three domains: a central receptor recognition domain involved in binding to outer membrane nutrient receptors; an N-terminal translocation domain, which, through its interactions with several periplasmic proteins, causes the outer membrane to be breached allowing the C-terminal cytotoxic domain to enter the periplasm. This latter activity is most often a voltage-gated ionophore (colicins A, B, Ia, E1, and N, for example), which associates with the inner membrane as a molten globule, and then depolarizes the cell (4–6). Cell death ensues through the efflux of potassium and other ions out of the cell.

Of the many different types of colicin that have been identified, perhaps the most unusual are the nuclease family of E. coli colicins (reviewed in Ref. 7). Nuclease colicins require the outer membrane vitamin B$_{12}$ receptor BtuB as well as the porin OmpF and the Tol proteins (located in both the periplasm and inner membrane) for import. This complex machinery is needed to translocate the cytotoxic domain across both membranes of Escherichia coli in an energy-independent process in order that they reach their cytolic targets, ribosomal RNA for colicin E3 (8, 9), tRNA anticodons for colicin E5 (10), and chromosomal DNA for colicins E2 and E7–E9 (11–14). Little is known of the mechanisms of membrane penetration by this family of nuclease colicins.

Colicin-producing bacteria protect themselves by co-synthesizing an immunity protein that neutralizes the activity of the toxin (15). For pore-forming colicins, immunity is needed to prevent cell death from toxin molecules penetrating a producing organism, since the ionophore is only active from the periplasmic side of the inner membrane. Thus, the immunity protein takes the form of a membrane protein that prevents the insertion of an incoming colicin into the inner membrane (16). By contrast, nuclease colicins can kill cells the moment their synthesis is complete and so have evolved a highly efficient immunity system to combat suicide (17, 18). The 9.5-kDa immunity protein Im9, for example, folds into its distorted four-helical bundle structure with a rate constant of 2200 s$^{-1}$; thus, folded immunity protein appears in <1 ms once SOS induction of the colicin operon is activated (17). The folded Im9 then associates with the colicin E9 DNase at the rate of diffusion to form an inactive complex with an equilibrium dissociation constant of 9.3 × 10$^{-17}$ M, one of the highest affinity protein–protein interactions yet reported (19). As well as exhibiting high affinity binding, colicin-immunity protein complexes are also very specific since non-cognate immunity proteins such as Im2 and Im8 bind (and inactivate) the DNase of colicin E9 6–8 orders of magnitude less tightly than the cognate Im9 (20).

Following colicin immunity protein association, the heterodimeric complex is released into the extracellular medium through the action of the bacteriocin release protein (7). The

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‡ To whom correspondence should be addressed. Tel.: 44-1603-593221; Fax: 44-1603-592250; E-mail: c.kleanthous@uea.ac.uk.
complex is able to bind to outer membrane receptors and penetrate and kill an unprotected cell, but only after the bound immunity protein is released during the translocation process (21).

Recent crystal structures of the 15-kDa DNase domains of colicins E9 and E7 in complex with their cognate immunity proteins Im9 and Im7, respectively, revealed that the immunity protein does not bind within the active site cleft of the DNase but adjacent to it (17, 22). From these studies it was argued that inactivation of the colicin occurs through steric and electrostatic occlusion of substrate DNA, consistent with solution data demonstrating the inability of the complex to bind dsDNA, even though the active site cleft of the DNase is wide enough to accommodate substrate (17). The two structures also revealed that colicin DNases are metalloproteins, containing single, tetrahedrally coordinated transition metals within their active sites, located more than 10 Å away from the protein-protein interface. Zinc was found in the E7 structure, while nickel was observed in the E9 structure (Refs. 22 and 17, respectively). In both studies, the role of the metal ion was unknown but suggested to be either structural and/or catalytic. Of particular interest in the E9 DNase work was the first structural description of the so-called HNH motif, a sequence motif found in homing endonucleases that had previously been identified in colicins (23, 24). These enzymes are encoded by self-splicing introns or inteins that promote the homing of genetic elements containing the genes for the nucleases into intronless or inteinless alleles in prokaryotes and eukaryotes (25). The motif covers 32 amino acids at the C terminus of the E9 DNase domain and encompasses the bound metal ion and three histidine ligands (Fig. 1). The occurrence of a metal ion in the active site of the E9 DNase was unexpected, leaving its role open to speculation. Indeed, there have been no reports in the literature on the potential role of transition metals in the HNH family of homing endonucleases.

Due to the differing ways in which the DNase domains of colicin E7 and E9 were produced in the two recent structural studies, different metal centers were found in each protein bound by the same amino acid residues (Fig. 1). The E7 DNase–Im7 complex was expressed as a histidine-tagged complex (in which the Im7 protein carried the tag) and the structure solved by molecular replacement using the previously solved Im7 crystal structure (22, 26). Atomic absorption experiments indicated that zinc was the predominant metal in this complex, although nickel could also be detected (22). In contrast, the E9 DNase–Im9 complex was solved by multiple anomalous dispersion phasing, in which either one or both of the subunits was labeled with selenomethionine, by preparing each of the components separately and then forming and crystallizing the complex (27). The E9 DNase was purified as described by Garinot-Schneider et al. (28), by co-expressing the domain with histidine-tagged Im9 and purifying the complex on a nickel-affinity column, followed by denaturation of the domain, to achieve its separation from the tightly bound Im9 protein, and refolding by dialysis. This form of the enzyme contains only nickel, as determined by atomic absorption (17). E9 DNase purified by this route is active as an endonuclease and binds stoichiometric amounts of Im9, which can be expressed separately and to high yield in bacteria in the absence of the DNase (14). Even though the complexes of the E9 and E7 DNases with their immunities were prepared by different methods, the resulting crystal structures are very similar, indicating that denaturation of the DNase does not affect the final structure. Where the structures differ, however, is in the metal that is bound within the HNH motif and the identity of the fourth ligand: phosphate in the E9 structure, water in E7.

**FIG. 1. HNH motif of the E9 DNase showing the bound nickel ion (17).** The metal ion is tetrahedrally co-ordinated to 3 histidines and a phosphate molecule. In the original structure (solved to 2.05 Å resolution; accession no. 1bxi) His131 was not unambiguously located in the electron density, but three histidines have since been shown to bind to the Ni^{2+} by NMR (50). The equivalent of His131 in the zinc-bound E7 DNase structure of Ko et al. (22) is also a coordinating ligand for the metal. Residues from the E9 DNase that make up the HNH motif itself are His103–Asn118–His127. Asn118 (not shown) forms hydrogen bonds across the two β-strands with the main-chain atoms of residue 106. The figure was prepared using the program MOLSCRIPT (51).

The aim of the present work was to define the role of the transition metal site within the HNH motif of colicin endonucleases. Through a series of activity and biophysical experiments using the E9 DNase domain, we compared the properties of apo- and holoenzyme; based on these results we suggest how the creation of apo-enzyme might be important in the translocation of these toxins across bacterial membranes.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Protein Determinations of E9 DNase Domain—**Plasmid pPJ353 (encoding the E9 DNase domain and Im9 with a C-terminal histidine tag) was transformed into E. coli BL21 (DE3) and cells grown on Luria- Bertani broth. The E9 DNase and the mutant His277 → Ala E9 DNase were purified as described by Garinot-Schneider et al. (28). In addition, to produce metal-free apo-protein, the E9 DNase was unfolded in 3 M guanidine hydrochloride (GdnHCl; BDH) and pre-incubated with a 4-fold excess of EDTA and gel-filtered in the presence of 3 M guanidine hydrochloride and 50 mM Tris/ HCl buffer, pH 7.0. The denaturant was then removed by extensive dialysis against 50 mM potassium phosphate buffer, pH 7.5. A 0.25 mg/ml solution of unlabeled pUC18 was used as substrate under defined metal regimes. Circular dichroism (CD) spectroscopy was performed at 20 °C using a Jasco spectropolarimeter. The protein concentration was 0.25 mg/ml in 50 mM potassium phosphate buffer, pH 7.5. A 0.02-cm cuvette was used and far UV spectra recorded from 190 to 260 nm. Measurements of intrinsic tryptophan fluorescence emission were made using a Shimadzu RF5000 spectrofluorimeter, thermostatted at 25 °C and in filtered 50 mM Tris/HCl buffer, pH 7.0, using an excitation wavelength of 295 nm. Emission spectra were recorded from 300 to 450 nm. The excitation bandwidth was set to 10 nm, and 3-ml quartz cuvettes were used. The protein concentration was 27 μg/ml (1.8 μM). GdnHCl-dependent denaturation of the E9 DNase domain was also investigated by tryptophan fluorescence emission spectroscopy, wherein the protein (9 μg/ml, 0.6 μM) with
or without added metal was incubated with different GdnHCl concentrations, equilibrated for 2 h at 25 °C, and fluorescence measurements taken as described above. In fluorescence experiments using the dye 8-anilino-1-naphthalene-1-sulfonic acid (ANS), emission was measured using an excitation wavelength of 365 nm and emission spectra recorded from 390 to 650 nm. Quartz cuvettes (3 ml) were used containing 1.5 ml of Tris/HCl buffer, pH 7.5, and 13.2 μM ANS, open-circle DNA; I, linear DNA. Apo-E9 DNase was prepared as described under “Experimental Procedures.” A, E9 DNase incubated with DNA alone. B, E9 DNase with 10 mM MgCl₂. The chosen concentration of MgCl₂ was based on previous studies of the magnesium dependence of E9 DNase activity (27). C, E9 DNase with 10 mM MgCl₂ and 10 μM ZnCl₂. D, E9 DNase with 10 μM ZnCl₂.

### RESULTS

The Metal Center of the HNH Motif Is Not a Catalytic Device in the Colicin DNase—Transition metals such as zinc and nickel can perform catalytic functions in enzyme active sites (33), and the presence of one or other in the HNH motif of this family of endonucleases suggested their involvement in catalysis. We therefore investigated the effect of these metals on the ability of the E9 DNase to cleave plasmid DNA (Fig. 2). Prior to the x-ray structures of colicin DNases, previous studies on the effects of metal ions on E9 DNase cleavage of DNA did not take into account the possibility that the enzyme contained bound metals (29). Indeed, it is likely that previous protein preparations used in enzymic assays were mixtures of both metal-loaded and apo-enzyme. Hence, the question of transition metal involvement in catalysis was re-examined under defined metal-loading conditions by comparing the plasmid nicking activities of apo- and holoenzyme (Fig. 2). Apo-enzyme was prepared by incubating the enzyme with EDTA in either the folded or unfolded state (using guanidine hydrochloride as a denaturant) followed by removal of the chelating agent (and denaturant) by gel-filtration chromatography or dialysis (see “Experimental Procedures”). The results were essentially identical regardless of the method used to generate the metal-free form of the enzyme. Atomic absorption analyses indicated the absence of any bound metal (data not shown). At pH 7.5 and at 29 °C, apo-E9 DNase showed no endonuclease activity in the absence of added cations (Fig. 2A). The addition of Mg²⁺ ions (10 mM) readily caused nicking of the substrate by the apo-enzyme (Fig. 2B), eventually degrading the DNA completely (data not shown). The inclusion of Zn²⁺ (10 μM is sufficient to saturate the single metal site; see below) with Mg²⁺ did not yield faster rates of DNA cleavage (Fig. 2C), but rather resulted in slight inhibition of the Mg²⁺-dependent DNase activity. Inhibition by zinc was even more pronounced at elevated temperatures where the DNase is more active (data not shown). Incubation of the enzyme with 10 μM zinc alone (Fig. 2D) induced no cleavage of the DNA, even at relatively high concentrations (0.5 mM; data not shown). In contrast, the addition of 10 μM Ni²⁺ increased (by ~2-fold) the Mg²⁺-dependent activity of the E9 DNase (data not shown). The present data demonstrate that the transition metal bound in the HNH motif of colicin endonucleases is not essential for the random hydrolysis of plasmid DNA substrates and indeed may even inhibit this activity, which is dependent on magnesium ions.

### Structural Changes Accompanying Metal Binding to the HNH Motif of Colicin E9—In order to investigate further the role of transition metals in the function of colicin endonucleases, we analyzed the effect of transition metal ion binding on both secondary and tertiary structure. Circular dichroism (CD) experiments have shown previously that the E9 DNase, prepared as described above, is a folded domain, and analytical ultracentrifugation has shown the domain to be monomeric in solution (29). In the present study, we found that small but significant changes occur in the far UV CD spectrum of the E9 DNase on binding Ni²⁺ (Fig. 3A). The most significant change occurred at 210 and 230 nm, and these changes could be reversed by the addition of EDTA (data not shown). The alterations in secondary structure also result in changes to tertiary structure. The intrinsic tryptophan emission fluorescence of the E9 DNase (which contains two tryptophan residues) shows a slight enhancement on removing bound Ni²⁺ or Zn²⁺, while the λ_{max} for the fluorescence emission (334 nm) remains unchanged (Fig. 3B). This indicates that the effects of removing the metal ion are not the result of global denaturation, which instead leads to a quench in the tryptophan fluorescence (see Fig. 5B) and a shift in the emission maximum to 350 nm. This is also consistent with near UV circular dichroism spectra showing that relatively minor changes result from the removal of bound metal (data not shown). Taken together these data imply that apo-protein retains a fold similar to that of the holoenzyme and that the structural changes that occur as a result of removing the bound metal are probably localized around the metal site. Although removal of bound metal ion causes relatively minor changes to the secondary and tertiary structure of the E9 DNase, this nonetheless results in an increase in the exposure of non-polar groups as deduced by experiments with the dye ANS. As can be seen in Fig. 4, apo-E9 DNase shows a significant ANS fluorescence emission at ~490 nm, which on binding transition metals (Ni²⁺ is shown in Fig. 4, but identical results were obtained with Zn²⁺) is quenched approximately 2-fold, indicating the burial of these exposed hydrophobic regions. The
Conformational Stability Changes of the E9 DNase Domain on Binding Metals—The increase in ANS binding of apo-E9 DNase relative to holoenzyme suggested that the conformational stability of the domain may be affected by metal ions. We therefore investigated this possibility using proteolysis, chemical denaturation, and calorimetry. Proteolytic susceptibility of proteins is often used as an indicator of thermodynamic stability since unstable proteins are better proteolytic substrates than their stable counterparts. Hence, the proteolytic susceptibility of apo-E9 DNase domain was compared with that in the presence of zinc (Fig. 5A). Identical results were obtained for nickel and cobalt (data not shown). Removal of the bound metal elevated the proteolytic susceptibility of the domain to the point where the apo-enzyme was completely digested with 1% trypsin in under 10 min. Addition of 1 eq of zinc to the enzyme significantly increased its resistance to proteolysis, consistent with the metal increasing the stability of the domain. Control experiments indicated that metals did not affect the activity of trypsin against a non-metal-containing protein (data not shown). Notwithstanding the overall increase in proteolytic resistance of the metal-bound form of the enzyme, a single tryptic cleavage did occur at the N terminus of the E9 DNase at Arg5 (deduced from sequencing of the blotted fragment) (Fig. 5A). This corresponds to the tryptic cleavage site identified by Wallis et al. (34) in the original identification of the DNase domain of colicin E9 and so was not unexpected.

Changes in domain stability were also analyzed by guanidine hydrochloride denaturation experiments in which denaturation of apo- and holoenzyme were monitored by tryptophan fluorescence emission spectroscopy. The fluorescence of the domain is quenched ~2-fold on complete denaturation, providing a convenient measure of unfolding (Fig. 5B). Low concentrations of guanidine hydrochloride enhance the fluorescence of the domain (~ metal ions), suggesting a possible interaction between GdnHCl and the DNase that has been documented for other proteins (35). The fluorescence of the fully denatured protein was the same regardless of its metal loading, indicating that the fluorescence of the unfolded states of the apo- and holoenzyme are the same. The most significant difference between holo- and apo-enzyme was the concentration of GdnHCl required to unfold the E9 DNase since metal-loaded enzyme required substantially more denaturant (~0.4 M) to unfold the protein compared with the apo-enzyme. In addition, the holoenzyme denaturation profile was non-cooperative, unlike that of the apo-enzyme, which showed a single cooperative transition (Fig. 5B).

Changes in DNase domain stability as a result of metal ion binding to the HNH motif of the E9 DNase monitored by ANS fluorescence. To 13 µg either wild type E9 DNase or a His127→Ala mutant dissolved in Tris/HCl buffer, pH 7.5, was added 20 and 40 µM ANS, respectively, at 23 °C and the fluorescence emission recorded at 495 nm (λex = 365 nm) following the addition of increasing molar ratios of nickel. The signal for wild type enzyme showed a 2-fold quench in the fluorescence at approximately 1-to-1 metal-to-protein ratio, whereas the His127→Ala mutant showed no evidence of metal binding.

The increase in ANS binding of apo-E9 DNase to which 100 µM Ni²⁺ had been added (single line). Protein solutions were dissolved in 50 mM phosphate buffer, pH 7.5, and spectra collected at 25 °C. The changes induced by metal ion binding were fully reversible by the addition of EDTA (data not shown). To ensure that the ANS fluorescence derived from dye binding to apo-E9 DNase and that the resulting quench was indeed due to metal binding to the HNH motif, a control experiment was carried out using a single site mutant of the DNase in which one of the metal co-ordination sites, His127, was mutated to alanine (Figs. 1 and 4 and Ref. 28). The His127→Ala mutant also binds ANS, but in this case transition metals did not cause a change in fluorescence. This verified the identity of this residue as a metal co-ordination site and indicated that the ANS fluorescence quench experiments are indeed monitoring changes to protein tertiary structure as a result of transition metal ion binding to the HNH motif of the E9 DNase.

The increase in ANS binding of apo-E9 DNase (0.5 mg/ml; 33 µM), shown in triangles, overlaid with the spectrum of the enzyme to which 100 µM Ni²⁺ had been added (single line). Protein solutions were dissolved in 50 mM phosphate buffer, pH 7.5, and spectra collected at 25 °C. The changes induced by metal ion binding were fully reversible by the addition of EDTA (data not shown). To ensure that the ANS fluorescence derived from dye binding to apo-E9 DNase and that the resulting quench was indeed due to metal binding to the HNH motif were also investigated by DSC (Fig. 3).

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FIG. 3. Metal binding to the HNH motif causes minor structural changes to the E9 DNase. A, far UV-CD spectra of apo-E9 DNase (0.5 mg/ml; 33 µM), shown in triangles, overlaid with the spectrum of the enzyme to which 100 µM Ni²⁺ had been added (single line). Protein solutions were dissolved in 50 mM phosphate buffer, pH 7.5, and spectra collected at 25 °C. The changes induced by metal ion binding were fully reversible by the addition of EDTA (data not shown). B, tryptophan fluorescence emission spectra (λem = 295 nm) of E9 DNase (1.8 µM) with and without added Ni²⁺ (100 µM) in 50 mM Tris/HCl buffer, pH 7.0, at 25 °C.
is very low, since it has a melting temperature ($T_m$) of 36 °C, in keeping with its susceptibility toward proteolytic digestion. Second, binding of zinc to the domain increased the $T_m$ by 22 °C to 58 °C, consistent with the increased resistance to proteolytic digestion and guanidine hydrochloride denaturation that accompany metal ion binding. Finally, this increase in thermodynamic stability also resulted in a substantial increase in the enthalpy of unfolding, consistent with both stabilization of the native fold by metal ion binding and with the strong temperature dependence of unfolding enthalpies (positive $\Delta C_p$ effect) characteristic of globular proteins (36). The low stability of the metal free E9 DNase domain is likely to be of importance to the biological mechanism of toxicity of endonuclease colicins, and this is addressed under “Discussion.”

The Affinity of DNase-Metal Complexes—While it was clear that first row transition metals bind to the DNase domain of colicin E9, the affinity of these complexes had not been established. In order to confirm the stoichiometry of metal ion binding and determine equilibrium dissociation constants ($K_d$ values) for E9 DNase-metal complexes, ITC was used, and these data are presented in Fig. 6 and Table I. Control experiments (data not shown) indicated the absence of any detectable binding of magnesium ions and the absence of transition metal binding to the His127Ala mutant of the DNase, the latter experiment again confirming the location of metal ion binding site to the HNH motif in the E9 DNase active site. The binding of three metals (Ni$^{2+}$, Zn$^{2+}$, and Co$^{2+}$) to the E9 DNase domain was investigated. In all three cases, the enthalpy of metal binding was substantial (>16 kcal/mol) and the stoichiometry of binding close to 1-to-1, in agreement with the stoichiometry obtained from the ANS experiments (Fig. 4). Complete single ligand binding isotherms were obtained for both Co$^{2+}$ (shown in Fig. 6) and Ni$^{2+}$, for which the dissociation constants were 1.8 and 0.68 μM, respectively (Table I). Zinc binds very much more tightly to the domain than can be obtained from ITC...
Table I

| Metal | $K_d$ (molar ratio) | $\Delta H^\circ$ (kcal mol$^{-1}$) |
|-------|---------------------|----------------------------------|
| Cobalt | $1.8 \pm 0.01 \times 10^{-6}$ | $0.962 \pm 0.032$ |
| Nickel | $0.68 \times 10^{-6}$ | $0.901$ |
| Zinc  | $1 \times 10^{-9}, 4 \times 10^{-9}$ | $0.961 \pm 0.037$ |

Nickel 0.68

3 quoted

of the E9 DNase for zinc is higher than can be estimated by ITC; the parentheses are standard errors from duplicate observations. The affinity for substrate or hydrolytic water (37). In the E7 structure this site is occupied by water, while phosphate is found in E9. Water coordination to the metal points to its possible activation to produce hydroxide, in a fashion similar to other hydrolytic zinc enzymes such as the single strand-dependent nuclease P1, where the hydroxide produced is postulated to attack the scissile phosphodiester bond (38). However, we see no significant zinc or nickel-dependent endonuclease activity for the colicin DNase domains, implying that DNA binding specificity may be associated with a separate DNA binding module. Such two domain architecture has indeed been demonstrated for the phage enzyme I-TevI, a member of the GIY-YIG family of homing endonucleases (41).

Although inconsistent with a role in catalysis, the changes in proteolytic degradation and chemical and thermal denaturation that result from removal of the zinc atom in the HNH motif of the DNase domain of colicin E9 argue for a structural role in the enzyme. Interestingly, the homing endonuclease I-PpoI from the His-Cys family also contains structural zinc atoms (42). It is clear from the recent crystallographic data that the HNH motif encodes a zinc-finger-like structure (Fig. 1). The role of the metal ion in a classic zinc finger is structural, but this is ordinarily accomplished with four protein ligands, including cysteine and histidine residues (33). The question arises as to why a structural zinc site within colicin endonucleases should have evolved with only three protein ligands? The answer may lie in the need to remove the metal ion prior to membrane translocation of the DNase domain, and the generally tighter binding of metal that is seen for four coordinate zinc sites versus three.

Zinc Is the Physiological Metal for Colicin Endonucleases—Our data demonstrate that colicin endonucleases are metallo-proteins that bind 1 eq of transition metals within the HNH motif, in agreement with the two recent crystal structures of the E7 and E9 DNases. Isothermal titration calorimetry data (Fig. 6 and Table I) showed that Zn$^{2+}$ has by far the highest affinity for the HNH motif compared with the other transition metals tested (Ni$^{2+}$ and Co$^{2+}$). Zinc is also in greater abundance inside bacterial cells than either nickel or cobalt, and since endonuclease colicins are released from producing organisms in a folded form in complexes with their immunity proteins, it is highly likely that these will be bound with zinc at the active site. We conclude, therefore, that zinc is most likely the physiological metal ion for the DNase colicins, and possibly HNH endonucleases in general.

Translocation of Colicin A—The tetrahedral geometry of the transition metal ion in the active site of colicin endonucleases is suggestive of a role in catalysis since this fits the classic description of a catalytic metal site in an enzyme; three protein ligands with one site free for substrate or hydrolytic water (37). In the E7 structure this site is occupied by water, while phosphate is found in E9. Water coordination to the metal points to its possible activation to produce hydroxide, in a fashion similar to other hydrolytic zinc enzymes such as the single strand-dependent nuclease P1, where the hydroxide produced is postulated to attack the scissile phosphodiester bond (38). However, we see no significant zinc or nickel-dependent endonuclease activity for the colicin E9 DNase using double-stranded DNA substrates, in both plasmid nicking assays (Fig. 2) and spectrophotometric Kunitz assays, in which calf thymus DNA is used (29). Indeed, zinc seems to inhibit the DNase activity of colicin E9 (Fig. 2C). Since transition metal ion binding increases the stability of the domain, this inhibition might be a consequence of reduced flexibility in the protein. While our data do not support a role for the metal in catalysis, we cannot discount the possibility that the true substrate for the enzyme is a specific DNA sequence (as yet unidentified) whose cleavage requires zinc bound in the active site. There is no evidence for the existence of a specific DNA recognition sequence for colicin DNases, although their similarity to HNH endonucleases, rare cutting enzymes with substrate recognition sequences 15–40 bases in length, would be consistent with the recognition of a specific DNA target sequence. But HNH enzymes usually encounter only one recognition sequence per $10^6$ to $10^7$ base pairs (39) and so it seems unlikely that the cytotoxic activity of a nuclease colicin would be dependent on such rare endonucleolytic events. Also, HNH enzymes such as I-TevIII from phage (40) are significantly larger (by 2-fold) than colicin DNase domains, implying that DNA binding specificity may be associated with a separate DNA binding module. Such two domain architecture has indeed been demonstrated for the phage enzyme I-TevI, a member of the GIY-YIG family of homing endonucleases (41).

The Role of Transition Metals in the Mode of Action of Endonuclease Colicins: A Model—Based on our present data, we propose a mechanism for cytotoxicity that combines earlier work and ideas on the translocation of pore-forming colicins across bacterial membranes (3) with the postulated structural role of the bound metal ion in the DNase (Fig. 7). As described above, it is likely that DNase colicins are released from cells already harboring a metal ion, thereby enhancing the stability of the DNase domain in the harsh conditions of the extracellular environment (Fig. 7A). Through the other two domains of the toxin, the colicin binds both its primary receptor (the BtuB protein) and the Tol protein complex, possibly through the porin OmpF (Fig. 7B) (3, 7). We postulate that both the immunity protein and metal ion are jettisoned at this point, possibly due to the interaction with the receptor or due to the close proximity of the phospholipid head groups of the outer membrane. Zinc can bind to membranes (45), while Im9 is very negatively charged and may be repelled from the membrane, thus destabilizing the complex sufficiently to displace the immunity protein; it has been estimated that the dissociation rate constant for the E9 DNase-Im9 complex must be accelerated by 4 orders of magnitude for cell death to occur on a time scale consistent with the kinetics of colicin-mediated toxicity (21). The appearance of unbound immunity protein in the extracellular medium has been observed for the RNase colicin cloacin DF13, but has yet to be demonstrated for DNase colicins (46).

Removal of the bound metal ion from the HNH motif destabilizes the DNase domain of the colicin and renders it competent for translocation across lipid bilayers (Fig. 7C). Although at present it is unknown whether the DNase of endonuclease colicins needs to be unfolded to enter cells, this seems likely based on previous work on pore-forming colicins (47, 48). Colicins are secreted by, and are active against, the enterobacteriaceae, which live in the intestinal tracts of mammals as well as in other habitats. Considering that the melting temperature for the apo-DNase domain is 36 °C, this would imply that, at the normal body temperature of a mammal (or growth temperature for laboratory strains of E. coli), more than 50% of metal-free
comes exposed to random cleavages, leading ultimately to cell death.

In conclusion, the HNH motif is a structural element within the active site of colicin DNases that most likely binds zinc. While it is unclear if zinc serves a catalytic role in homing endonucleases, it does not seem to have catalytic function in colicins. Instead the metal ion serves to stabilize the domain, its removal possibly initiating membrane translocation of the DNase into a bacterial cell.

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