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Mechanism and Cleavage Specificity of the H-N-H Endonuclease Colicin E9

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Colicin endonucleases and the H-N-H family of homing enzymes share a common active site structural motif that has similarities to the active sites of a variety of other nucleases such as the non-specific endonuclease from *Serratia* and the sequence-specific His-Cys box homing enzyme I-*Ppo*I. In contrast to these latter enzymes, however, it remains unclear how H-N-H enzymes cleave nucleic acid substrates. Here, we show that the H-N-H enzyme from colicin E9 (the E9 DNase) shares many of the same basic enzymological characteristics as sequence-specific H-N-H enzymes including a dependence for high concentrations of Mg^{2+} or Ca^{2+} with double-stranded substrates, a high pH optimum (pH 8-9) and inhibition by monovalent cations. We also show that this seemingly non-specific enzyme preferentially nicks double-stranded DNA at thymine bases producing 3′-hydroxy and 5′-phosphate termini, and that the enzyme does not cleave small substrates, such as dinucleotides or nucleotide analogues, which has implications for its mode of inhibition in bacteria by immunity proteins. The E9 DNase will also bind single-stranded DNA above a certain length and in a sequence-independent manner, with transition metals such as Ni^{2+} optimal for cleavage but Mg^{2+} a poor cofactor. Ironically, the H-N-H motif of the E9 DNase although resembling the zinc binding site of a metalloenzyme does not support zinc-mediated hydrolysis of any DNA substrate. Finally, we demonstrate that the E9 DNase also degrades RNA in the absence of metal ions. In the context of current structural information, our data show that the H-N-H motif is an adaptable catalytic centre able to hydrolyse nucleic acid by different mechanisms depending on the substrate and metal ion regime.

Keywords: homing endonuclease; DNA; metal ions; mechanism; toxin

Introduction

Homing endonucleases are found throughout biology. Encoded by group I and group II introns and inteins they promote the homing of the genetic elements coding for them into intronless/inteinless allelic sites. Traditionally, these ubiquitous enzymes are classified into families based on active site amino acid sequence identities with four main classes: LAGLIDADG, GIY-YIG, His-Cys box, and H-N-H.[1,2] In keeping with their biological importance, numerous studies have been reported on these enzyme families, with the LAGLIDADG, His-Cys box and GIY-YIG families the best studied but with few mechanistic reports for any H-N-H

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Abbreviations used: ds, double-stranded; E9 DNase, endonuclease domain of colicin E9; TEA, triethanolamine; $T(npp)_2$, thymidine-3′,5′-di-(*p*-nitrophenyl)-phosphate; TpA, thymidylyl (3′→5)-2′-deoxyadenosine; ss, single-stranded; ANS, 8-anilinonaphthalene-1-sulfonic acid.

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enzyme. In the present paper we analyse the nucleic-acid cleavage specificity and metal dependence of the H-N-H endonuclease from colicin E9, a bacterial toxin that kills *Escherichia coli* cells through a non-specific DNase activity, and assign putative functionality to many of its amino acid residues.

A characteristic feature of homing endonucleases is that their DNA-binding sites are extensive (15-40 bp) but degenerate, with double-strand cleavage occurring some distance away and generally leaving a 3′ overhang.[2] The enzymology of homing enzymes has been enhanced by structural and mechanistic studies on homologous endonucleases found in different biological settings. The non-specific, catabolic DNA/RNA nuclease from *Serratia marcescens*, for example, is a homologue of the His-Cys box enzyme I-*Ppo*I from *Physarum polycephalum*. Although both are dimers there is no structural similarity between these enzymes except at their active sites, cleaving DNA by the same basic mechanism.[3,4] Another example of active site similarity between homing enzymes and other endonucleases is that of the H-N-H family of enzymes and colicin endonucleases.[5] Colicins are plasmid encoded toxins released by some strains of *E. coli* following SOS-mediated induction. Cell entry by these bactericidal agents occurs through the parasitisation of outer membrane proteins normally used in nutrient uptake, such as BtuB in the case of E group colicins, and periplasmic proteins used either in nutrient uptake or for maintaining the integrity of the cell envelope (reviewed by Lazdunski *et al*.[6]). Enzymatic E group colicins are 60 kDa toxins that translocate their C-terminal cytotoxic domains across the inner membrane in order to elicit cell death through the hydrolysis of nucleic acid. Four H-N-H endonuclease colicins have been identified, E2, E7, E8 and E9, all of which target the bacterial chromosome. Here, the focus is the 15 kDa, monomeric endonuclease domain of colicin E9 (E9 DNase) domain from colicin E9, an H-N-H enzyme that is the subject of ongoing enzymological studies in our laboratory.[7,8]

The only crystal structures of H-N-H DNases currently available are those of colicins E7 and E9 bound to their cognate immunity proteins Im7 and Im9, respectively.[9,10] Immunity proteins bind and inhibit colicins in the producing host. Homology to homing enzymes in colicin DNases is restricted to 12 residues of the C-terminal 32 residue H-N-H motif itself. The structural work on the E9 DNase suggested that the classical description of homing enzymes into four families be re-evaluated since the H-N-H motif is structurally homologous to the active sites of both I-*Ppo*I, a His-Cys box enzyme, and *Serratia* nuclease even though these enzymes share almost no sequence similarity with E9.[11] Rather more unexpectedly, this analysis also highlighted that the three enzymes position a metal ion in the same location. However, where this is a magnesium ion in *Serratia* and I-*Ppo*I, the site is occupied by a transition metal ion in the structures of the H-N-H enzymes; zinc in the case of the E7 DNase and nickel in the E9 DNase, the latter a consequence of the purification protocol. Kühlmann *et al*.[11] proposed that these enzymes constitute a new group of metal-dependent endonucleases called the ''ββα-Me'' family, identifying the structural elements involved in the motif and the metal ion. The same motif (referred to as a ''β-finger'') is also found in bacteriophage T4 endonuclease VII, which is thought to be mechanistically similar to both *Serratia* and I-*Ppo*I (see the Discussion).[12] These structural similarities have been extended yet further with the observation that the basic motif encompassing the active sites of colicin and homing endonucleases is found in many enzyme families, some of which are not even nucleases, but are related by the property that they bind metal ions in a so-called ''treble clef finger''.[13]

Little is known of the mechanism by which H-N-H endonucleases cleave nucleic acid substrates. This can in part be attributed to the absence of extensive sequence identities between the H-N-H enzymes and its structural homologues, but is also due to the enigmatic role of metal ions in colicin DNases, given that they bind transition metals in a site where enzymes such as *Serratia* and I-*Ppo*I bind a magnesium ion.[9,10,14,15] Here, we focus on the mode of action of the DNase H-N-H enzyme from colicin E9 and define some of the basic conditions for optimal cleavage of DNA. We show that the enzyme exhibits base specificity for double-stranded (ds) DNA as well as cleaving both single-stranded (ss) DNA and ssRNA. The catalytic activities of colicin endonucleases are discussed in the context of the structural information on the H-N-H motif in its different metal-liganded states and its similarity to the active sites of other endonucleases. Finally, we discuss the possibility that colicin DNases are capable of hydrolysing DNA by two catalytic mechanisms, each with a distinct metal-cofactor requirement.

Results

pH and cation dependence of E9 DNase activity

We have demonstrated that the E9 DNase shows no activity against double-stranded DNA substrates in the presence of zinc ions even though zinc binds to the H-N-H motif of the enzyme with nanomolar affinity.[8,16] In addition, we have shown that the enzyme (at nanomolar protein concentrations) shows highest activity against supercoiled DNA substrates in the presence of Mg^{2+} or Ca^{2+},[8] but the metal ion concentration dependence of this activity was not addressed nor were the optimal cleavage conditions assessed. These are reported in Figure 1. We investigated the Mg^{2+} and Ca^{2+} dependence of the E9 DNase by two assay methods, one using calf thymus DNA in the spectrophotometric Kunitz assay, the other a plasmid nicking assay using radiolabelled pUC18 (see Pommer *et al*.[8] for further details). From both assays it

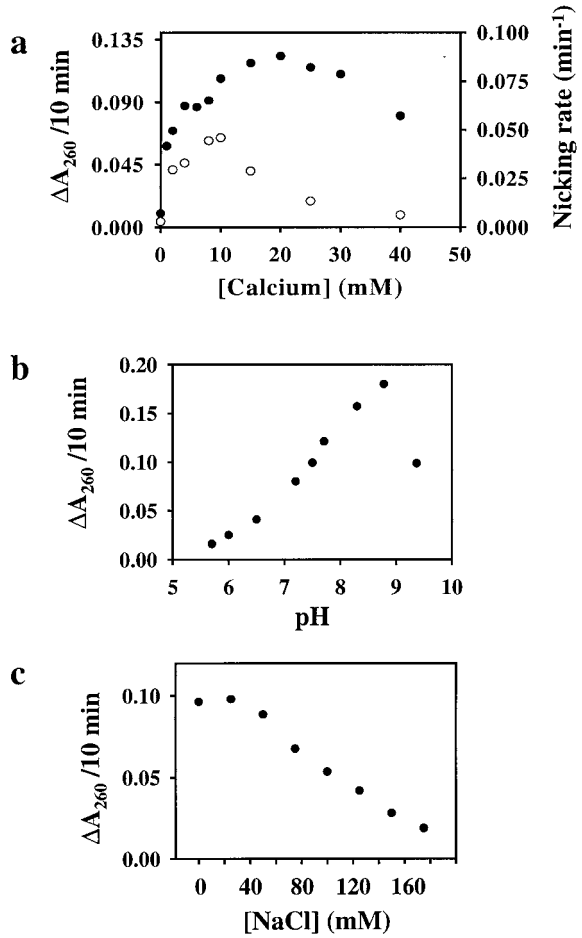


Figure 1. Basic enzymological characterisation of the H-N-H motif of the E9 DNase. (a) Effect of calcium ions on endonuclease activity monitored by the spectrophotometric Kunitz assay (closed symbols, left hand axis) and a plasmid nicking assay (open symbols, right hand axis) in bis-Tris propane at pH 8.5 and 25 °C as described by Pommer *et al*.[8] The DNase concentration used in the two assays was 1.5 μg/ml (Kunitz assay) and 15 ng/ml (nicking assay). See Materials and Methods for further details. (b) pH optimum for the enzyme assessed in 50 mM Hepes buffer using the Kunitz assay at 25 °C and 4 μg/ml E9 DNase. Substitution of Tris-HCl or bis-tris propane buffers for Hepes did not have any influence on the pH maximum (data not shown). (c) Effect of NaCl on E9 DNase activity (1.5 μg/ml) determined by the Kunitz assay in 50 mM bis-tris propane (pH 8.5) in the presence of 20 mM Mg^{2+}.

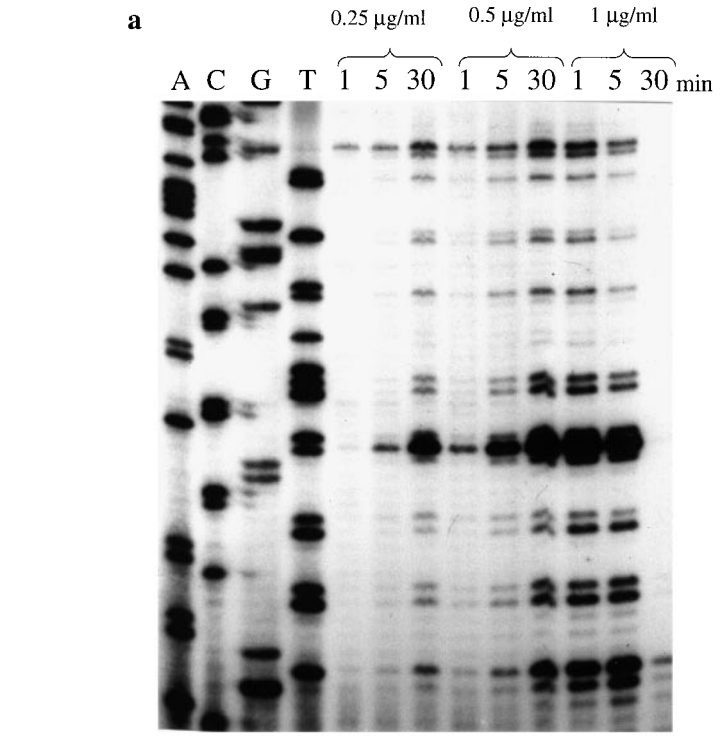
is clear that the enzyme shows a broad metal dependence with an optimum between 10-20 mM, depending on the substrate used. The data in Figure 1(a) are those for Ca^{2+}; very similar results were obtained for Mg^{2+} (not shown). We found that the pH optimum for the enzyme is 8-9 using the Kunitz assay, and that the inclusion of NaCl inhibited the activity of the enzyme, most likely due to the effect of monovalent cations since chloride salts of buffers have no effect on the enzyme (data not shown) (Figure 1(b) and (c)). These results are similar to those reported recently for I-*Cmoe*I, a site-specific H-N-H endonuclease from *Chlamydomonas moewusii*.[17] Hence, enzymes with an H-N-H active site motif likely cleave nucleic acid substrates by similar mechanisms regardless of their DNA recognition specificity, a situation reminiscent of the mechanistic similarities between the non-specific endonuclease *Serratia* and the highly specific His-Cys box enzyme I-*Ppo*I.

The E9 DNase displays dsDNA base specificity

Members of the ββα-Me family of endonucleases have a wide range of DNA base specificities; *Serratia* nuclease shows no sequence preference but prefers double-stranded *A*-form nucleic acids,[18,19] the T4 endonuclease VII junction resolvase is structure dependent,[12] while I-*Ppo*I is highly specific for its homing site.[20] To address whether the H-N-H E9 DNase possesses any sequence specificity toward dsDNA, the Mg^{2+}-dependent hydrolysis of the 160 bp *tyrT* promoter element was analysed alongside corresponding sequencing reactions (Figure 2(a)). The *tyrT* sequence has often been used to investigate the cleavage preferences of non-specific endonucleases (see, for example, Drew & Travers[21]). The E9 DNase was able to cleave at approximately one third of the 324 potential sites within the *tyrT* promoter sequence, cleaving tracts of GC poorly and having a preference for cleaving after thymine (Figure 2(b)); 56 % of sites cleaved were at T, 20 % at G, 14 % at A and 10 % at C.

Analysis of E9 DNase cleavage products

It has not been reported whether the products of DNA cleavage by H-N-H endonucleases contain 5′ or 3′ phosphates, a critical element in the understanding of their cleavage mechanism. Hence, we addressed this issue using the E9 DNase, looking at the digestion products of λ-DNA in the presence of Mg^{2+}. Following extended digestion, the sample was divided into two parts, one was treated with calf thymus alkaline phosphatase, to remove 5′ phosphate groups, while the other was left untreated. Both samples were then reacted with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (Figure 3). The rationale for this experiment is that if an endonuclease yields 5′ phosphate groups its products can only be radiolabelled if treated first with alkaline phosphatase. If an enzyme yields 5′ hydroxyl groups, radioactivity becomes incorporated by the kinase regardless of the alkaline phosphatase treatment. Cleavage products of the restriction endonucleases *Eco*RI and *Hin*dIII are known to produce 5′ phosphate groups and so these were included as controls in this experiment (Figure 3). Although there is a low level of incorporated radioactivity in the alkaline-phosphatase-untreated samples, observed for both the E9 DNase and the restriction enzymes



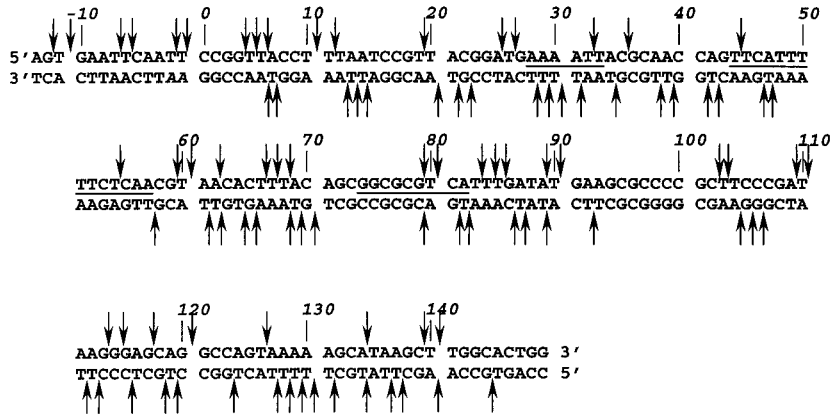


Figure 2. Cleavage of the *tyrT* promoter element by the E9 DNase. (a) [5′-^{32}P]-end-labelled *tyrT* DNA was incubated for varying times (one to 30 minutes) and with varying amounts of the E9 DNase in the presence of 0.5 mM Mg^{2+} and the products separated by gel-electrophoresis alongside sequencing reactions and visualised on a phosphorimager (see Materials and Methods for further details). The gel data show the E9 DNase to have a clear preference for cleaving at T residues, where in addition more intense cut sites are generated than at non-T sites. (b) Summary of the *tyrT* cleavage data with arrows indicating positions where cleavage by the E9 DNase was detected. The four underlined regions are those that are largely refractory to cleavage by DNase I.[21] The sequence shown in (a) corresponds to the first 53 nucleotides of the top DNA strand.

and most likely due to exchange of 5′ phosphate groups, this experiment strongly suggests that the E9 DNase cleavage products contain 5′ phosphate and 3′-OH termini. The same cleavage products are seen in the catalysed reactions of *Serratia* nuclease and I-*Ppo*I.

The E9 DNase does not cleave small nucleic acid substrates

The non-specific endonuclease from *Serratia* can digest DNA down to the level of mononucleotides, a consequence of its biological role in degrading extracytoplasmic nucleic acids for metabolism by

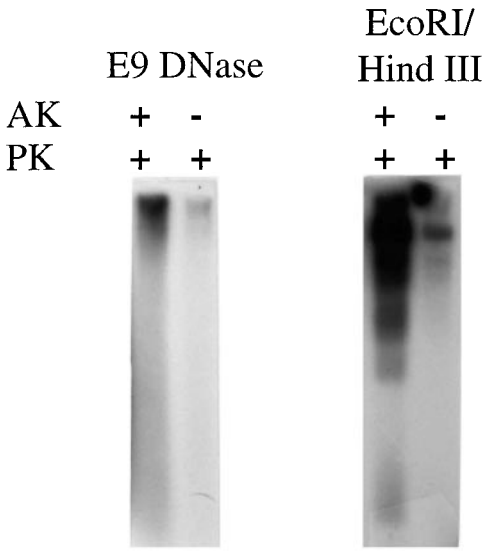


Figure 3. Colicin E9 DNase cleavage of DNA generates 5′ phosphate groups and 3′-OH groups. Phage lambda DNA (2.5 μg) was incubated either with the E9 DNase (left hand panel) or the restriction enzymes *Eco*RI & *Hin*dIII (right hand panel) for 20 minutes at 37 °C in 10 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$ and the digested DNA labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (PK), with or without prior treatment by calf intestinal alkaline phosphatase (AK), and the DNA visualised by autoradiography following separation on a 1.2 % agarose gel. Endonucleolytic cleavages leaving 5′-OH groups would be labelled with or without prior treatment with alkaline phosphatase while cleavages generating 5′ phosphate groups will only be labelled following this treatment.

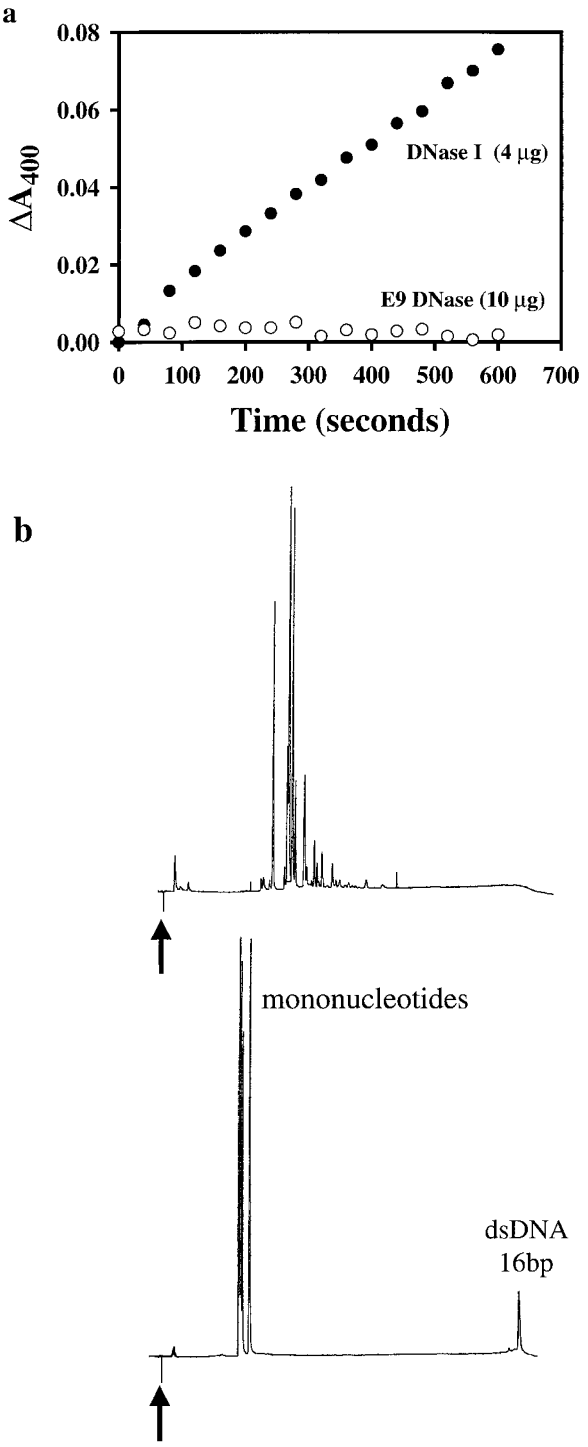


Figure 4. The E9 DNase does not cleave small substrates. (a) Spectrophotometric assay at 400 nm showing that at pH 8.0 and at 25 °C the E9 DNase does not release nitrophenolate anion from the synthetic substrate $T(npp)_2$ under conditions where DNase I readily shows activity. See Materials and Methods for further details. (b) Anion exchange HPLC profiles, eluted using an ammonium acetate gradient, of a palindromic 16 bp DNA sequence before (bottom) and after (top) incubation with the E9 DNase in 10 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$ at 37 °C for 20 hours. In order to establish the level of digestion by the E9 DNase the migration positions of each of the four 5′-phosphate mononucleotides were also established (bottom trace). The arrow in each profile indicates the injection point.

the organism.[22,23] Consistent with this activity is its ability to also cleave nucleotide nitrophenyl esters such as thymidine-3′,5′-di-(*p*-nitrophenyl)-phosphate ($T(npp)_2$),[3] which have also proved to be convenient substrates for kinetic and mechanistic studies of other non-specific endonucleases such as DNase I.[24] Cleavage of $T(npp)_2$ by DNase I generates the *p*-nitrophenolate anion which can be followed spectrophotometrically at 400 nm. We therefore investigated the cleavage of $T(npp)_2$ by the E9 DNase, using DNase I as a positive control (Figure 4(a)). Nitrophenolate anion was not generated by the E9 DNase, in contrast to the reported activities of I-*Ppo*I and *Serratia* both of which will cleave $T(npp)_2$,[3] and which have active sites that are structurally homologous to the E9 DNase.[11] Hence, although there are similarities between H-N-H enzymes such as E9 and other nucleases it is clear that there are also differences between them, particularly in terms of the substrates that they can hydrolyse.

To investigate this further we tested the ability of the E9 DNase to digest the simple dinucleotide substrate thymidylyl (3′ → 5′)-2′-deoxyadenosine (TpA) in the presence of Mg^{2+} but could detect no

activity with this substrate (data not shown). This was confirmed by HPLC analysis of E9 DNase cleavage products following incubation, for 20 hours at 37 °C in the presence of Mg^{2+}, with 16 bp duplex DNA (see Materials and Methods for details) that did not yield mononucleotide products even though the starting material was digested to completion (Figure 4(b)). Taken together these results show that, in the presence of Mg^{2+}, the E9 DNase will not cleave mononucleotide phosphoryl esters or dinucleotide substrates, implying that substrate size likely plays an important role in the ability of the enzyme to hydrolyse DNA.

dsDNA and ssDNA binding to the E9 DNase monitored by 8-anilinonaphthalene-1-sulfonic acid (ANS) fluorescence

The absence of catalytic activity by the E9 DNase against small nucleotide substrates could be due to their inability to bind to the enzyme and so DNA binding to the E9 DNase was investigated, capitalising on work showing that metal binding to the H-N-H motif causes substantial changes to the fluorescence of the extrinsic fluorophore ANS.[16] These changes occur as a result of the increase in stability metal binding causes and the concomitant expulsion of ANS from hydrophobic surfaces of the DNase. For these studies, we used a catalytically inactive mutant, E9 DNase H127A, identified through a random mutagenic screen,[25] and initially investigated the binding of dsDNA. dsDNA caused a ~30 % quench in the ANS-derived fluorescence on binding to E9 DNase H127A, saturating at a stoichiometry of approximately two protein molecules per 12mer duplex (Figure 5(a)). Since E9 DNase is able to bind ssDNA (see below) it is possible that the fluorescence changes were due to DNase-induced melting of the duplex and the binding of single-strands. However, this is unlikely since the melting temperature of the duplex used (40 °C) is significantly greater than the temperature at which the experiment was conducted (25 °C), and since the intercalating dye YOPRO-1, which is only fluorescent when intercalated into dsDNA, gave a high fluorescence when added to the dsDNA-E9 DNase H127A complex that was lost only when active endonuclease (DNase I) was added to the mixture (data not shown).

Early studies on the DNase of colicin E2 indicated that it was able to cleave single-stranded DNA,[26] although this activity, demonstrated using single-stranded phage DNA, was not described in detail nor reported for other DNase colicins. Hence, using ANS fluorescence spectroscopy, we sought to determine whether the E9 DNase could bind single-stranded oligonucleotides. In preliminary experiments we found that, as with dsDNA, ssDNA causes a quench in ANS-derived fluorescence but that this quench was dependent on the length of DNA and that the binding was stoichiometric at micromolar protein concentrations (data not shown). Hence, we analysed the binding of equimolar concentrations of ssDNA of varying length to the E9 DNase H127A mutant (13.2 μM) and quantitated the fluorescence quench (Figure 5(b)). The ssDNA used was randomised during synthesis so that each of the four bases was represented in every position in order to exclude any base information. The data demonstrate that small oligonucleotides (<five bases) bind poorly if at all to the E9 DNase, consistent with the lack of hydrolytic activity toward dinucleotides and $T(npp)_2$ (Figure 4), and that the optimal length for binding is >ten bases. The stoichiometry and affinity of ssDNA binding to the E9 DNase were determined by isothermal titration calorimetry

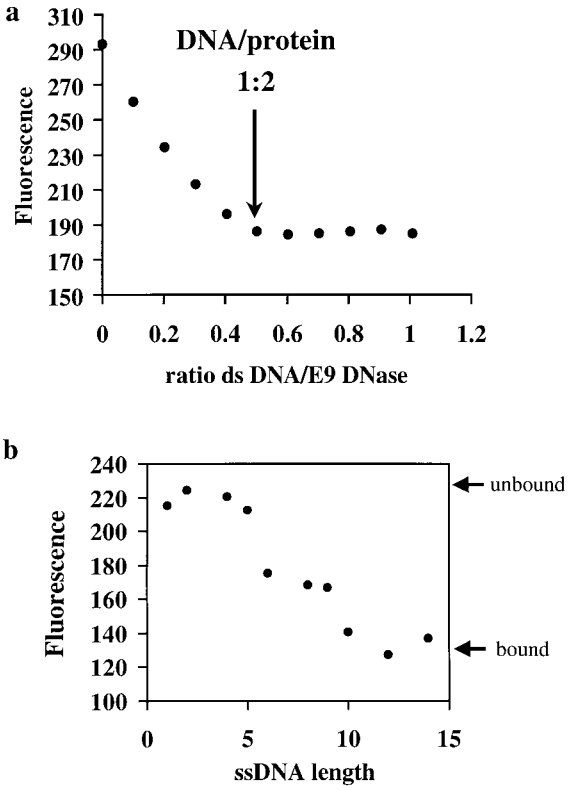


Figure 5. dsDNA and ssDNA binding to the E9 DNase followed by extrinsic fluorescence quenching. DNA binding to the E9 DNase was monitored by the quench in ANS fluorescence (λ^{ex} 365 nm/λ^{em} 490 nm) following the addition of ANS (40 μM) to the inactive mutant, E9 DNase H127A (0.2 mg/ml; 13.2 μM) in Tris-HCl buffer at pH 7.5 and 25 °C. (a) ANS fluorescence with increasing molar ratio of 12mer dsDNA-to-protein showing that approximately two molecules of E9 DNase bind the duplex. (b) ANS fluorescence change as a result of adding randomised sequences of equimolar ssDNA of increasing length (see Materials and Methods) showing that the E9 DNase prefers to bind substrates >ten nucleotides in length. Under these conditions, ssDNA (10mer and above) binds to the enzyme stoichiometrically (data not shown).

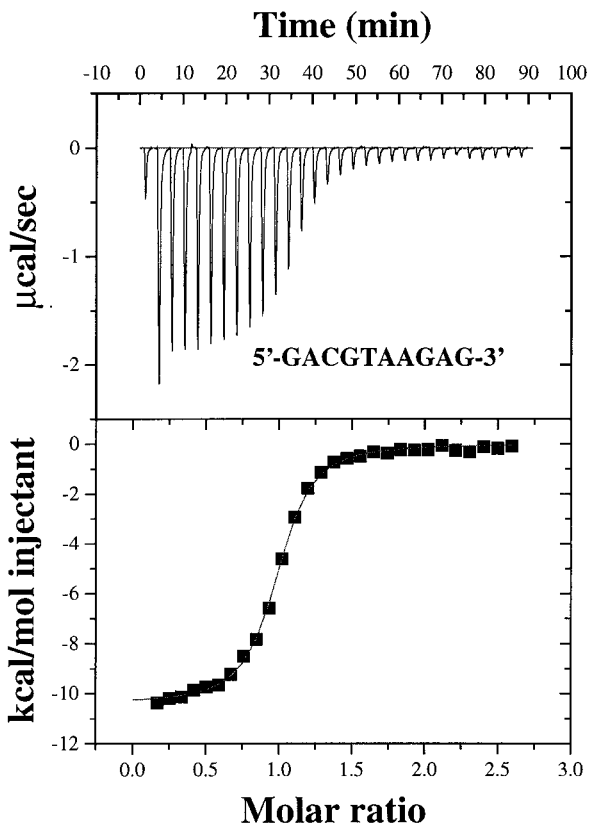


Figure 6. Monitoring ssDNA binding to the E9 DNase by isothermal titration calorimetry. Figure shows the binding of 10mer single-stranded DNA of defined sequence (insert) to the inactive mutant E9 DNase H127A. The top panel shows the calorimetric response of 30, 10 μl injections of E9 DNase (0.53 μM) to ssDNA (50 μM) in 50 mM TEA buffer (pH 7.5) and 25 °C. The bottom panel shows integrated injection heats for the above data fitted to a single, non-cooperative binding model and the continuous line represents the theoretical isotherm. Thermodynamic parameters from this fit are shown in Table 1.

(Figure 6 and Table 1). Here, we compared the binding of single-stranded DNA of random sequence (12mer) with DNA of defined sequence (10mer) and found similar affinities ($K_d \sim \mu M$) and 1:1 binding for both, consistent with the ANS data showing ssDNA >10 bases is optimal for binding.

ssDNA cleavage requires a transition metal ion

The question of whether E9 DNase cleaves ssDNA and with what metal dependence was addressed using a 23 bp ^{32}P-labelled ssDNA fragment (Figure 7). Very limited cleavage of the oligonucleotide was observed in the presence of 10 mM Mg^{2+} in a 15 minute incubation. Addition of Zn^{2+} did not support catalysis of ssDNA and did not enhance the ability of Mg^{2+} to catalyse its hydrolysis, whereas Ni^{2+} (K_d, 0.7 μM[16]) yielded the highest activity toward ssDNA. These experiments show that E9 DNase will cleave ssDNA in the presence of metal ions, with the highest activity associated with Ni^{2+} and only weak activity associated with Mg^{2+}. This order of reactivity is the same as that observed using calf thymus DNA in the spectrophotometric Kunitz assay but the reverse of metal ion reactivity in the nicking of supercoiled plasmids, where Mg^{2+} (or Ca^{2+}) is the preferred metal over Ni^{2+}.[8] Interestingly, the nanomolar protein concentration required to observe an appreciable activity in the plasmid nicking assay is significantly lower than that required for either the Kunitz assay or the cleavage of ssDNA (100 and 10,000-fold, respectively) suggesting that these later activities are "star" activities and may not be central to the cytotoxicity of colicins which are active against bacteria at nanomolar concentrations.

E9 DNase will cleave ssRNA

Non-specific endonucleases such as *Serratia* nuclease,[18] NucA,[27] and Mitogenic Factor secreted by *Streptococcus pyogenes*.[28] which cleave ss and dsDNA will also cleave ssRNA in the presence of Mg^{2+}. Therefore, potential RNase activity for the E9 DNase was investigated by incubating the enzyme with a synthetic, fluorescently-labelled ssRNA 10mer under a variety of metal regimes. We found that the enzyme does indeed cleave RNA and that this activity could be ascribed to the E9 DNase and its H-N-H motif since it was inhibited by Im9 binding and by the H-N-H mutation His127Ala (Figure 8(a)). Surprisingly, however, we found that the enzyme cleaved RNA in the absence of divalent cations and that this activity was not stimulated either by Mg^{2+} or Zn^{2+}, but was stimulated by Ni^{2+} (Figure 8(b)). This is in contrast to the work of Schaller & Nomura[26] on colicin E2

Table 1. Thermodynamic parameters for ssDNA binding to the E9 DNase H127A mutant determined by isothermal titration calorimetry at pH 7.5 and 25 °C

ssDNA sequence	n	K_d (M)	ΔH (kcal/mol)
GAC-GTA-AGA-G	0.89, 0.96	0.4, 0.7×10^{-6}	−12.0, −10.2
Randomised 12mer	1.06	2.3×10^{-6}	−13.0

The thermodynamic parameters for a randomised 12mer are very similar to those of a 10mer of defined sequence (two independent measurements on two protein preparations are shown). For further details see the legend to Figure 6. (1) cal = 4.184 J.

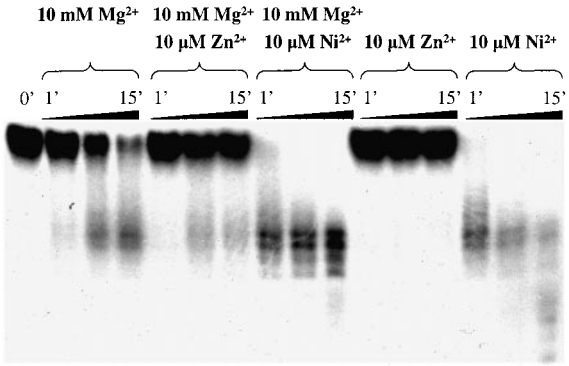


Figure 7. Cleavage of ssDNA by the E9 DNase requires transition metal ions. 23mer ssDNA was end-labelled with ^{32}P and incubated with the E9 DNase (0.2 mg/ml) over a 15 minute time course under a variety of metal regimes and in TEA buffer (pH 7.5) at 37 °C (see legend to Figure 8). The products were separated using a 20% polyacrylamide gel containing 8 M urea and visualised using a phosphorimager. The data show that Zn^{2+} does not support ssDNA cleavage whereas Ni^{2+} yields rapid digestion, while Mg^{2+} is a poor cofactor for this substrate.

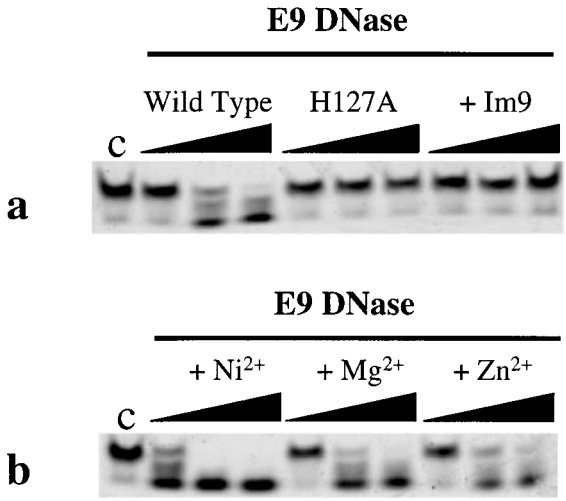


Figure 8. The E9 DNase cleaves RNA in the absence of metal ions. Carboxy-fluoresceine-labelled 10mer ssRNA (GAC GUA AGA G) was incubated with the E9 DNase (0.15 mg/ml) in 50 mM TEA buffer (pH 7.5) for 30 minutes at 30 °C and the products analysed by a phosphorimager following separation by acrylamide gel electrophoresis. Three time points are indicated (15 seconds, 15 minutes and 30 minutes), with ''c'' identifying the migration of substrate RNA in the gel. (a) The RNase activity of the enzyme occurs in the absence of metal ions and is abolished by Im9 binding and by a histidine-to-alanine mutation at residue 127, the latter result indicating that the H-N-H motif of the enzyme is responsible for this activity. (b) The effect of metal ions on the RNase activity of the E9 DNase. The concentrations of the Ni^{2+} and Zn^{2+} used (10 µM) were above their respective K_d values[16] while that of Mg^{2+} (10 mM) is optimal for DNA cleavage (Figure 1).

where no RNase activity could be detected against phage RNA. However, these early studies used lower concentrations of the toxin (20 µg/ml) than used here with the E9 DNase domain (150 µg/ml), emphasizing that this is a weak RNase activity.

Discussion

Enzymological similarities and differences between the E9 DNase and other nucleases

The non-specific endonuclease from colicin E9 shares many of the same basic enzymological characteristics as sequence-specific H-N-H homing endonucleases such as I-*Cmoe*I as well as with the His-Cys box enzyme I-*Ppo*I, including a requirement for high concentrations of Mg^{2+}, a pH optimum ~8.5 and inhibition by monovalent cations. We show, for the first time, that an H-N-H endonuclease releases 5′ phosphate and 3′-OH termini, which is also the case for *Serratia* nuclease and I-*Ppo*I. Moreover, the E9 DNase will cleave ssDNA, ssRNA and dsDNA, demonstrating a lack of specificity for the sugar moiety of nucleic acid, which is also the case for *Serratia* nuclease. These similarities to other nucleases suggests that, in the presence of Mg^{2+}, the E9 DNase may cleave nucleic acid by a similar mechanism to that used by I-*Ppo*I and *Serratia* nuclease, two enzymes that have been very well characterised in terms of their catalytic activity, and this is addressed below.

The similarities and differences between the E9 DNase and other nucleases, revolving around substrate preferences and base specificity, may point to their evolutionary ancestry. Not only does the E9 DNase act on a wide range of polynucleotides but it also shows a very strong preference for cutting at T bases in dsDNA. This specificity is presumably a reflection of the biological role of the enzyme, digestion of the relatively A/T rich *E. coli* genome. Pronounced selectivity for a single base is rare with deoxyribonucleases since any preference seen with low-specificity DNasess is usually ascribed to gross structural features of the nucleic acid. For example, both the *Serratia* nuclease[19] and DNase I[21] cut d(A).(T) tracts poorly, probably due to the rigidity of such sequences.[29] Staphylococcal nuclease is an example of a low specificity nuclease that has a preference for T bases, in exposed single-stranded regions of DNA,[30] but produces 3′ phosphate groups and 5′ hydroxyl termini which is not the case with the E9 DNase. Base-specific cleavage is more common with ribonucleases (e.g RNase T_1 at G; RNase U_2 at A; RNase A at C/U) suggesting that staphylococcal nuclease and E9 DNase, both of which are active on RNA, may have evolved from base-selective RNases. In relation to this, it is interesting to note that cation-independent cleavage of RNA but cation-dependent cleavage of DNA by a single nuclease active site is reminiscent of the RNase α-sarcin from

Aspergillus which cleaves RNA in the absence of metal ions but requires Mg^{2+} to cleave DNA.[31] Finally, since colicin E9 expression is induced by DNA damage through the SOS response, the E9 DNase may have evolved from a DNA repair endonuclease, a possibility given credence by the observation that H-N-H endonuclease domains have been found in mismatch repair enzymes.[32]

E9 DNase nucleic acid binding characteristics

Unlike enzymes such as *Serratia* nuclease, the E9 DNase does not cleave dinucleotides or simple nucleotide derivatives since these bind poorly if at all to the enzyme (Figure 5). The colicin E9 DNase can bind ssDNA in a sequence- independent manner preferring DNA above a certain length (>ten bases). Gel-shift experiments using plasmids have shown that the E9 DNase H127A mutant can bind dsDNA.[10,25] This is confirmed by the ANS fluorescence binding data presented here but which also show that at least two molecules of the E9 DNase can bind a 12mer duplex. Given the dimensions of the major and minor grooves of *B*-form DNA, the dimensions of the E9 DNase (40 Å × 25 Å × 25 Å) and its preference for binding relatively large substrates, it seems unlikely that two molecules of the enzyme could be accommodated simultaneously in the same DNA groove of a 12mer. More likely, enzyme monomers bind to the phosphate backbone of each DNA strand thus explaining the binding stoichiometry and the ability of the enzyme to bind ssDNA with 1:1 stoichiometry.

The fact that the E9 DNase prefers to bind (and hydrolyse) DNA above a certain length has two important consequences for our understanding of colicin action. First, it implies that the free energy of binding is derived largely from phosphate recognition and that this may play a role in the hydrolysis of DNA, observations consistent with the natural substrate for colicin DNases being the bacterial chromosome. Second, this has ramifications for the way in which DNase colicins are inhibited in bacteria. Suicide is prevented in colicin-producing cells through the action of an immunity protein which binds with very high affinity (the K_d for Im9 binding the E9 DNase is 10^{-16} M at pH 7 and 25 °C[33]) to an Immunity Protein Exosite (IPE) that lies adjacent to the H-N-H motif, with the immunity protein making no direct interactions with catalytic residues.[10,34,35] The immunity protein is thought to inhibit the enzyme primarily through steric hindrance and electrostatic repulsion of substrate binding and this is supported by modelling studies,[10] and by the absence of conformational changes in the active site on immunity binding as reported by NMR spectroscopy.[36] Hence, it would be a disadvantage to a colicin-producing organism for the enzyme to retain endonuclease activity against small, single-stranded oligonucleotide substrates since the immunity protein would not inhibit their binding. The results presented here show that the E9 DNase shows no activity against dinucleotides and binds ssDNA less than ten bases poorly, observations consistent with the exosite inhibitory mechanism of immunity proteins.

Since there are no structures of the E9 DNase bound to nucleic acid, an important question remains as to whether the H-N-H motif represents the site of DNA binding. Three lines of evidence strongly suggest the motif as the site of DNA binding in the E9 DNase: (1) The motif forms the major part of a positively charged cleft in the protein where phosphate readily binds, and acidic mutations within this cleft abolish DNA binding to E9 (T. Georgiou and C.K., unpublished results); (2) DNA is known to bind to the equivalent site in I-*Ppo*I;[37] (3) The intrinsic tryptophan fluorescence of the E9 DNase is sensitive to ligand binding, with transition metal binding to the H-N-H motif quenching the fluorescence but immunity protein binding to the IPE yielding a fluorescent enhancement.[16,38] ssDNA and dsDNA both quench the intrinsic tryptophan fluorescence on binding to the E9 DNase which is consistent with the H-N-H motif being the site of binding (A.H.K. & C.K., unpublished results). It should also be noted that DNA binding to the H-N-H motif is metal independent, as is that to I-*Ppo*I,[20] since the E9 DNase H127A variant used in these studies does bind transition metal ions.[16] This is in contrast to the reports of Drouin *et al*.[17] on the H-N-H homing endonuclease I-*Cmoe*I where EDTA treated protein failed to gel-shift target DNA. However, caution has to be taken when treating H-N-H enzymes with EDTA since NMR experiments in our laboratory on the E9 DNase have shown that it readily binds EDTA[39] and that this is sufficient to inhibit DNA binding (data not shown).

Structures of the H-N-H motif in different liganded states identify a catalytic tetrad

Three structures of colicin DNases are currently available, two for the E9 DNase (at 2.05 and 1.7 Å resolution), shown in Figure 9, and one for the E7 DNase at 2.4 Å resolution, each bound to their immunity protein, Im9 and Im7, respectively.[9,10,40] The active sites of all three complexes, which are near identical in terms of their amino acid sequence, differ in terms of their metal ligation, with the E9 structures showing the apo- and Ni^{2+}-liganded forms and the E7 structure that of the Zn^{2+}-bound form. Superposition of the two transition metal complexes (not shown) reveals that the structures are very similar, with the metal ions in both adopting distorted tetrahedral geometries. However, there are subtle differences between the two complexes that are revealing: (1) The Ni^{2+} ion has only two clearly identifiable protein ligands (His102 and His127) with the third (His131) positioned poorly to bind the metal (Figure 9(b)), although solution NMR experiments identify three histidine ligands to the Ni^{2+} ion.[39] The E7 DNase crystal structure, on the other hand, shows clearly

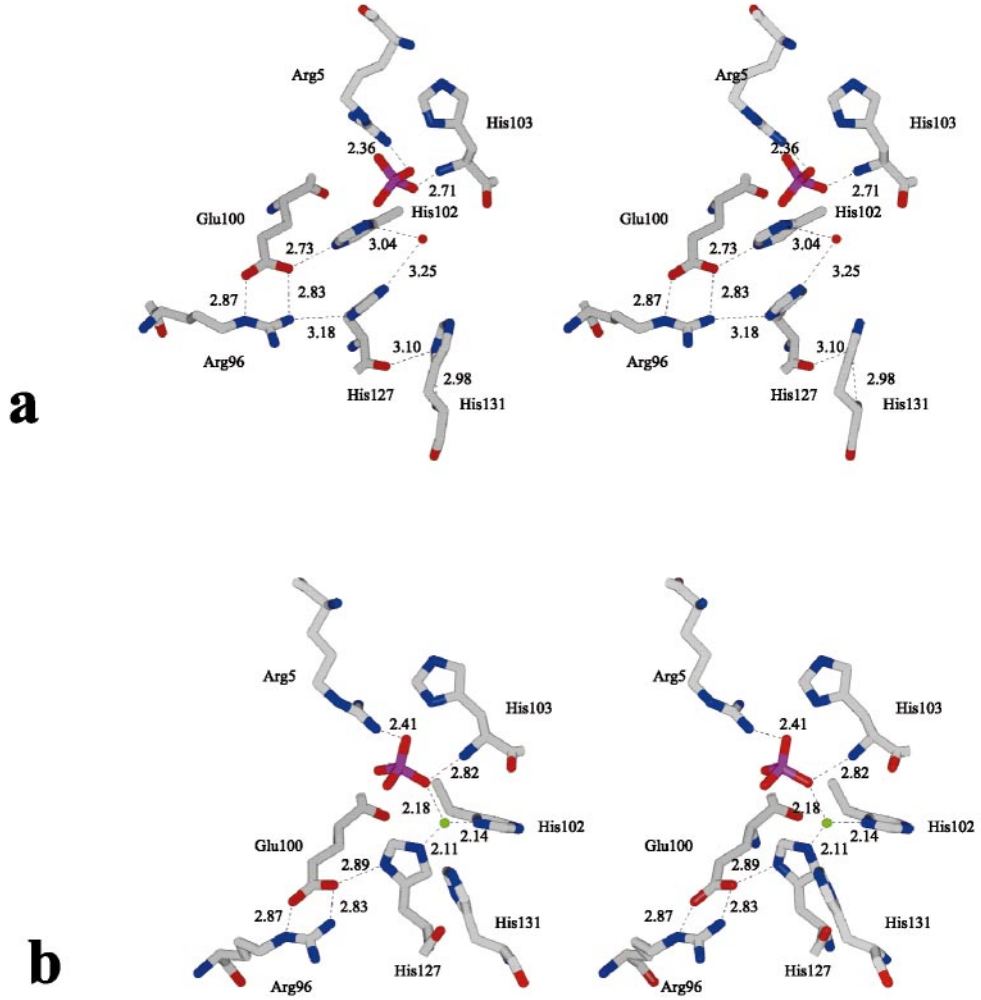


Figure 9. Stereo representations of the active site hydrogen bonding network within the H-N-H motif of the E9 DNase with and without bound Ni^{2+}. In each case the structure of the E9 DNase was solved in complex with the immunity protein Im9, which does not bind in the active site. (a) Apo-E9 DNase (at 1.7 Å resolution; accession code, 1emv) from Kühlmann *et al.*[40] The red sphere is a bound water molecule. (b) Ni^{2+}-bound structure (at 2.05 Å resolution; accession code, 1bxi) from Kleanthous *et al.*[10] The green sphere is the nickel ion. A bound phosphate molecule (red and purple) is present in both structures.

that the zinc ion is coordinated by all three histidine residues. These differences in coordination chemistry are reflected in the affinities of the metals for the E9 DNase since the K_d for Zn^{2+} is nM while that for Ni^{2+} (and Co^{2+}) is μM;[16] (2) Ni^{2+}-liganded E9 DNase has a bound phosphate molecule that occupies one of the coordination sites to the metal ion but this phosphate is replaced by water in the Zn^{2+}-loaded enzyme; (3) a consequence of these differences is that the Ni^{2+}-bound E9 DNase has one free coordination site (taken by His131 in Zn^{2+}-bound E7 DNase) and this may explain why nickel (and cobalt) is active as a metal cofactor while zinc, a catalytic metal ion in other nucleases,[41] does not support DNase activity in the H-N-H motif of colicins (see below).

The presence of phosphate in the active site of the E9 DNase likely denotes the position either of the scissile bond or, more likely, the product 5′ phosphate generated by the enzyme, and this phosphate molecule is bound even in the absence of metal ions (Figure 9(a)). The hydrogen bonding pattern in the active site of the apo-E9 DNase is substantially different, although the overall structure of the active site changes little.[40] Particularly striking are changes to the imidazole groups of His102, which rotates by 180°, and His127, both of which form new hydrogen bond interactions. His102 hydrogen bonds to Glu100 while His127 (previously hydrogen bonded to Glu100) interacts with Arg96 which in turn forms a salt bridge with Glu100, and so together comprise a catalytic tetrad. Three of these residues are known to be essential for catalysis[25] and so the entire tetrad is likely to be central to the catalytic mechanism of colicin DNases.

The H-N-H motif of the E9 DNase is an adaptable catalytic centre

In the following sections we attempt to reconcile the different activities of the E9 DNase with the structural information available, and suggest that the H-N-H motif adopts different catalytic strategies depending on the metal ion regime. We postulate that the apo-form of the E9 DNase represents that which undergoes Mg^{2+}-dependent hydrolysis of dsDNA, most likely by a mechanism similar to that described for *Serratia* nuclease and I-*Ppo*I.[14,15,42] This mechanism is unusual for Mg^{2+}-dependent endonucleases in that a histidine residue, rather than a magnesium ion, activates the hydrolytic water molecule through general base catalysis (Figure 10(a)). This residue is His103 in the E9 DNase, which superimposes closely with the equivalent histidine residues in *Serratia* (His89) and I-*Ppo*I (His98).[11] Stabilisation of the pentacoordinate transition state in these enzymes is accomplished by a magnesium ion liganded to the protein through the oxygen atom of a single asparagine residue (Asn119 in both *Serratia* and I-*Ppo*I). The equivalent position in colicin DNases is His127 (Figure 9). Considering that histidine co-ordination of magnesium has never to our knowledge been observed in an enzyme, this raises doubts as to whether His127 could fulfil such a role. However, we note that there is precedent for His coordination of a calcium ion in squid diisopropylfluorophosphatase[43] and that Ca^{2+} is as effective a cofactor in E9 DNase dsDNA hydrolysis as Mg^{2+} and likely to bind in the same site. Indeed, the equivalent position is occupied by a calcium ion in T4 endonuclease VII, albeit bound by an asparagine residue as in *Serratia* and I-*Ppo*I.[12] The case for His127 as the Mg^{2+}/Ca^{2+} ligand is made stronger by the fact that there are few potential oxygen ligands for magnesium within the E9 DNase H-N-H motif, especially since the asparagine identified in the motif serves a structural role.[10] Lastly, Arg5 contacts the bound phosphate in the E9 DNase and is equivalent to Arg61 in I-*Ppo*I (and Arg57 of *Serratia*) where its role is in stabilising the product 5′ phosphate.

One of the striking differences between the active sites of I-*Ppo*I and *Serratia* and that of the E9 DNase is the preponderance for histidine residues in E9, four compared to one, with the two that denote the H-N-H motif (His103 and His127, described above) highly conserved and the remaining two (His 102 and His131) conserved in most cases.[5] We now speculate on the potential roles of these latter residues. The role of His131 is the most enigmatic but may be involved in binding to the backbone of substrate DNA. The hydrogen bond of His102 to Glu100 in the apo-form of the enzyme is likely to raise its pK_a which, considering the pH optimum for the enzyme is pH 8-9, argues for a role as a general acid, protonating the 3′-oxygen leaving group (Figure 10(a)). This would be in contrast to I-*Ppo*I, where a magnesium-bound water molecule protonates the leaving group,[14] but is consistent with T4 endonuclease VII where a histidine residue is postulated to take this role.[12] Invoking the participation of a general acid and a general base both of which are histidine residues, and strategically placed above and below the bound phosphate in the E9 DNase apo-structure (Figure 9(a)), may provide an explanation for the metal-independent RNase activity of the E9 DNase. However, it remains to be established whether the hydrolytic products of RNA cleavage by the E9 DNase are the same as those of DNA hydrolysis or indeed if a cyclic phosphate intermediate is formed, a characteristic feature of many RNases.

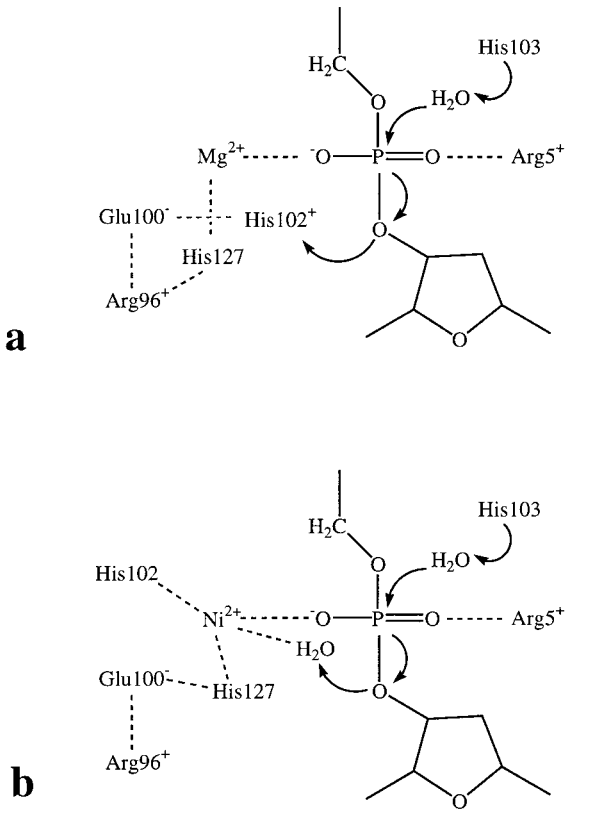


Figure 10. Putative mechanisms for the hydrolysis of DNA catalysed by the H-N-H motif in the presence of, (a) Mg^{2+} and (b) Ni^{2+} based on the structures of the E9 DNase in the apo and Ni^{2+}-liganded forms.[10,40] In (a) the catalytic tetrad at the centre of the motif serves to depress the pK_a of His127, which is postulated to act as a ligand for Mg^{2+}, and raise the pK_a of His102, predicted to be a general acid involved in protonating the 3′ oxygen leaving group. In (b) the binding of Ni^{2+} causes the reorganisation of the tetrad into a triad where now His127 is hydrogen bonded to Glu100 and His102 coordinates the nickel ion. It is proposed that a metal-activated water molecule bound to the metal ion takes on the role of protonating the 3′ oxygen, the fourth coordination site used to activate the scissile phosphodiester bond.

The binding of Ni^{2+} to the E9 DNase H-N-H motif results in the catalytic tetrad of the apo-enzyme converting to a triad. His102 moves >6 Å to be co-opted as a ligand for the transition metal along with His127 which moves 3 Å (Figures 9(b) and 10(b)). Assuming that the activation of the hydrolytic water remains the role of His103 (its position in the Ni^{2+}-bound and apo- structures does not change), then the problem arises as to how the 3′ oxygen becomes protonated since His102 (the putative general acid) is no longer available. We postulate that a Ni^{2+}-coordinated water molecule fulfils this role, taking one of four coordination sites to the metal ion, the remaining site used to bind and activate the scissile phosphodiester bond (Figure 10(b)). This mechanism would explain why zinc does not support catalysis in colicin DNases since the bound transition metal would need to retain two free metal coordination sites for catalysis to proceed whereas only one is available in the tightly bound zinc complex.[9] Another consequence of which metal ion is used by colicin DNases is substrate preference since magniesum ions are optimal for double-stranded substrates while transition metals such Ni^{2+} and Co^{2+} are optimal for ssDNA, although the basis for these preferences remains obscure at present.

In summary, the mechanism of DNA hydrolysis by colicin DNases in the presence of transition metals is almost certainly different to that in the presence of Mg^{2+} because of the side-chain movements that accompany transition metal ion binding. We postulate that the main difference between these mechanisms is how the 3′ oxygen is protonated, by a histidine residue in Mg^{2+}-dependent hydrolysis but a metal-activated water molecule when transition metals such as Ni^{2+} and Co^{2+} are bound to the enzyme; the absence of two free coordination sites in the Zn^{2+}-bound complex likely explaining why it does not support the hydrolysis of nucleic acid. The structural rearrangements observed in colicin active sites begin to explain how a single protein scaffold can recruit either a magnesium or transition metal ion for the hydrolysis of phosphodiester bonds and emphasize that the histidine-rich H-N-H motif of homing enzymes and colicins is a remarkably adaptable catalytic centre.

Materials and Methods

Bacterial strains and media

Plasmid pRJ353 (encoding the E9 DNase domain and Im9 with a C-terminal histidine tag) was transformed into *E. coli* BL21 (DE3) pLysS and cells grown on Luria-Bertani broth, as described.[25]

Protein purification and protein and DNA quantitation

The E9 DNase was purified as described by Garinot-Schneider *et al.*[25] and adapted by Pommer *et al.*[16] Since Ni^{2+}-affinity chromatography is used in the purification of the E9 DNase and Ni^{2+} binds to the enzyme, all protein preparations were treated with 10 mM EDTA in Tris-HCl buffer (pH 7.5) to remove bound metal ions and then dialysed against the same buffer but containing 200 mM NaCl. Protein was finally lyophilized from water and stored at −20 °C. The absence of transition metal contamination in all E9 DNase preparations was verified as described[16] using ANS fluorescence. Protein was quantitated by absorbance spectrophotometry at 280 nm as described,[7] while DNA concentrations were quantitated by absorbance at 260 nm.

Kunitz and ^{3}H-plasmid nicking assays of E9 DNase activity

Colicin E9 endonuclease activity was routinely measured using the Kunitz assay,[44] where the change in hyperchromicity of calf thymus DNA was measured spectrophotometrically at 260 nm, or a plasmid nicking assay where the rate of nicking was quantitated by separating the cleavage products of supercoiled [^{3}H]pUC18 by agarose gel-electrophoresis and determining the amount of supercoil remaining by scintillation counting.[8,45] The E9 DNase shows non-linear kinetics in the Kunitz assay when hydrolysing calf thymus DNA in the presence of Mg^{2+} or Ca^{2+},[9] and so the hyperchromicity data were analysed as the change in absorbance at 260 nm after a ten minute incubation. Where indicated the pH, salt and metal ions in these assays was altered to determine the effect on E9 DNase activity.

Identification of the colicin E9 DNase cleavage sites

Radiolabelled *tyrT* promoter DNA was used to determine the positions of cleavage by the E9 DNase.[21] *tyrT* was PCR amplified using primers with *Eco*RI and *Hin*dIII adapters and cloned using these sites in both M13mp18 and M13mp19, which makes it possible to identify positions of cleavage in both strands of the DNA.[46] Once the phage single-strand was obtained, double-strand was made using 1 pmol of M13 universal primer which was 5′-end labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP. After annealing to the template, the primer was extended by treatment with Klenow enzyme and all four dNTPs. The double strand DNA was then incubated with the E9 DNase in 5 mM Tris-HCl (pH 8.0) in the presence of 0.5 mM $MgCl_2$ and 0.5 mM $MnCl_2$ at 37 °C for one to 30 minutes and the products of digestion run in parallel with four sequencing lanes obtained using the same primer and according to the manufacturers method (Amersham-Pharmacia). Reactions were stopped with stop solution (95 % (v/v) formamide, 20 mM EDTA, 0.05 % (w/v) bromophenol blue and 0.05 % (w/v) xylene cylanol FF) and results visualised with autoradiography for 24-36 hours at −70 °C with intensifying screens and bands scanned using a phosphoroimager (Molecular Dynamics).

Analysis of the terminal nucleotide of endonuclease-generated fragments

λ-DNA (2.5 μg) was incubated for 20 minutes in 10 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$ in a final volume of 20 μl with either the E9 DNase (40 μg) or *Eco*RI/*Hin*dIII at 37 °C. The reactions were stopped by phenol-extraction (phenol/chloroform/isoamyl alcohol, 25:24:1 (v/v)). After splitting each digest into two, 1 μg was incubated with one unit of calf intestinal alkaline

phosphatase (CIAP) (Boehringer Mannheim) with the manufacturers recommended buffer for one hour at 37 °C. Three phenol extractions (phenol/chloroform/isoamyl alcohol (25:24:1 (v/v)) were carried out in order to stop CIAP activity. The dephosphorylated DNA was then precipitated with 0.1 volume of sodium acetate (3 M) and three volumes of ethanol and washed with 70 % (v/v) ethanol. Both CIAP treated and control fragments were labelled at the 5′-termini using T4 polynucleotide kinase and 1 μl [γ-^{32}P]ATP for 40 minutes at 37 °C. After two ethanol precipitations (0.1 volumes sodium acetate (3 M) and three volumes ethanol) the precipitates were washed twice with 70 % ethanol. The product was resuspended in 20 μl of TE-buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and samples separated on a 1.2 % (w/v) agarose gel and analysed by autoradiography, carried out for three hours at −70 °C with an intensifying screen.

Digestion of T(npp)$_2$ and TpA

The hydrolysis of T(npp)$_2$ was carried out as described by Liao[24] in a volume of 0.5 ml containing 25 mM Tris-HCl (pH 8), 1 mM $CaCl_2$, 10 mM $MnCl_2$ and T(npp)$_2$ at a concentration of 15 mM. The reaction was initiated by the addition of E9 DNase or DNase I. The rate of cleavage was determined from the increase in absorbance at 400 nm typical for the cleavage product, *p*-nitrophenol in its deprotonated form. Assays were performed at 25 °C over ten minutes. The hydrolysis of TpA ($\varepsilon_{260} = 2.34 \times 10^4\ M^{-1}\ cm^{-1}$) was carried out in a volume of 25 μl containing 5 mM TpA, 10 μg of E9 DNase domain, 25 mM Tris-HCl (pH 7) and 10 mM $MgCl_2$ at 37 °C. The progress of the digestion was followed by HPLC, using a C18 reverse phase column at 55 °C, where 2 μl of the digest was loaded onto the column. Elution was achieved with a gradient formed from buffer A (0.1 M triethylammonium acetate (pH 6.5), 3 % (v/v) CH_3CN) and up to 80 % buffer B (as buffer A but with 65 % CH_3CN) in 40 minutes with a flow rate of 1 ml/min. Elution was monitored by measuring the absorbance at 254 nm.

Hydrolysis of a 16 bp palindromic oligonucleotide

A 16 bp palindromic sequence (GAA TTC GAT CGA ATT C; MWG Biotech) was used to investigate the digest limit of E9 DNase with dsDNA. The 20 hour digest was carried out at 37 °C in 30 μl of 10 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$ and DNA. E9 DNase (10 μg) was added to start the digestion and 2 μl of the reaction mixture was analysed using an anion exchange column, PA-100 column (Dionex), coupled to a Gilson HPLC apparatus at 25 °C. The fragments were eluted using a gradient formed from buffer A (10 % CH_3CN) and buffer B (10 % CH_3CN + 1.5 M ammonium acetate (pH 7.5)); up to 80 % B in 40 minutes, at a flow rate of 1 ml/min. Elution was monitored by measuring the absorbance at 254 nm and the profile compared to the retention time of the four 5′ phosphate mononucleotides on the same column under identical conditions.

ANS fluorescence spectroscopy

Fluorescence experiments using the dye ANS were performed with an excitation wavelength of 365 nm and monitoring the emission in the range, 390-650 nm. Quartz cuvettes (3 ml) were used and the reactions contained 1.5 ml TEA buffer (pH 7.5), 13.2 μM E9 DNase H127A and 40 μM ANS. For all experiments the DNA (supplied by MWG Biotech) was made up in AnalaR water and added in 1-2 μl aliquots. A 12mer duplex of defined sequence was used (CCA GGT AGC CAG) as well as single-stranded sequences which were randomised for each base at every position during synthesis (two to 14 bases in length).

Isothermal titration calorimetry

Isothermal titration calorimetry was carried out essentially as described by Pommer *et al*.[16]

Digestion of ssDNA and ssRNA

^{32}P-labelled 23-mer (GTT TTC CCA GTC ACG ACG TTG TA; MWG Biotech) was labelled with T4 kinase and [γ-^{32}P]ATP and used as a substrate. For the detection of RNA cleavage, 5′-carboxyfluorescein labelled 10mer RNA (GAC GUA AGA G; Cruachem Ltd) was used as a substrate. In both cases, digests were carried out in 50 mM triethanolamine buffer (pH 7.5), in 10 μl and at 37 °C for ssDNA, and in 30 μl and at 30 °C for ssRNA containing E9 DNase (2 and 4.5 μg, respectively) under a variety of metal regimes, and resolved by acrylamide gel-electrophoresis and visualised using a phosphorimager.

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