Enzymological characterization of the nuclease domain from the bacterial toxin colicin E9 from *Escherichia coli*

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The cytotoxicity of the bacterial toxin colicin E9 is due to a non-specific DNase that penetrates the cytoplasm of the infected organism and causes cell death. We report the first enzymological characterization of the overexpressed and purified 15 kDa DNase domain (E9 DNase) from this class of toxin. CD spectroscopy shows the E9 DNase to be structured in solution, and analytical ultracentrifugation data indicate that the enzyme is a monomer. The nuclease activity of the E9 DNase was compared with the well-studied, non-specific DNase I by using a spectrophotometric assay with calf thymus DNA as the substrate. Both enzymes require divalent metal ions for activity but, unlike DNase I, the E9 DNase is not activated by Ca^{2+} ions. Somewhat surprisingly,

the E9 DNase shows optimal activity and linear kinetics in the presence of transition metals such as Ni^{2+} and Co^{2+} but displays non-linear kinetics with metals such as Mg^{2+} and Ca^{2+} . Conversely, Ni^{2+} and other transition metals showed poor activity in a plasmid-based nicking assay, yielding significant amounts of linearized plasmid, whereas Mg^{2+} was very active, with the main intermediate being open-circle DNA. The results suggest that, on entry into bacterial cells, the E9 DNase is likely to exhibit primarily Mg^{2+} -dependent nicking activity against chromosomal DNA, although other metals could also be utilized to introduce both single- and double-strand cleavages.

INTRODUCTION

Colicins are a large and varied family of plasmid-encoded bacterial toxins that are produced in response to DNA-damaging agents such as UV and mitomycin C [1]. They kill related strains of bacteria by first binding to specific outer-membrane receptors and then translocating into the cell to deliver a toxic activity, which generally takes the form of ionophores [2–4], RNases [5,6] or DNases [7,8]. Colicins are classified by the cell surface receptor that they use to initiate cell death. Our work has centred on the E group colicins [9,10], which utilize the BtuB receptor in concert with the porin OmpF to penetrate cells [11,12]. BtuB is normally required by the bacterium for the import of vitamin B₁₂. After binding to the BtuB receptor the colicin toxin is then able, by an unknown mechanism, to translocate into the periplasm of the bacterium, a process that in the E group colicins requires several periplasmic Tol proteins (A, B, Q and R) [13]. Tol-mediated toxicity is common to all A group colicins (of which the E colicins are a subgroup) and is also used by filamentous bacteriophage to infect cells, whereas group B colicins (such as colicin Ia and colicin B) utilize the Ton system comprising TonB, ExbB and ExbD [9,14].

Although colicins can use a number of different receptors and two different routes to penetrate the periplasm, as well as displaying a range of different cytotoxic activities, they tend to share a similar domain architecture [15]. This consists of domains responsible for receptor binding, translocation and cytotoxicity, all of which are encoded in a single polypeptide chain, usually 50–70 kDa in size. Although the mechanism by which colicins can translocate into cells remains unresolved, a recent important breakthrough came with the description of the crystal structure for the entire colicin Ia molecule, a group B pore-forming toxin that uses the FepA protein as its receptor [16]. Structures for

isolated pore-forming domains had previously been obtained (see, for example, [17]) but these did not show the other domains in the toxin. The colicin Ia structure showed that the domains are separated by very long α -helices that could conceivably span the entire length of the periplasm; this suggested that the toxin could remain attached to its receptor while it delivers the toxin domain to the inner membrane, in agreement with previous models based on the proteolytic susceptibility of receptor-bound toxins [18].

The E group nuclease colicins all share the same domain structure. A central region houses the receptor-binding domain; the N-terminus of the protein is required for translocation into cells [15]. The extreme C-terminus encodes the DNase domain, which can be removed proteolytically from the rest of the toxin [15,19].

Colicin-producing organisms protect themselves against the action of the toxin by co-synthesizing a small inhibitor protein, known as an immunity protein [8,20,21]. It is known that in the enzymic colicins these 9.5 kDa immunity proteins form a stoichiometric complex with the nuclease domains, thus neutralizing their activity. These toxins are released into the extracellular medium as heterodimeric complexes and it is as complexes that they begin their entry into a susceptible cell. Work from our laboratory [22] has shown that the affinity of the DNase toxin colicin E9 for its cognate immunity protein (Im9) is 0.1 fM, which is one of the tightest protein–protein interactions yet reported. With such a high affinity it is unclear how (or indeed where) the two proteins separate to allow the toxin to penetrate a susceptible cell and digest its DNA.

The DNase domain of colicin E9 (E9 DNase)–immunity protein system has proved to be a valuable model with which to investigate specificity in protein–protein interactions [10]. While cognate colicin–immunity protein interactions are tight, non-cognate associations can also occur but with much weaker

Abbreviations used: Bistris-propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; E9 DNase, DNase domain of colicin E9; Im9, immunity protein specific for colicin E9.

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affinities. Hence the E9 DNase will bind all the DNase-specific immunity proteins but with affinities that span the affinity range from millimolar to femtomolar [23]. The specificity differences of the immunity proteins for DNase colicins are governed by a single helix (helix II) in these four-helix proteins [24,25]. Interestingly, however, this is not the only region that contacts the DNase. The results of heteronuclear NMR experiments [26] and alanine scanning mutagenesis on Im9 [27] indicate that the main DNase-binding interactions come from approximately ten amino acid residues: five are variable in sequence in the immunity protein family (helix II) and five are conserved (helix III); it is the conserved sites that dominate the DNase binding energy. We have given the name 'dual recognition' to this form of recognition, where conserved sequences act as the anchor of a protein-protein interaction and variable residues modulate these interactions, to emphasize the dual nature of the binding site [10,27].

In contrast with the wealth of information that is available for the mode of action of DNase immunity proteins, relatively little is known about the DNases themselves. No crystal structures are yet available for a DNase colicin, nor even for an isolated DNase domain that can be expressed and purified separately from the rest of the toxin [19]. Information about colicin DNase active-site residues and amino acids involved in immunity specificity have therefore relied heavily on mutagenesis experiments. Curtis and James [28] used sequence alignments and site-directed mutagenesis to identify several residues that have a role in determining immunity specificity, whereas Garinot-Schneider et al. [29] used random mutagenesis to identify active-site groups in the colicin E9 DNase domain. Three invariant amino acids (Arg-544, Glu-548 and His-575) were identified that, when mutated to alanine, neutralized colicin toxicity and inactivated enzymic activity.

The hydrolysis of phosphodiester bonds in nucleic acids by nucleases often requires a divalent metal ion as a cofactor to stabilize the pentaco-ordinate phosphate transition state or activate the nucleophilic water molecule [30]. It is known that the DNases of the E group of colicins require divalent metals [7] but there is no information on which metals can be used and their relative activities, contrary to what is known for bovine pancreatic DNase I, one of the most widely studied of all the metal-dependent nucleases [31]. Interestingly, DNase I participates in protein-protein interactions, as does colicin E9. It forms stoichiometric complexes with monomeric actin, which inhibits its activity [32,33]. The dissociation constant for this complex is approx. 1 nM [34], eight orders of magnitude weaker than that observed for the E9 DNase-Im9 complex.

The activity of DNase I is measured routinely with the spectrophotometric Kunitz assay [35]. This assay involves the use of highly polymerized calf thymus DNA as a substrate of undefined molecular mass. DNA-cleaving activity is measured by an increase in absorbance at 260 nm resulting from a decrease in the hyperchromicity of DNA bases as a consequence of denaturation and depolymerization of the DNA. It is thought that extensive degradation of DNA is required to obtain a measurable signal in this assay, although the number of cleaved phosphate bonds per unit change in absorbance is not known [36]. The DNase from colicin E9 is known to generate a signal by this assay [29] but there has been no kinetic or mechanistic analysis, and in particular its dependence on DNA and metal ion concentration have not been investigated.

Here we report the first enzymological characterization of the expressed and purified E9 DNase domain. We have analysed its secondary structure composition, its oligomeric structure and the metal dependence of the endonuclease activity. In particular, we

compare the activity of the E9 DNase to the well-studied, non-specific endonuclease DNase I.

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 *Escherichia coli* BL21 (DE3) and cells were grown on Luria-Bertani broth, as described by Garinot-Schneider et al. [29].

Protein purification and protein determinations

The E9 DNase was purified as described by Garinot-Schneider et al. [29] and quantified by absorbance spectrophotometry as described by Wallis et al. [22].

CD spectroscopy

CD spectroscopy was performed at 20 °C with a JASCO spectropolarimeter. For the determination of secondary structure composition, the protein concentration was 0.98 mg/ml in water; a quartz cuvette with 0.1 mm path length was used. To investigate dilution effects, quartz cuvettes with differing path lengths were used, ranging from 0.1 to 5 mm. Spectra were recorded from 180 to 260 nm and curves were fitted with the Selcon procedure, from which the relative amounts of helix, sheet and coil were quantified [37].

Analytical ultracentrifugation

Experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics and an An60Ti rotor. Samples (110 µl) were placed in the sample cells of an Epon charcoal-filled six-channel centrepiece. The reference cells were loaded with 125 µl aliquots of the buffer. Experiments were performed at 20000 rev./min at 20 °C. The partial specific volume of E9 DNase (0.729 ml/g) was calculated from the amino acid composition by the method of Cohn and Edsall [38].

Equilibrium data were collected at 298 nm in step-scan mode with a separation of 0.001 cm. Five readings were averaged for each scan and a baseline scan was taken at 360 nm to correct for optical imperfections. Readings were taken at 4 h intervals until no difference could be detected between consecutive scans. The equilibrium distributions from three different loading concentrations (0.8, 1.7 and 3.3 mg/ml) were analysed simultaneously with the Nonlin curve-fitting algorithm [39]. Experiments were conducted with a two-buffer system: 50 mM Mops, pH 7.0, containing 1 mM dithiothreitol, and 50 mM phosphate, pH 7.0, containing 1 mM dithiothreitol in the absence and presence of 2 mM EDTA.

Kunitz assay

Calf thymus DNA (50 µg/ml) was used as substrate in a final volume of 1 ml in a quartz cuvette equilibrated at 25 °C. Depending on the experiment, one of two buffer systems was used: Hepes (10–50 mM) or 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis-tris-propane) (50 mM) at pH 7.5–8.5. Divalent metals (Mg^{2+} , Ca^{2+} , Mn^{2+} , Co^{2+} , Zn^{2+} or Ni^{2+}) were included at concentrations ranging from 0 to 40 mM. Enzymic reactions were initiated by the addition of E9 DNase, usually 0.1–10 µg per assay, and the hydrolysis of DNA was monitored spectrophotometrically at 260 nm with a Phillips PU 8730 spectrophotometer. Data were downloaded and processed with Microsoft Excel.

³H-plasmid-nicking assay

The preparation of tritiated plasmid (pUC18) was as described previously [23]. Assays were performed in either 50 mM Bistris-propane buffer, pH 8.5, or Tris/HCl buffer, pH 7.5. A typical assay contained BSA (0.3 mg/ml), divalent metals (0–30 mM) and 1.5 µg of DNA (specific radioactivity 9 nCi/µg) in a final volume of 30 µl. Reactions were started by the addition of E9 DNase (0.5–10 ng) and incubated at 37 °C for up to 25 min. Aliquots (4.6 µl) were removed and added to stop mix (containing EDTA) before electrophoresis in a 1.2% (w/v) agarose gel. Bands were excised and incubated with 0.5 ml of 5 M sodium perchlorate at 67 °C for at least 2 h, after which 10 ml of scintillation fluid was added and the level of radioactivity was measured by scintillation counting. The loss of supercoiled DNA was recorded as a function of time from that in the original preparation of substrate DNA (80–90%). First-order rates of supercoil nicking were estimated as described previously [23].

RESULTS AND DISCUSSION

Structure of the E9 DNase

The purified 15 kDa E9 DNase domain was analysed by far-UV CD spectroscopy at various protein concentrations from 0.02 to 1.0 mg/ml (Figure 1a). All the spectra could be superimposed,

Figure 1 Isolated 15 kDa DNase domain from colicin E9 is structured and monomeric in solution

(a) Far-UV CD spectra of the E9 DNase at a variety of protein concentrations (0.02–1.00 mg/ml) recorded in water at 20 °C. Quartz cuvettes of increasing path length, ranging from 0.1 to 5 mm, were used for each new concentration. (b) Equilibrium analytical ultracentrifugation analysis of the E9 DNase (see the Materials and methods section for details) at pH 7.0 and 20 °C, showing that the protein is a monomer in solution. The equilibrium distributions from three different loading concentrations (△, 0.8 mg/ml; □, 1.7 mg/ml; ○, 3.3 mg/ml) were analysed. The data are plotted as apparent weight-average molecular mass distribution against concentration.

indicating that there was no change in the secondary structure of the protein, for example through self-association, as a function of concentration. The secondary-structure composition of the E9 DNase was estimated as 30% α -helix, 20% β -sheet and 50% coil.

When the immunity protein from the RNase toxin colicin E3 is removed, the toxin dimerizes [40]. It was important to ascertain whether a similar self-association could occur when the E9 DNase domain was separated from its endogenous immunity protein; purified E9 DNase was therefore analysed by equilibrium ultracentrifugation (Figure 1b). The equilibrium distributions from different loading concentrations were analysed simultaneously with the Nonlin curve-fitting algorithm [39]. The native molecular mass was determined as 15600 ± 800 Da, which shows reasonable agreement with the monomer molecular mass for the E9 DNase of 15088 Da. Data were also analysed by calculation of the apparent weight-average molecular mass at concentrations throughout the sample cells and, as shown in Figure 1(b), no self-association was observed at protein concentrations up to 4 mg/ml.

Our previous work on immunity binding has shown that Im9 binds stoichiometrically and with equal affinity to both colicin E9 and the isolated E9 DNase [19,22], showing that truncating the colicin protein at residue 450 and expressing the C-terminal 134 residues did not affect immunity binding [29]. One complication arising from these studies is that the association kinetics of both colicin E9 and the E9 DNase domain binding Im9 are biphasic in stopped-flow experiments, the physical basis of which could be due to changes in the aggregation state of the DNase [22]. The present results indicate that the isolated 15 kDa E9 DNase domain, in the absence of immunity protein, shows no evidence of self-association, although this does not discount the possibility that its aggregation state could change in the presence of substrate DNA. This seems unlikely, however, given that the translocation of a single molecule of colicin endonuclease into a bacterial cell is thought to be sufficient to bring about cell death [1].

Comparison of the endonuclease activities of the E9 DNase and DNase I

Preliminary experiments indicated that the optimal pH for the E9 DNase in the Kunitz assay was approx. pH 8.0 (results not shown), whereas the optimal condition for DNase I has previously been reported to be approx. pH 7 [41]. Comparing the two enzymes under optimal conditions of pH for each, it was clear that the E9 DNase does not show the same rate behaviour in the presence of Mg^{2+} as does DNase I because the increase in absorbance at 260 nm was non-linear throughout the time course (Figure 2). This precluded detailed kinetic experiments for this metal. Reasoning that accessibility of cleavage sites might be a problem with such a highly polymerized substrate, we sonicated the DNA before the addition of enzyme to produce smaller fragments but this did not result in linear kinetics. Neither did the addition of BSA, which is often used to stabilize proteins (results not shown). DNase I Kunitz assays frequently display a lag phase at the beginning of the assay (Figure 2) and this has been interpreted by Bernardi [42] to indicate that the initial products of DNA cleavage do not give rise to hyperchromism. It is interesting to note then that, although the E9 DNase in the presence of Mg^{2+} produces non-linear kinetics, it does not display such a lag phase, suggesting that many or all the products of cleavage produce a 'hyperchromicity effect'. Lastly, we determined the E9 DNase concentration dependence by using the absorbance change after 10 min as a relative value and at a fixed

Figure 2 E9 DNase displays non-linear kinetics in Kunitz assays in the presence of Mg^{2+} ions

The substrate (50 $\mu g/ml$ calf thymus DNA), temperature (25 $^{\circ}C$) and assay volume (1 ml) were the same for each assay. For the DNase I assay, 50 mM Mes buffer, pH 7.0, containing 4.2 mM $MgCl_2$, was used; the protein concentration was 0.14 $\mu g/ml$. For the E9 DNase, 50 mM Bistris-propane, pH 8.0, containing 20 mM $MgCl_2$, was used; the protein concentration was 10 $\mu g/ml$. Assays were initiated by the addition of enzyme and the absorbance change at 260 nm was recorded.

DNA concentration (50 $\mu g/ml$). This concentration dependence also displayed non-linearity (results not shown).

DNase I requires Ca^{2+} as an essential structural cofactor and the presence of a second divalent metal for catalytic activity [31], and it has been well documented that the Ca^{2+} -selective chelating agent EGTA inhibits DNase I completely [43]. Subsequent additions of excess Ca^{2+} reverse this inhibition. These effects have been reconstructed in Figure 3. Attempting the same experiment with the E9 DNase produces very different results: the enzyme shows the same activity with or without added Ca^{2+} . This experiment indicates that, unlike DNase I, the E9 DNase domain does not contain an essential Ca^{2+} -binding site. This experiment and that shown in Figure 2 also highlight another difference between the two enzymes, whether the assay conditions are identical or optimal for each enzyme, DNase I is more than 50-fold more active than the E9 DNase domain against calf thymus DNA.

Metal and DNA dependence of the E9 DNase using the Kunitz assay

The E9 DNase does not show any DNA cleavage activity in the absence of divalent metal ions but the dependence on the concentration of divalent metals has not previously been investigated [7,8]. By measuring the ΔA_{260} over a 10 min period and using 50 $\mu g/ml$ DNA in the Kunitz assay, we found that the concentration dependence for Mg^{2+} and Ca^{2+} ions is bell-shaped with a broad maximum at approx. 20–25 mM [44]. Therefore the activity of the enzyme is inhibited at high metal ion concentrations, which is also a characteristic of DNase I cleavage of DNA [43].

Nucleases such as DNase I can often use a number of other divalent cations, particularly transition metals, in place of Mg^{2+} [31,43]. Because the physiological metal cofactor of the E9 DNase (or indeed any colicin endonuclease) is unknown, we used the Kunitz assay to investigate the possibility that other metals could elicit activity from the enzyme (Figure 4a). We found that, as for DNase I, other metals can be substituted for Mg^{2+} in this assay but the resulting activity is different from that with Mg^{2+} .

Figure 3 E9 DNase is not activated by Ca^{2+} ions

The top panel shows the dependence of DNase I activity (0.1 $\mu g/ml$ protein) on Ca^{2+} ions; the bottom panel shows the Ca^{2+} -independent activity of the E9 DNase (10 $\mu g/ml$ protein). Symbols: ●, ◆, assays with 0.2 mM Ca^{2+} present throughout the assay; □, ○, assays in which 0.2 mM Ca^{2+} was added at the indicated time. In both sets of experiments, 50 $\mu g/ml$ calf thymus DNA was used in 10 mM Hepes buffer (pH 8.0) at 25 $^{\circ}C$ containing 5 mM $MgCl_2$ and 0.1 mM EGTA. DNase I activity (top panel) tails off when all the substrate DNA has been digested. Under these conditions, this situation is not reached with the E9 DNase (bottom panel).

Of the four transition metals that were studied, Mn^{2+} , Ni^{2+} , Co^{2+} and Zn^{2+} , only Zn^{2+} did not result in E9 DNase Kunitz activity. Moreover, the activities with Ni^{2+} and Co^{2+} were approximately an order of magnitude greater than that seen with Mg^{2+} (Figures 2 and 4a). Rather more surprising, however, was that, in contrast with the curved rates seen for the E9 DNase Kunitz assays in the presence of Mg^{2+} , the activities in the presence of the transition metals all resulted in linear rates. The relative order of these activities at the single metal ion concentration tested (1 mM) was $Ni^{2+} > Co^{2+} > Mn^{2+}$. This is different from the pattern of transition metal dependence for DNase I where (with the exception of Mn^{2+}) these metals are less active than Mg^{2+} in the Kunitz assay and their order of reactivity is reversed [43].

Because nickel resulted in the highest E9 DNase Kunitz activity of all the metals tested we analysed this activity further. It was possible to measure initial rates of DNA hydrolysis and so we could determine the E9 DNase concentration dependence, which was also linear (insert to Figure 4b). The dependence of the nuclease activity on both DNA and metal ion concentration under steady-state conditions was also analysed. Somewhat surprisingly, variations in the amount of DNA added to the Kunitz assays (10–50 μg DNA) did not result in any change in

Figure 4 E9 DNase shows higher Kunitz activity and linear kinetics in the presence of transition metals

(a) Kunitz assays in the presence of a variety of transition metals. Assays were conducted at 25 °C and contained 50 mM Bistris-propane, pH 7.5, and 1 mM metal; they were initiated with 0.5 $\mu\text{g/ml}$ E9 DNase. (b) The dependence of Kunitz activity on the concentration of nickel. Data were fitted by using the Michaelis–Menten equation within the ENZFITTER program. The insert shows the linear dependence on E9 DNase concentration of the Ni^{2+} -dependent nuclease activity under the same conditions.

the rate of DNA cleavage (results not shown), implying that the K_m for DNA under these conditions is markedly lower than can be achieved in the assay. This is not true of DNase I, because its DNA dependence can be analysed by the Kunitz assay (see, for example, [41]). The Ni^{2+} dependence of the E9 DNase Kunitz activity could, however, be measured and showed classic Michaelis–Menten behaviour; the K_m for Ni^{2+} was estimated as 0.93 mM (Figure 4b).

Metal dependence of E9 DNase investigated with a plasmid-nicking assay

It is well documented that the mechanism of DNA cleavage catalysed by DNase I is strongly dependent on which metal is used in the reaction [31]. Transition metals such as Mn^{2+} result in a higher proportion of double-strand breaks, whereas single-strand breaks predominate with main-group elements such as Mg^{2+} [43,45], which is thought to reflect distinct DNA-chelation properties of the different metals. Because we had observed different rate profiles in the Kunitz assay for the E9 DNase in the presence of Mg^{2+} and Ni^{2+} , we sought to investigate the pattern of DNA cleavages with different metals by using a substrate more easily analysed than the linear, highly polymerized DNA

Figure 5 Mg^{2+} ions are optimal for supercoiled plasmid-nicking activity of the E9 DNase

(a–c) Agarose gels (1.2%, w/v) of tritiated pUC18 DNA in the presence of E9 DNase over a 25 min time course: no metals added (a); in the presence of 10 mM Mg^{2+} and 15 ng/ml enzyme (b); in the presence of 1 mM Ni^{2+} and 75 ng/ml enzyme (c). Each panel also indicates the migration of supercoiled (S), open-circle (O) and linear (L) DNA. (d) The bands from these gels were excised and the level of radioactivity was quantified as described in the Materials and methods section. Only data for the loss of supercoil DNA are presented, shown as the percentage of the starting level of radioactive supercoiled plasmid. Symbols: $\square$, no metal; $\circ$, Mg^{2+} ; $\bullet$, Ni^{2+} .

from calf thymus. We therefore chose a plasmid-based nicking assay system because the reaction products (relaxed and linear DNA) can be excised from agarose gels and (by using tritiated supercoiled plasmid DNA) quantified. The same assay has been used to investigate the cleavage mechanism of DNase I [45] and we have previously used it to analyse the effects of non-cognate immunity protein binding on E9 DNase activity [23].

$[\text{H}]p\text{UC18}$ was incubated with the E9 DNase (see the Materials and methods section) (Figure 5). In the absence of divalent metal ions, no activity was observed, whereas in the presence of 10 mM Mg^{2+} the E9 DNase (0.5 ng; 1 nM) was very active, completely nicking the supercoiled plasmid within 20 min. Pseudo-first-order rate constants for supercoil nicking (see [23] for details) were obtained over a range of Mg^{2+} concentrations to show the metal dependence of the nicking activity. Although not as broad as the Kunitz data, this dependence was also bell-shaped and was maximal at a lower concentration of Mg^{2+} (approx. 10 mM [44]).

Reactions with Mg^{2+} produced very little linearized DNA under these assay conditions. Conversely, 1 mM Ni^{2+} under the same conditions showed little activity in this assay (results not shown). Indeed, the enzyme concentration had to be raised 5-

fold before appreciable nicking activity was observed, but even then much of the starting supercoiled DNA remained after 20 min (Figure 5c). Quantifying these activities by scintillation counting (Figure 5d) showed that the E9 DNase plasmid nicking activity is approx. 10–20 times greater in the presence of Mg^{2+} than Ni^{2+} , the opposite of that seen in the Kunitz assay. The patterns of E9 DNase plasmid nicking were also quite different for Ni^{2+} and for Mg^{2+} (Figure 5). In the presence of Mg^{2+} , the primary intermediate was the open-circle form of the plasmid and the linear form appeared in appreciable amounts only as this was digested further. In the presence of Ni^{2+} , however, the open-circle and linear forms appeared in equal proportions, indicating a higher proportion of double-strand breaks with this metal ion. Similar poor cleavage of supercoiled DNA was observed for other transition metals such as Co^{2+} (results not shown).

Conclusions

The 15 kDa E9 DNase is a monomeric, metal-dependent endonuclease that shows both similarities to and differences from the well-studied endonuclease DNase I. Unlike DNase I, the E9 DNase does not require Ca^{2+} ions for activation. Both enzymes can substitute transition metals for Mg^{2+} but the relative activities with these differ. Whereas DNase I yields linear kinetics with all metals in Kunitz assays, only transition metals produce linear kinetics for the E9 DNase and this activity is significantly higher than that seen with Mg^{2+} . This might be due to a higher proportion of double-strand breaks with transition metals; this was tested for by analysing DNA cleavage patterns of supercoiled plasmid DNA. The results confirm that in the presence of metals such as Ni^{2+} the E9 DNase produces a high proportion of double-strand breaks in supercoiled DNA, but this activity is much lower than with Mg^{2+} , which predominantly nicks the DNA on one strand. The cytotoxic DNase of colicin E9 can therefore utilize a number of metal ions to cleave chromosomal DNA, the identity of which governs whether the enzyme produces single- or double-strand breaks.

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