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The zinc ion in the HNH motif of the endonuclease domain of colicin E7 is not required for DNA binding but is essential for DNA hydrolysis

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ABSTRACT

The HNH motif was originally identified in the subfamily of HNH homing endonucleases, which initiate the process of the insertion of mobile genetic elements into specific sites. Several bacteria toxins, including colicin E7 (ColE7), also contain the 30 amino acid HNH motif in their nuclease domains. In this work, we found that the nuclease domain of ColE7 (nuclease-ColE7) purified from *Escherichia coli* contains a one-to-one stoichiometry of zinc ion and that this zinc-containing enzyme hydrolyzes DNA without externally added divalent metal ions. The apo-enzyme, in which the indigenous zinc ion was removed from nuclease-ColE7, had no DNase activity. Several divalent metal ions, including Ni^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}, Cu^{2+} and Zn^{2+}, re-activated the DNase activity of the apo-enzyme to various degrees, however higher concentrations of zinc ion inhibited this DNase activity. Two charged residues located at positions close to the zinc-binding site were mutated to alanine. The single-site mutants, R538A and E542A, showed reduced DNase activity, whereas the double-point mutant, R538A + E542A, had no observable DNase activity. A gel retardation assay further demonstrated that the nuclease-ColE7 hydrolyzed DNA in the presence of zinc ions, but only bound to DNA in the absence of zinc ions. These results demonstrate that the zinc ion in the HNH motif of nuclease-ColE7 is not required for DNA binding, but is essential for DNA hydrolysis, suggesting that the zinc ion not only stabilizes the folding of the enzyme, but is also likely to be involved in DNA hydrolysis.

INTRODUCTION

The HNH motif was first identified based on the consensus sequence observed in several group I intron-encoded homing endonucleases (1–3). These homing endonucleases make DNA breaks at specific sites and initiate the homing process, which moves mobile introns from intron-containing alleles to intron-less alleles of cognate genes (4–6). Subsequent sequence comparison in gene data banks revealed a family of these type of proteins widespread in all phylogenic kingdoms, including bacteriophage, bacteria, virus, yeast mitochondria and plant chloroplasts. The biggest subfamily among the HNH proteins are site-specific homing endonucleases, such as the group I homing endonucleases I-*Hmu*I (7), I-*Hmu*II, I-*Hmu*III (8), I-*Tev*III (9) and IA2 (10); and the group II homing endonucleases Cpc (11), Avi (11), PetD (12), COXI al1 and COXI al2 (13). Other HNH proteins with known functions include the bacteria restriction enzyme McrA (14), repair enzyme MutS (13), and protein toxins that contain DNase activity, such as DNase-type colicins (15,16) and pyocins (17).

The E-group DNase-type colicins, including ColE2, ColE7, ColE8 and ColE9, are protein toxins secreted by *Escherichia coli* that kill other *E.coli* and closely related bacteria (18). They share high sequence identity and all contain an HNH motif in their cytotoxic nuclease domains. After these colicins traverse the membranes of bacteria cells, they digest DNA chromosomes in target cells and lead to cell death (16). An immunity protein (Im) bound specifically to the nuclease domain of colicin (nuclease-ColE) is co-expressed with colicin to inhibit its DNase activity, and thus protects the host cell from the cytotoxic effect. The crystal structures of two DNase-type colicins, nuclease-ColE7 in complex with immunity E7 protein (Im7) (19) and nuclease-ColE9 in complex with Im9 (20), have been reported. These structures revealed the topology of the HNH motif of two antiparallel β-strands linked to a C-terminal α-helix with a metal ion, similar to that of a classic zinc finger motif (Fig. 1). A Zn^{2+} ion is located at the center of the HNH motif in ColE7 and a Ni^{2+} ion is located at the center of the HNH motif of ColE9. The zinc ion in the HNH motif of ColE7 is bound to three conserved histidine residues and one water molecule in a distorted tetrahedral geometry.

Since all the HNH family proteins with known function carry endonuclease activity, it is very likely that the HNH motif is involved in DNA hydrolysis. However, how the HNH

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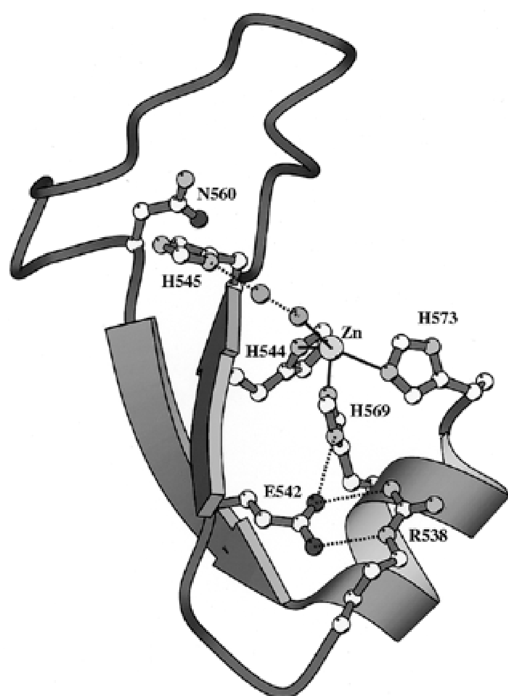

Figure 1. The crystal structural model of the HNH motif in the DNase domain of ColE7 (19). This motif has a topology similar to that of the classical zinc finger motif with two antiparallel β-strands linked to an α-helix by a Zn^{2+} ion. The Zn^{2+} ion is bound to three histidine residues, His544, His569 and His573, and a water molecule in a distorted tetrahedral geometry.

motif mediates its function in DNA hydrolysis and the role of the zinc ion in the HNH motif are still elusive. Divalent metal ions have been shown to play essential roles in activating DNase activity in many restriction enzymes and nucleases (21–23). A divalent metal ion may function as (i) a general base to activate the attacking water (24), (ii) a Lewis acid to stabilize the pentacovalent phosphate group (24,25), or (iii) a general acid to activate a water molecule, which provides a proton for the leaving group (25). The metal ion in the HNH motif of ColE9 may play a structural role to stabilize the HNH motif, as was suggested by the finding that zinc-bound nuclease-ColE9 is more thermally stable than the apo-nuclease-ColE9 (26). However, a structural comparison between the active sites of the *Serratia* nuclease, the His–Cys box homing nuclease I-*Ppo*I and the HNH motif of ColE9, revealed a similar ββα folding, with the divalent metal ions in the three proteins superimposed at a similar position (27). The metal ions in the *Serratia* nuclease and I-*Ppo*I have been proposed to be involved in catalytic pathways (25,28,29), implying that the zinc ion in the HNH motif also participates in DNA hydrolysis. A recent review article compared the structures of several nuclease-type colicins, and the authors suggested that the metal-ion-binding site in the HNH motif is the DNase-active site based solely on the finding that a phosphate ion is directly bound to the Ni^{2+} ion in the crystal structure of nuclease-ColE9 (30). Therefore, it is necessary to further clarify the role of divalent metal ions in the HNH motif of DNase-type colicins for non-specific DNA hydrolysis.

In this work, we characterize the requirement of a divalent metal ion in the HNH motif of ColE7 for DNA hydrolysis and DNA binding. We found that the nuclease-ColE7 is active in buffers with low concentrations of zinc ions but higher concentrations of zinc ions function as inhibitors for the DNase activity. A variety of divalent metal ions are active cofactors for nuclease-ColE7. The apo-nuclease-ColE7 without a metal ion bound at the HNH motif, binds to DNA, but does not hydrolyze DNA. Our results suggest that the zinc ion in the HNH motif of ColE7 is likely to be involved in DNA hydrolysis.

MATERIALS AND METHODS

Purification of the DNase domain of ColE7

The expression vector pQE-30 (Qiagen, Germany) containing a six-histidine affinity tag at the N-terminus of the cloning site was used to overexpress nuclease-ColE7/Im7 complex. The detailed procedure for the construction and protein purification of the complex was previously described (19,31). The nuclease-ColE7 was further dissociated from Im7 by lowering the pH of the buffer to 3 to denature the purified complex and the two proteins were applied to a SP-Sepharose column (Amersham, USA) and eluted with buffers of pH 3 and 7, respectively. Greater than 98% homogeneity of the nuclease-ColE7 was obtained as shown in sodium dodecylsulfate-poly-acrylamide gel electrophoresis (SDS–PAGE). The mass spectrometry analysis gave a molecular weight of 16 708 Da as compared with the calculated molecular weight of 16 707 Da (molecular weight of nuclease-ColE7 plus zinc). The contents of metal elements in the nuclease-ColE7/Im7 complex and the free nuclease-ColE7 were measured on a Jarrell-Ash model ICP 9000 inductively coupled plasma atomic emission spectrophotometer. The protein concentrations were estimated based on the absorbance at 280 nm (ε_{280} = 22 190 M^{-1} cm^{-1} for the nuclease-ColE7/Im7 complex and 12 665 M^{-1} cm^{-1} for the free nuclease-ColE7).

Optimization of the enzymatic activity of nuclease-ColE7

Plasmid DNA, pUC18, was used as a substrate for the DNase activity test. The supercoiled DNA of pUC18 was obtained by the alkaline lysis method (32). For the determination of the optimal temperature for DNase activity of nuclease-ColE7, 100 nM of the purified DNase domain and 0.1 µg pUC18 DNA were incubated for 20 min in a digestion buffer containing 10 mM $MgCl_2$ and 10 mM Tris–HCl (pH 7.25) in a series of designated temperatures (from 0 to 70°C). The digested patterns of the pUC18 DNA at distinct incubation temperatures were examined by 0.8% agarose gel electrophoresis. The degrees of completion of DNA digestion were measured by a Personal densitometer SI and Image QuaNT (Molecular Dynamics, Sunnyvale, USA) after agarose gel electrophoresis. The reactions were repeated three times and were stopped with the gel loading buffer containing 250 mM EDTA, 0.25% bromophenol blue and 30% glycerol. The optimal pH for the enzymatic reaction of nuclease-ColE7 was determined in a similar way in that 6 µM of the purified nuclease-ColE7 and 6.4 nM of pUC18 DNA were incubated for 1 or 5 min in the digestion buffers of pH ranging from 3 to 10 at 37°C. These reactions were stopped with the addition of EDTA to a final concentration of 250 mM and 600 µAU protease K (Qiagen). The sample was then loaded onto a running Novex® 4–20%

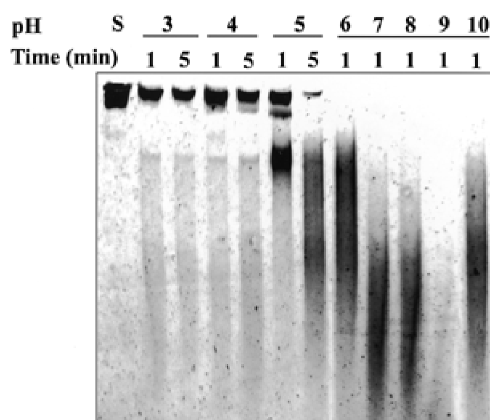

Figure 2. The pH-dependent DNase activities of nuclease-ColE7. The DNase activity of nuclease-ColE7 in solutions with different pH varying from 3 to 10, was determined based on the DNA digestion patterns observed in a 4–20% TBE polyacrylamide gel using pUC18 plasmid DNA as the substrate. The DNase activity is indicated by the decreased amount of substrate DNA (the lane labeled with S). The nuclease-ColE7 has the optimal activity at ~pH 9 in which most of the DNA substrates were digested.

TBE polyacrylamide gel (Invitrogen). The digestion pattern is shown in Figure 2.

EDTA treatment of the purified nuclease-ColE7

To examine the effect of a single divalent metal ion in the activation of nuclease-ColE7, preparation of a divalent metal-free nuclease domain is necessary. A 10 ml solution of the purified nuclease domain (0.17 mg/ml) was incubated with 1 M divalent metal chelating agent EDTA at room temperature for 1 h. The EDTA-treated nuclease-ColE7 was dialyzed against 1 l of Tris–HCl buffer overnight at 4°C with three changes of buffer. A residual EDTA concentration of ~1 µM remained in the solution which would extract any contaminated divalent metal ions in solution. After dialysis, the volume of EDTA-treated protein was concentrated to 3 ml (0.4 mg/ml) with an Ultrafree-0.5 centrifugal filter (Millipore, Bedford, MA, USA). The enzymatic activity of the EDTA-treated nuclease-ColE7 was determined by the method described in the following section.

Characterization of the effects of divalent metal ions on DNase activity

Eight divalent metal ions (Ni^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}, Cu^{2+} and Zn^{2+}) were used for these tests. The concentrations of divalent metal ions used for activation of nuclease-ColE7 with or without EDTA treatment ranged from 1 pM to 50 mM. A supercoiled form of pUC18 DNA (0.1 µg) and purified nuclease-ColE7 (100 nM) were used for the enzymatic reaction throughout the experiments. The procedure for the activity assay is described in the above section 'Optimization of the enzymatic activity of nuclease-ColE7'. The digestion patterns of DNA were stained with ethidium bromide and monitored by agarose gel.

Measurement of the DNA cleavage using the fluorophore-labeled oligonucleotides

The 30 base pair (bp) long oligonucleotide labeled with a fluorophore of hetrachloro-6-carboxyfluorescein and a quencher of BHQ-1™ (DNaseAlert™ QC system DNA substrate; Ambion Inc., USA) was used to measure the DNase activity of nuclease-ColE7. Fluorescence measurements were routinely carried out by mixing 1 µl of 6 µM nuclease-ColE7, 10 µl of 100 mM Tris–HCl buffer (pH 8.0), 10 µl of 2 µM DNase-Alert™ QC system DNA substrate and various concentrations of $ZnCl_2$ or $MgCl_2$ (BioChemika grade; Fluka, Switzerland) in a plate-well incubated at 40°C. The increased fluorescence emission intensity resulting from the DNA cleavage was measured on a NUNC™ black 96-well plate with a Fluoroskan Ascent plate reader (equipped with a 538 nm excitation filter, a 584 nm emission filter and a thermostatted temperature option; ThermoLabsystems, Finland). Fluorescence kinetic data were collected at 60 s intervals over a period of 80 min at 40°C. All the assay component volumes (100 µl) remained the same for all the experiments. The initial velocity was calculated only from the data points from the first 3–8 min after enzyme addition. Data were analyzed using SigmaPlot 6.0 (SPSS Inc., Chicago, IL, USA).

The holo-nuclease-ColE7 used in this assay (see Fig. 5C) was obtained by supplement of zinc ions during purification processes and extra zinc ions were removed by a desalting column (Amersham) followed by dialysis against Milli-Q water. The apo-enzyme of nuclease-ColE7 was obtained using a Chelex® 100 chelating ion exchange resin (Bio-Rad Laboratories, Switzerland) to remove Zn^{2+}, followed by elution with 1 M imidazole (BioChemika grade; Fluka). Apo-nuclease-ColE7 was then dialyzed against metal-free water.

Construction of mutant nuclease-ColE7 and DNase-activity assay

PCR-mediated site-directed mutagenesis was performed as previously described (33). Plasmid pColE7-K317 (34) was used as a template for PCR-mediated site-directed mutagenesis to create mutations at the active site of the DNase domain of ColE7. The crystal structure of the DNase domain of ColE7 in complex with Im7 (19) showed that the three residues Arg538, Glu542 and His569 are situated near the metal-binding site of the DNase domain and that His569 is directly bound to the zinc ion. In order to verify the critical residues for the DNase activity of the domain, a strategy was used to generate mutants at the designated residues: R538A, E542A and R538A + E542A. The resultant mutagenic plasmids were designated as pColE538, pColE542 and pColE538–542, respectively. The sequences of the mutagenic oligonucleotide primer pairs are listed as follows:

(i) R538A: T2A-538U, 5′-TCAGGGAAG<u>GCA</u>ACTTCAT-TCG-3′; T2A-538D, 5′-AAGTT<u>GC</u>CTTCCCTGAAACATC-3′

(ii) E542A: T2A-542U, 5′-TTCATTC<u>GCG</u>CTTCATCATGAG-3′; T2A-542D, 5′-ATGAAG<u>CGC</u>GAATGAAGTTCTC-3′

(iii) R538A + E542A: T2A-53842U, 5′-GAAG<u>GCA</u>ACTTC-ATTC<u>GCG</u>CTTCATC-3′; T2A-53842D, 5′-AAG<u>CGC</u>GAA-TGAAGTT<u>GC</u>CTT-3′

The sites for the change of residues are underlined.

For analysis of the DNase activity of the mutant nuclease-ColE7, 0.1 µg of supercoiled pUC18 plasmid DNA was incubated with 100 nM of each of the purified mutant nuclease-ColE7 in the presence of 10 mM $MgCl_2$ and 10 mM Tris–HCl, pH 7.25 for 20 min at 37°C. The methods used for gel electrophoresis of the digested DNA and determination of the relative nuclease-ColE7 activity by densitometry were the same as

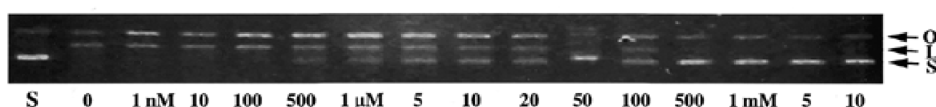

Figure 3. The effect of various concentrations of zinc ion on the DNase activity of nuclease-ColE7. A plasmid DNA pUC18 (0.1 μg) was used as the substrate to react with 100 nM nuclease-ColE7. The topological changes of the substrate DNA were monitored by 0.8% agarose gel electrophoresis. The supercoiled (S) form of pUC18 plasmid DNA alone was used as negative control (lane S). The effects of enzymatic reactions with various concentrations of zinc ions were monitored by the conversion of the supercoiled form DNA to its corresponding open circular (O) or linear (L) form. The concentration of zinc ion was increased from 1 nM to 10 mM as indicated on the bottom of each lane.

described in the above section 'Optimization of the enzymatic activity of nuclease-ColE7'.

Gel retardation assays

A 27 bp DNA was synthesized and purified by a chromatographic method using Nensorb™ resin (DuPont, USA). A solution containing 50, 5.0 or 0.5 μM of nuclease-ColE7 and 6.7 μM dsDNA in 50 mM HEPES (pH 7.5), 150 mM NaCl, with or without 1.25 mM EDTA was incubated for 2 min at 25°C. 180 μAU protease K (Qiagen) was added in a reaction mixture and incubated at 54°C for 1 h to digest nuclease-ColE7. These samples were then loaded onto a running Novex® 20% TBE polyacrylamide gel (Invitrogen) with a length of 12 cm, equilibrated in 1× TBE and subjected to 200 V for 45 min at room temperature. Gels were stained with SYBR Green I (Molecular Probes, the Netherlands) and monitored by FUJI Science Imaging System LAS-1000 Plus (Fuji Photo Film, Japan).

RESULTS

Zinc ion binds with a one-to-one stoichiometry to ColE7

The zinc contents in the nuclease-ColE7/Im7 complex and in the free nuclease-ColE7 were measured by atomic emission spectroscopy. The zinc ion was bound with a one-to-one stoichiometry (complex:zinc = 1:0.97) to the nuclease-ColE7/Im7 complex. The amount of other metal ions was negligible. Only residual amounts of zinc ions were associated with the free nuclease-ColE7 with a molar ratio of nuclease-ColE7:zinc = 1:0.05. It is likely that the zinc ions were dissociated from nuclease-ColE7 during the protein purification steps, which separated the nuclease-ColE7 from Im7 by partial denaturation of the complex in low pH buffers. It has been shown that Zn^{2+} has the highest affinity for ColE9 as compared with two other transition metal ions Ni^{2+} and Co^{2+} (26). Therefore, it is well accepted that the DNase-type colicins are zinc-dependent enzymes and one zinc ion is bound to one protein molecule.

Optimization of DNase activity

The endonuclease activity of nuclease-ColE7 was assayed using pUC18 plasmid DNA as a substrate at different temperatures and over a range of pHs. The optimal temperature for the DNase activity of nuclease-ColE7 was ~50°C. The nuclease-ColE7 started to display weak DNase activity at 4°C, which gradually increased to reach its peak at 50°C (data not shown). The DNase activity of nuclease-ColE7 was tested from pH 3 to 10 and the optimal pH for enzymatic reaction was ~9 (see Fig. 2). The DNase activity appeared ~pH 5.0 and gradually increased to a maximum at pH 9, but was inhibited at higher pH (pH >10). Preheating the nuclease-ColE7 at temperatures between 4 and 37°C for up to 12 h did not affect the enzyme activity. However, when nuclease-ColE7 was pretreated at 50°C for 1 h, its DNase activity decreased substantially.

The zinc-containing nuclease-ColE7 is active at low but inactive at higher Zn^{2+} concentrations

Activation of a Zn^{2+}-containing nuclease-ColE7 was characterized by the topological changes of the substrate DNA (Fig. 3). Without externally added divalent ion, the Zn^{2+}-containing nuclease-ColE7 converted the supercoiled form of DNA into open circular and linear forms of DNA (Fig. 3). In this experiment, the concentration of nuclease-ColE7 was ~1 nM, and the concentration of zinc ion was increased from 1 nM to 10 mM. Nuclease-ColE7 was most active when the concentration of zinc ion was <1 μM, and it became inactive at higher zinc concentrations, i.e. when the molar ratio of zinc ion to nuclease-ColE7 was >1000-fold, the zinc ion started to inhibit DNase activity. When the Zn^{2+} concentration reached 500 μM, that is ~10^5-fold to the nuclease-ColE7, the zinc ion completely inhibited the DNase activity.

A variety of divalent metal ions activate the DNase activity of ColE7

To characterize the DNase activity of nuclease-ColE7 in the presence of a single metal ion, purified nuclease-ColE7 was treated first with EDTA to remove the zinc ion associated with the enzyme. However, ~1 μM of EDTA remained in the protein solution, which would extract any residual metal ions to ensure there was no contamination. We found that the EDTA-treated DNase domain completely lost its enzymatic activity (data not shown). However, the activity of the EDTA-treated nuclease-ColE7 resumed to various degrees when different divalent ions were introduced into the assay system (Fig. 4). Ni^{2+} and Mg^{2+} were the most potent divalent ions in activating EDTA-treated nuclease-ColE7: the enzyme completely digested DNA when the metal ion concentration exceeded the residual EDTA concentration at ~1 μM. On the other hand, Co^{2+}, Mn^{2+}, Ca^{2+} and Sr^{2+} required a higher concentration (1 mM) for activation. Activation of the enzyme by Cu^{2+} and Zn^{2+} was the least obvious. These two metal ions only activated the conversion of the supercoiled form of the substrate DNA to the respective open circular form.

The DNase activity of nuclease-ColE7 was further assayed by a more sensitive fluorescent method using smaller oligonucleotides covalently bound with fluorescent dye and quencher as a substrate (35,36). The degradation of the flurophore-labeled oligonucleotides by nuclease-ColE7 resulted in the increase of fluorescence emission intensity, which was monitored by a spectrofluorometer. We found that the DNase activity of nuclease-ColE7 gradually decreased with the

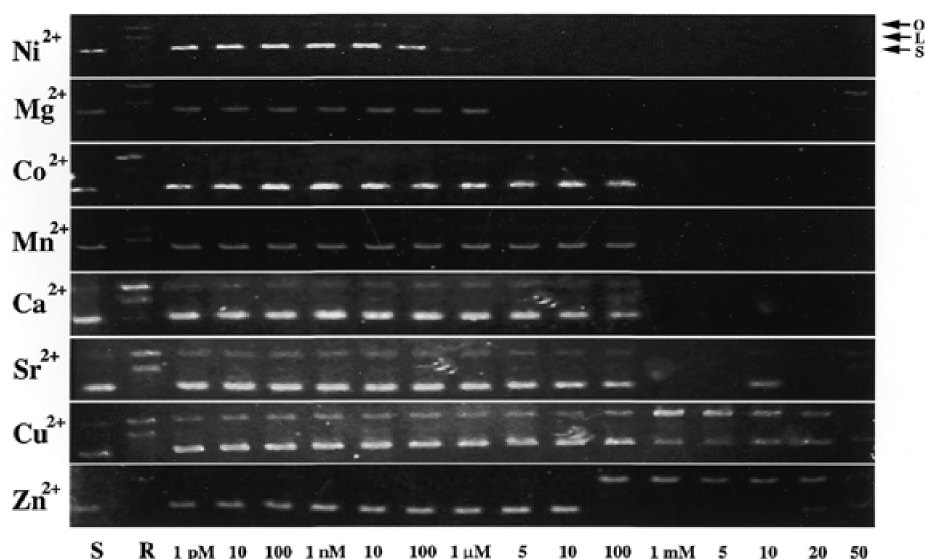

Figure 4. The effect of various divalent metal ions in the activation of EDTA-treated nuclease-ColE7. Supercoiled pUC18 DNA was used as a negative control (lane S). Digestion of the substrate DNA by the Zn^{2+}-containing nuclease-ColE7 with no externally added divalent ion was used as a reference (lane R). EDTA-treated nuclease-ColE7 was used for digestion of the substrate DNA in the presence of various concentrations (1 pM to 50 mM) of divalent metal ions as indicated in the last row.

increase of Zn^{2+} concentration and the enzyme completely lost its DNase activity when the Zn^{2+} concentration (>55 μM) is over ~1000-fold larger than that of nuclease-ColE7 (60 nM) (Fig. 5A). This inhibitory effect produced by Zn^{2+} ions is consistent with the results from the topological analysis using plasmid DNA as substrates described in the previous section. On the other hand, Mg^{2+} activated the DNase activity of the Zn^{2+}-containing holo-nuclease-ColE7 with an initial increase in activity with increasing [Mg^{2+}], followed by a gradual decrease in activity (Fig. 5B). The decrease of activity at high [Mg^{2+}] has been observed for several Mg^{2+}-dependent nucleases and was explained by a model of metal-mediated substrate inhibition (37). The time course of fluorescence emission intensity caused by the DNase activity of nuclease-ColE7 in the presence of Zn^{2+}, Mg^{2+} or Zn^{2+} + Mg^{2+} is shown in Figure 5C. It clearly demonstrates that the apo-enzyme of nuclease-ColE7 had no DNase activity, Zn^{2+} inhibited the DNase activity, and Mg^{2+} activated the DNase activity of the Zn^{2+}-containing holo-enzyme.

Mutations at positions close to the metal-binding site eliminated the DNase activity

Two residues, Arg538 and Glu542, near the metal-binding site were mutated. In the crystal structure of nuclease-ColE7/Im7, Arg538 forms hydrogen bonds to Glu542 and His569, which binds directly to the zinc ion (Fig. 1). The mutation of the three conserved residues, Arg544 (equivalent to Arg538 in ColE7), Glu548 (equivalent to Glu542) and His575 (equivalent to His569) in ColE9 to alanine, generated inactive mutants *in vivo* and *in vitro* (38). However, we found that R538A (22%) and E542A (36%) contained residual DNase activity as compared with the wild-type nuclease-ColE7 using pUC18 plasmid DNA as the substrate (Fig. 6). The double mutant, R538A + E542A, had no observable DNase activity and all the DNA substrate remained intact. The immunity protein Im7 inhibited the DNase activity for these mutants, indicating that the mutants still retained their conformation. Glu542 is ~50% conserved and Arg538 is only slightly conserved (<10%) in the HNH family proteins, suggesting that they do not play major roles in DNA hydrolysis.

The metal-free enzyme is capable of binding to DNA

EDTA-treated nuclease-ColE7 has no DNase activity as demonstrated in the previous section. An earlier comparison of the circular dichroism spectra and the tryptophan emission fluorescence between the apo- and holoenzyme of the DNase domain of ColE9 showed that the apo-protein retains a fold similar to that of the holoenzyme (26). This finding implies that the zinc ion in the HNH motif of the DNase-type colicin is not required for protein folding. However, it is unknown whether the apo-enzyme can bind to DNA.

To address this important question, we used a 27 bp dsDNA as the substrate and found that the zinc-containing nuclease-ColE7 digested DNA, but that the metal-free nuclease-ColE7 only bound to DNA as shown in a gel-shift assay (Fig. 7). The concentration of the 27mer DNA was maintained at 6.7 μM while the concentrations of nuclease-ColE7 varied from 50 to 0.5 μM (lanes 1–3, 4–6 and 7–9). The 27mer DNA was digested in lanes 1, 2, 7 and 8 when EDTA was not present, but the DNA was up-shifted by the enzyme when EDTA was added as shown in lane 4. Protease K was added to digest nuclease-ColE7 in lanes 7–9. Compared with lanes 1–3, it appeared that nuclease-ColE7 binds to the smaller fragment DNA products in lane 1 and the DNA products were released from the enzymes in lane 7 after protease K digestion. These results demonstrate that not only is the zinc ion in the HNH motif not required for protein folding, it is also not required for DNA binding.

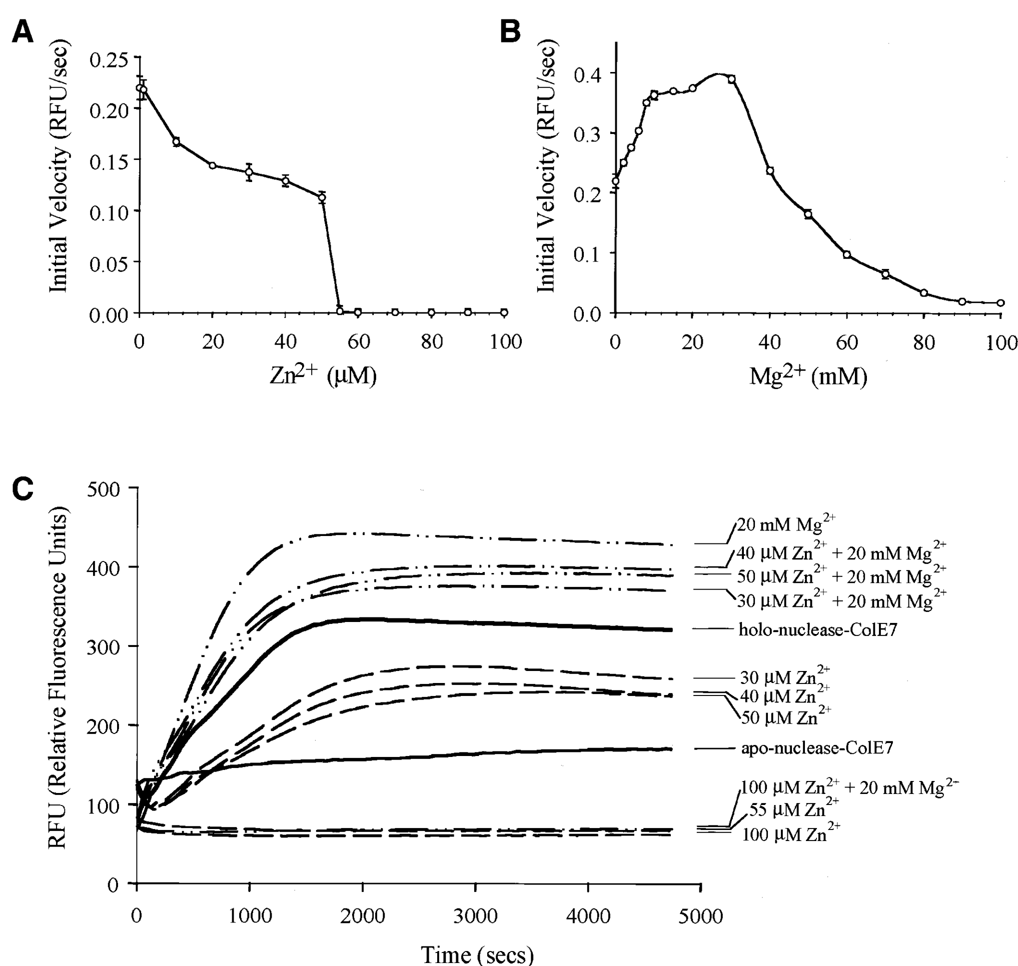

Figure 5. Dependence of nuclease-ColE7 DNase activity on the concentrations of Zn^{2+} and Mg^{2+} measured by a fluorescent method using the fluorophore and quencher-labeled oligonucleotide as a substrate. (**A**) The initial velocity of DNA cleavage for the holo-nuclease-ColE7 was varied with Zn^{2+} ion concentration. The zinc ion inhibited the DNA cleavage and nuclease-ColE7 had no detectable DNase activity when Zn^{2+} concentrations (55 μM) were more than ~1000-fold of enzyme concentration (60 nM). (**B**) The initial velocity of DNA cleavage for the zinc-containing holo-nuclease-ColE7 was varied with Mg^{2+} ion concentration. (**C**) The time course of fluorescence emission intensity caused by nuclease-ColE7 in buffers containing different divalent metal ions. The zinc-containing holo-nuclease-ColE7 had moderate activity; the apo-enzyme had no DNase activity; Zn^{2+} inhibited and Mg^{2+} (<40 mM) activated DNase activity of the holo-enzyme.

DISCUSSION

The zinc ion in the HNH motif of nuclease-ColE7 is not required for DNA binding

It has been shown before that the zinc binding to the apo-nuclease-ColE9 increased the proteolytic resistance and melting point of the enzyme; therefore, it was concluded that the transition metal ion in the HNH motif serves a structural role (26). However, in the present work, our results indicate that the zinc ion in the HNH motif of ColE7 also appears to be involved in the catalytic pathway. First, nuclease-ColE7 is bound to zinc ions in a one-to-one stoichiometry, and this holoenzyme is active for DNA hydrolysis. Removal of the zinc ion produces an apo-enzyme with no DNase activity. Secondly, mutations in the vicinity of the metal-binding site impair DNase activity. Thirdly, the apo-enzyme has no DNase activity, but it retains the ability to bind DNA. Since the zinc ion in the HNH motif is not required for DNA binding, but is required for DNA hydrolysis, it is very likely that the zinc ion is involved in DNA hydrolysis.

However, our DNA-binding results for nuclease-ColE7 are different from that of I-*Cmoe*I (39), which is a group I HNH family homing endonuclease encoded by the introns in the *Chlamydomonas moewusii* chloroplast *psb*A gene. I-*Cmoe*I requires a metal ion cofactor for DNA binding that it cannot bind to its DNA substrate in the presence of EDTA. The different results observed for the two HNH proteins may be due to the intrinsic difference in the two proteins: I-*Cmoe*I is a site-specific homing endonuclease, whereas nuclease-ColE7 is a non-specific enzyme. In addition, these two proteins only share sequence similarity in the HNH motif. It is also possible that the different experimental conditions used, including the concentrations of proteins, DNA substrates and EDTA, result in the differing binding activities. It will be instructive to find out whether a zinc ion is required for DNA binding for other homing endonucleases and bacteria toxins in the HNH family.

The Zn^{2+} dependence of ColE7 DNase activity

The metal-dependence study of colicin E9 showed that the addition of Zn^{2+} did not result in any DNase activity according

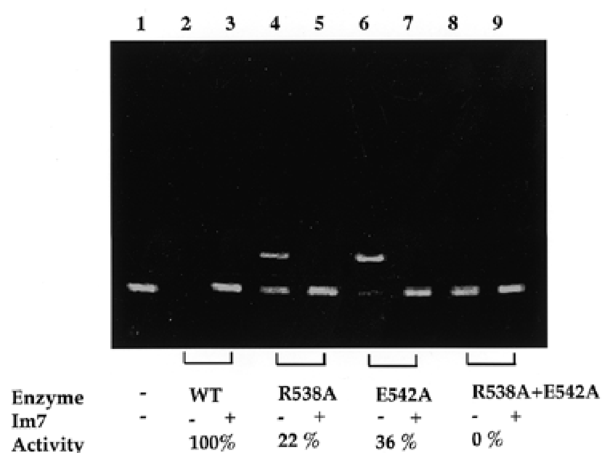

Figure 6. The DNase activity of nuclease-ColE7 is significantly reduced with mutations at Arg538 and Glu542. Either wild-type or mutated nuclease-ColE7 (100 nM) was incubated with 0.1 µg of the supercoiled form of pUC18 plasmid DNA. An equal molar concentration of Im7 inhibitor to that of nuclease-ColE7 was used to monitor the inhibitory effect of the inhibitor against the mutant-type nuclease-ColE7 protein. The upper and lower bands indicate the open circular and supercoiled form DNA, respectively. The relative DNase activities with respect to the wild-type enzyme measured by densitometry were 22% for R538A, 36% for E542A and 0% for R538A + E542A. The enzyme activity was completely inhibited with the addition of an equal molar concentration of Im7 inhibitor indicating these mutants still folded well. Lanes 1 and 2, pUC18 incubated without and with wild-type nuclease-ColE7, respectively. Lane 3, the DNA incubated together with wild-type nuclease-ColE7 and equal molar concentrations of Im7 inhibitor. Lanes 4, 6 and 8, the DNA incubated with nuclease-ColE7 mutant R538A, E542A and R538A + E542A, respectively. Lanes 5, 7 and 9, have the same conditions as lanes 4, 6 and 8 except for the addition of equal molar concentrations of Im7 inhibitor.

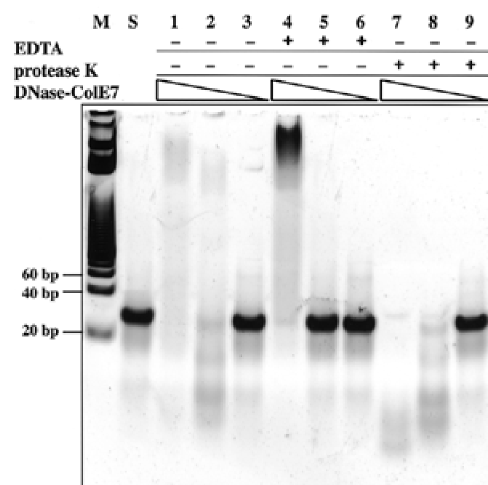

Figure 7. A gel retardation assay shows that nuclease-ColE7 hydrolyzed a 27mer DNA in the presence of zinc ions, but up-shifted the DNA in the absence of zinc ions. A solution containing 50 µM (lanes 1, 4, 7), 5 µM (lanes 2, 5, 8) or 0.5 µM (lanes 3, 6, 9) of nuclease-ColE7 was incubated with 6.7 µM of 27 bp of dsDNA for 2 min at 25°C with or without the presence of 1.25 mM EDTA. Protease K was used to digest nuclease-ColE7 after the incubation described above (lanes 7, 8, 9). Lane M, DNA markers; lane S, substrate 27 bp DNA.

to Kunitz assays (26). However, in the present work, supercoiled DNA was nicked to open-circular and linear forms when incubated with nuclease-ColE7 in a low Zn^{2+} concentration range. The EDTA-treated enzyme was reactivated by Zn^{2+}, but the DNase activity was inhibited when the enzyme was incubated in a buffer containing a higher concentration of Zn^{2+}. This phenomenon was further assayed using a fluorophore and quencher-labeled oligonucleotide as a substrate, and a similar result was observed that extra Zn^{2+} ion inhibited DNase activity. Therefore, the higher concentration of zinc ion (>1000 in molar ratio to enzyme) has an inhibitory effect on the DNase activity. The different results observed for ColE9 (26) might be due to the high Zn^{2+} concentration (10 µM as compared with ~1 nM of protein concentration) used in their experiments, which in turn produced an inhibitory effect on DNase activity.

Inhibition of enzyme activity by a relatively higher concentration of Zn^{2+} has been observed for several zinc-dependent enzymes, including metallo-β-lactamase (40) and methionine aminopeptidase (41). There are two metal-binding sites in metallo-β-lactamase, and Zn^{2+} binding to the second site produces the inhibitory effect. A previous study (42) and our present work showed that HNH endonucleases are more active in the presence of Mg^{2+}. Mg^{2+} prefers binding to oxygen-containing ligands, and thus far no structural example has shown that Mg^{2+} binds to three histidine residues (43). It seems unlikely that Mg^{2+} binds at the same zinc-binding site in ColE7. In I-*Ppo*I and *Serratia* nuclease, the Mg^{2+} binds to an asparagine residue and the scissile phosphate oxygen atoms, and Mg^{2+} likely participates in protonation of the 3′ oxygen leaving group (25,29,43–45). We suspect that the DNA hydrolysis by ColE7 is catalyzed by more than one metal ion, even though we observed only one metal-binding site in ColE7. We further noted that Mg^{2+} activated the DNase activity of nuclease-ColE7, but it cannot rescue the Zn^{2+}-suppressed activity that the enzyme had no activity in a buffer containing 100 µM of Zn^{2+} and 20 mM of Mg^{2+} (Fig. 5C). This result is consistent with the earlier prediction that Zn^{2+} can dislodge Mg^{2+} from its octahedral binding site (46). It will be interesting to investigate the possibility of a second metal-binding site for Mg^{2+} in ColE7 upon DNA binding.

Possible roles of zinc ion in the HNH motif

A conserved surface for nuclease-ColE7 is displayed in Figure 8, in which all the conserved residues in bacteria toxins, including ColE2, ColE7, ColE8, ColE9, Pyocin S1 and S2, are colored red, and the non-conserved residues are colored white. The protein–protein interaction surface for immunity proteins is clearly not conserved. This explains the specificity for each pair of colicin and immunity proteins since this surface has to be different for specific recognition by the cognate inhibitor. On the contrary, the DNA-binding site and DNase-active site are likely to be located within a conserved surface because all of these toxins carry a similar DNase activity. We found that the zinc ion in nuclease-ColE7 is indeed located in the middle of a conserved surface exposed to solvent and accessible for DNA hydrolysis.

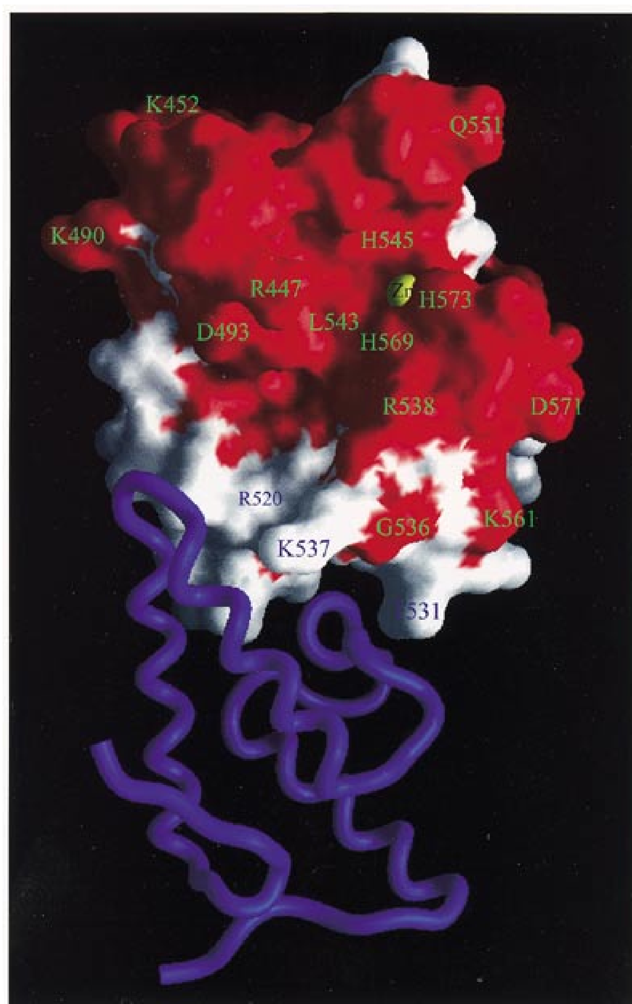

Figure 8. The conserved and non-conserved molecular surface of nuclease-ColE7. The molecular surface that contains the conserved residues in the six bacteria toxins in HNH family is colored red, and the non-conserved surface is colored white. Im7 is displayed in a ribbon model in blue. The protein interface is not conserved, therefore the immunity protein specifically recognizes its cognate colicin. On the contrary, the zinc ion is located in the middle of a conserved surface where it is likely that the DNA-binding site and DNase-active site are located.

What is the role of the zinc ion in the HNH motif of ColE7 in DNA hydrolysis? A comparison between the HNH motif and the active site of other nucleases may shed some light on the possible functions of the metal ion. Structural similarity has been observed between the active sites of ColE9, the non-specific nuclease from *Serratia* and the His–Cys box containing homing endonuclease I-*Ppo*I. An analogous ββα folding was identified in the three enzymes with divalent metal ions (Mg^{2+} in the *Serratia* nuclease and I-*Ppo*I, and Zn^{2+} in ColE7) and several conserved residues in each family superimposed at similar positions (27). The magnesium ion in I-*Ppo*I appears to be involved in stabilizing the phosphoanion transition state and in protonating the 3′ oxygen leaving group as shown in the crystal structures of I-*Ppo*I in complex with DNA substrates (25). The magnesium ion in the *Serratia* nuclease seems to play a similar role in stabilizing the phosphoanion transition state and helping to protonate the leaving group (28,29). The zinc ion in the HNH motif of ColE7 binds to a water molecule in the crystal structure of nuclease-ColE7/Im7, however, the metal ion in the same position in ColE9 binds to a phosphate ion. Our recent structural data (not shown) also verify that a phosphate ion is directly bound to the zinc ion when the complex crystals are first soaked in phosphate buffer before data collection. This finding indicates that the zinc ion in the HNH motif may be involved in stabilizing the phosphoanion transition state. Nevertheless, the exact role of the zinc ion in the HNH motif is still a matter for discussion and more information is needed to further define the mechanism for DNA hydrolysis catalyzed by the HNH family proteins at the atomic level.

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