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Endonuclease Active Site Plasticity Allows DNA Cleavage with Diverse Alkaline Earth and Transition Metal Ions

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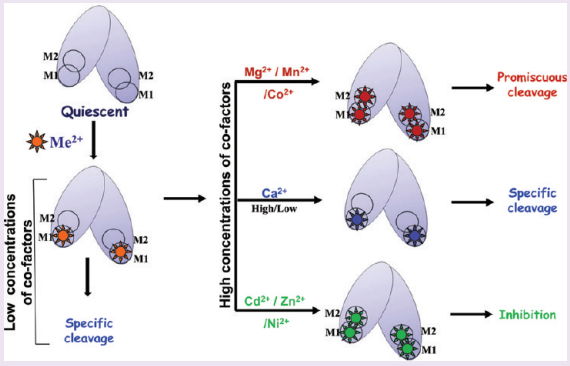
Supporting Information

ABSTRACT: A majority of enzymes show a high degree of specificity toward a particular metal ion in their catalytic reaction. However, Type II restriction endonuclease (REase) R.*Kpn*I, which is the first member of the HNH superfamily of REases, exhibits extraordinary diversity in metal ion dependent DNA cleavage. Several alkaline earth and transition group metal ions induce high fidelity and promiscuous cleavage or inhibition depending upon their concentration. The metal ions having different ionic radii and co-ordination geometries readily replace each other from the enzyme's active site, revealing its plasticity. Ability of R.*Kpn*I to cleave DNA with both alkaline earth and transition group metal ions having varied ionic radii could imply utilization of different catalytic site(s). However, mutation of the invariant His residue of the HNH motif caused abolition of the enzyme activity with all of the cofactors, indicating that the enzyme follows a single metal ion catalytic mechanism for DNA cleavage. Indispensability of His in nucleophile activation together with broad cofactor tolerance of the enzyme indicates electrostatic stabilization function of metal ions during catalysis. Nevertheless, a second metal ion is recruited at higher concentrations to either induce promiscuity or inhibit the DNA cleavage. Regulation of the endonuclease activity and fidelity by a second metal ion binding is a unique feature of R.*Kpn*I among REases and HNH nucleases. The active site plasticity of R.*Kpn*I opens up avenues for redesigning cofactor specificities and generation of mutants specific to a particular metal ion.



REases are excellent model systems for understanding sequence specificity, DNA−protein interactions, metal ion induced DNA binding or cleavage, and mechanisms of DNA hydrolysis. They are a diverse class of enzymes found ubiquitously among the prokaryotes. To date, nearly 4000 Type II REases are known with more than 290 different specificities.[1] Five different kinds of active sites are found in these enzymes, based on structural and biochemical evidence, namely, PD-(D/E)XK, PLD, GIY-YIG, HNH, and "halfpipe".[2−5] Almost all of these enzymes, except the phospholipase D family of REases, require Mg^{2+} that functions at one or more steps of DNA binding and phosphodiester bond cleavage.[1−6] Mg^{2+} is the cofactor of choice; replacement with other metal cofactors having similar properties usually affects DNA binding and/or cleavage. Notably, Ca^{2+} supports DNA binding but not cleavage, whereas Mn^{2+} affects the DNA cleavage specificity as well as activity.[5,6] By far, R.*Kpn*I is markedly different from other Type II REases as it exhibits robust DNA cleavage in the presence of Ca^{2+} and Zn^{2+}. Surprising characteristics of the enzyme were demonstrated in our previous studies; it is a highly promiscuous enzyme in Mg^{2+}-catalyzed reactions, unlike most other REases.[7] The promiscuous activity is further enhanced in the presence of Mn^{2+}. In contrast, the enzyme exhibits high fidelity in the presence of Ca^{2+} or Zn^{2+}.[7,8] The ability of R.*Kpn*I to utilize metal ions belonging to the alkaline earth group (Mg^{2+} and Ca^{2+}) to induce two distinct patterns of cleavage is a unique property not found with other REases. Transition group metals, Mn^{2+} and Zn^{2+}, also impart promiscuity and specific cleavage, respectively.

R.*Kpn*I is the prototype for the HNH REases.[4] HNH nuclease superfamily members, including nonspecific nucleases, homing endonucleases, and Holliday junction resolvases, are characterized by the presence of a $\beta\beta\alpha$ secondary structure motif that co-ordinates the active site metal ion.[9] The invariant first His of the HNH active site acts as the general base for the DNA cleavage reaction and by far is the most conserved residue within the motif.[6,10] The other two residues in the motif function in catalysis either by positioning the scissile phosphodiester bond for DNA hydrolysis or by metal ion co-ordination.[11] In R.*Kpn*I, H149 was shown to be required for the activation of the nucleophile, while the other active site residues D148 and Q175 co-ordinate with Mg^{2+}.[4]

The role of divalent metal ions in DNA binding and cleavage specificity for several REases belonging to the PD-(D/E)XK

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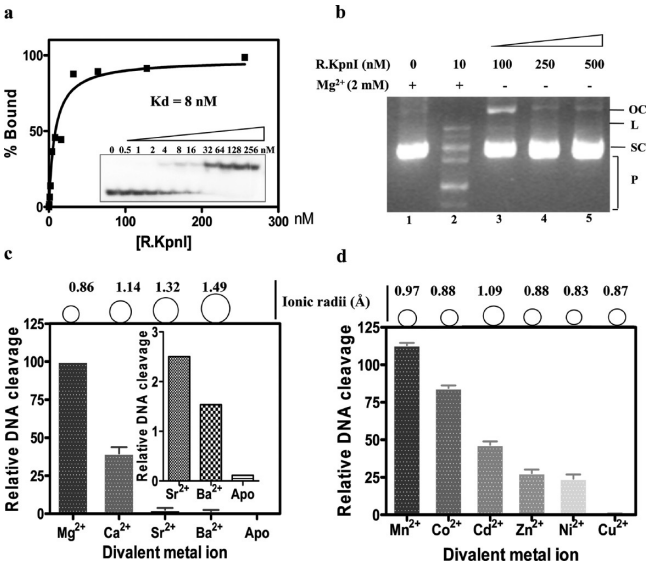

Figure 1. DNA binding and cleavage properties of EDTA-treated R.*Kpn*I. (a) EMSA. Different concentrations of EDTA-treated R.*Kpn*I (0 −256 nM) were incubated with end labeled duplex 1 (1 nM) containing GGTACC sequence in binding buffer (20 mM Tris-HCl pH 7.4, 25 mM NaCl and 5 mM 2-mercaptoethanol) on ice for 15 min. The graph depicts the percentage of bound oligonucleotide with EMSA gel in the inset. The data were fit to a hyperbolic curve using Graphpad Prism 4. (b) DNA cleavage. Different concentrations of EDTA-treated R.*Kpn*I (10−500 nM) were incubated with supercoiled pUC18 (14 nM) in buffer containing 10 mM Tris-HCl pH 7.4 at 37 °C for 1 h. Lane 1 is supercoiled pUC18 without enzyme. Lane 2 is DNA incubated with EDTA-treated enzyme and 2 mM Mg^{2+}. Lanes 3−5 are DNA incubated with increasing concentrations of enzyme in the absence of Mg^{2+}. SC, L, and OC indicate the positions of the supercoiled, linear, and open circular forms of the plasmid, respectively. Promiscuous DNA cleavage products are indicated by P. (c and d) DNA cleavage activity with various metal ions. The bar graphs depict the relative DNA cleavage in the presence of 0.5 mM (c) alkaline earth metals and (d) transition group metal ions. DNA cleavage in the presence of Mg^{2+} was normalized to 100%. Ionic radii of the metal ions at hexa co-ordination is depicted above the bar graphs and were taken from ref 35.

family has been established, but much needs to be understood for HNH REases.[5,12,13] Structural and biochemical studies on HNH nucleases have revealed that they share a common single metal ion mechanism for catalysis.[6] Although many members of the superfamily have the ability to cleave DNA with a few cofactors,[10,14,15] it is unclear whether different cofactors employ the same catalytic mechanism for a given enzyme. The ability of R.*Kpn*I to utilize different metal ions provides an opportunity to understand the role of metal ions in HNH nucleases and delineate the molecular basis of its active site plasticity. We have investigated cofactor adaptability for DNA binding and cleavage by the REase and demonstrated that the HNH active site of R.*Kpn*I is essential for DNA cleavage in the presence of a number of alkaline earth and transition metal ions. An additional ion bound to the enzyme regulates its cleavage specificity, inducing either promiscuity or inhibition of phosphodiester bond hydrolysis. We describe the mechanistic features underlying the two distinct metal ion-mediated reactions.

RESULTS AND DISCUSSION

R.*Kpn*I Exhibits Broad Cofactor Specificity. A number of alkaline earth and transition metal ions were tested for their ability to support DNA cleavage by R.*Kpn*I. To characterize the endonuclease activity with different metal ions, EDTA-treated enzyme was used. The enzyme formed a stable complex (K_d = 8 nM) with the 20 mer canonical duplex oligonucleotide in the absence of any cofactor, indicating that the EDTA treatment did not alter the tertiary structure of the protein (Figure 1a). As such, EDTA-treated enzyme did not exhibit endonuclease activity without the addition of metal ions even with excess enzyme (Figure 1b, lanes 3−5). However, with 2 mM Mg^{2+}, the enzyme regained the cleavage activity (Figure 1b, lane 2). In the absence of the enzyme, metal ions had little effect on the mobility of the DNA in the gels and did not catalyze DNA cleavage (Supplementary Figure S1). Depending on the cofactor supplemented, a varied degree of activity was observed (Figure 1c and d). The enzyme exhibited robust cleavage with both alkaline earth ($Mg^{2+}$and Ca^{2+}) and transition group metal ions (Zn^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, and Ni^{2+}) (Figure 1c and d). Efficiency of the enzyme activity with different metal ions was $Mn^{2+} > Mg^{2+} > Co^{2+} > Ca^{2+} > Cd^{2+} > Zn^{2+} = Ni^{2+} \gg Cu^{2+} > Sr^{2+} > Ba^{2+}$. The broad range of cofactor utilization for phosphodiester bond hydrolysis and accommodation of both alkaline earth and transition group metal ions are unique properties of R.*Kpn*I, suggesting plasticity of the active site.

When alkaline earth metal ions of increasing ionic radii were used, the cleavage activity of the enzyme decreased with the increase in ionic radius. The enzyme exhibited robust activity with ions having radii in the range of 0.86−1.14 Å; divalent ions with ≥1.32 Å ionic radii (Sr^{2+} and Ba^{2+}) showed very little activity, thus indicating an upper limit for the size of the cofactor at the active site. In support, competition of bound Mg^{2+} against

Table 1. DNA Binding Affinities and Kinetic Parameters of R.*Kpn*I in the Presence of Different Metal Ions[a]

GGTACC				
metal ion (mM)	K_d (nM)[b]	K_M (nM)	k_{cat} (min^{-1})	k_{cat}/K_M (M^{-1} s^{-1} × 10^5)
Apo	8 ± 1.6			
Mg^{2+} (2)	9 ± 1.4	19	3.24	28 ± 0.6
Ca^{2+} (2)	10 ± 2.3	18	1.80	16 ± 0.3
Sr^{2+}		nd	nd	nd
Ba^{2+}		nd	nd	nd
Mn^{2+} (0.25)	8 ± 3.4	20	3.60	30 ± 0.5
Co^{2+} (0.25)	12 ± 0.6	25	3.06	20 ± 0.3
Ni^{2+} (0.25)	15 ± 1.6	33	1.5	7 ± 0.9
Cu^{2+}		nd	nd	nd
Zn^{2+} (0.25)	6 ± 1.6	45	1.9	7 ± 0.4
Cd^{2+} (0.25)	12 ± 0.5	27	1.3	8 ± 0.3
GaTACC				
metal ion (mM)	K_d (nM)[b]	K_M (nM)	k_{cat} (min^{-1})	k_{cat}/K_M (M^{-1} s^{-1} × 10^4)
Mg^{2+} (2)	20 ± 1.5	87	0.12	2.3 ± 0.1
Mn^{2+} (2)	15 ± 3.5	124	1.38	18.5 ± 0.6
Co^{2+} (2)	21 ± 1.4	76	0.03	0.7 ± 0.1

[a] Values are determined from triplicates and are shown as mean ± SE. nd: not determined. [b] DNA binding affinities were determined by EMSA using H149A mutant.

Sr^{2+} or Ba^{2+} did not affect the Mg^{2+}-mediated activity indicating that the latter ions were not able to replace Mg^{2+} and occupy the active site (Supplementary Figure S2). Unlike alkaline earth metal ions with varied ionic radii, transition group ions used in the assays have similar ionic radii (0.86−1.09 Å). However, among transition group members, Cu^{2+} exhibited lower activity. Notably, members of this group in different metalloproteins have their respective preferential co-ordination geometries. Octahedral arrangement is preferred by Co^{2+} and Ni^{2+}, and both tetrahedral and octahedral by Cd^{2+} and Zn^{2+}. Though octahedral co-ordination by Ni^{2+} was thought to be uncommon earlier, many examples of metalloenzymes now indicate the contrary.[16,17] Since Cu^{2+} has a square planar mode of co-ordination geometry, it is conceivable that this affects the substrate positioning required for efficient DNA cleavage. In addition to the ionic radius, charge, and preferred co-ordination geometry of the metal ions, their affinity to the enzyme−DNA complex could also affect the DNA cleavage.

Steady-State Kinetic Analysis. Versatility of R.*Kpn*I to catalyze DNA cleavage with a variety of metal ions led us to examine its kinetic behavior. Kinetic constants K_M and k_{cat} were determined as described in Methods and are summarized in Table 1. The catalytic constants derived in the presence of Mg^{2+} were consistent with the previously reported values.[7] The Michaelis constant of the R.*Kpn*I−canonical DNA complex in the presence of metal ions ranged from 18 to 45 nM. Similarly, depending on the cofactor used, the enzyme exhibited different turnover numbers (k_{cat}). For example, with Mn^{2+} or Co^{2+}, kinetic parameters were similar to those of Mg^{2+}. Kinetic analyses with Zn^{2+} revealed k_{cat} of the enzyme to be 1.9 min^{-1}, which is comparable to Ca^{2+} (1.8 min^{-1}), indicating that the Zn^{2+}-mediated DNA cleavage is as efficient as the Ca^{2+}-dependent cleavage. In contrast, Ni^{2+}- or Cd^{2+}-bound enzyme exhibited lower k_{cat}. To understand the catalytic efficiency of the enzyme with various metal ions, specificity constants (k_{cat}/K_M) were determined. The specificity constant for the enzyme did not vary significantly in the presence of Mg^{2+}, Mn^{2+}, or Co^{2+}, though the values were 2- to 5-fold lower when Ca^{2+}, Cd^{2+}, Ni^{2+}, or Zn^{2+} were used. From these kinetic analyses, it is evident that the REase exhibits efficient DNA cleavage with a wide range of cofactors and the catalytic efficiency is dependent on the properties of the cofactor.

Comparison between cofactor preference of PD-(D/E)XK and HNH enzymes suggest the latter group to have a greater flexibility for metal ion co-ordination. A few PD-(D/E)XK REases exhibit dismal DNA cleavage when Mg^{2+} is substituted with other metal ions.[5,6] Although HNH enzymes bind different metal ions,[10,14] these nucleases often prefer only either alkaline earth or transition group metal ions for DNA cleavage. Colicins, for example, utilize a few first row transition metal ions such as Zn^{2+}, Co^{2+}, and Ni^{2+},[11,18] whereas I-CmoeI cleaves its DNA substrate in the presence of alkaline earth metal ions but not transition group.[19] The active site of R.*Kpn*I thus appears to be distinct since it accommodates a battery of divalent cofactors belonging to both alkaline earth and transition groups for DNA cleavage.

Catalytic Mechanism. In R.*Kpn*I, H149 represents the invariant first His of the HNH motif that activates a water molecule for an in-line nucleophilic attack on the scissile phosphodiester bond.[4] D148 and Q175 constitute the other two residues of the enzyme for the Mg^{2+}-mediated catalysis. The enzyme's ability to cleave DNA with both alkaline earth and transition group ions could imply the utilization of different mechanisms of catalysis,

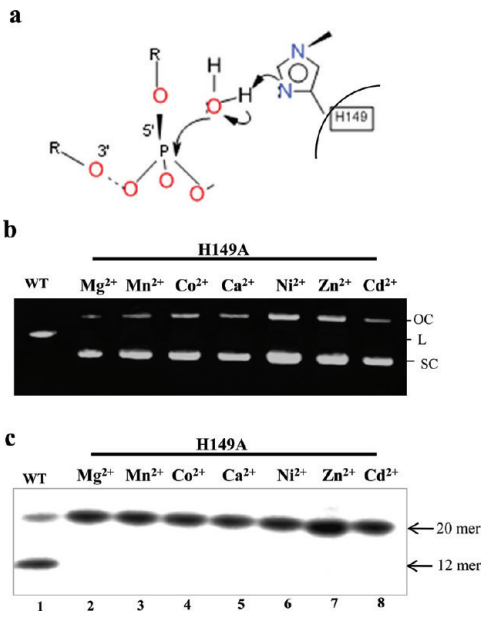

Figure 2. DNA cleavage activity of H149A mutant in the presence of different cofactors. (a) H149, general base of R.*Kpn*I activates the nucleophilic water molecule to facilitate phosphodiester bond cleavage. (b, c) H149A (100 nM) was incubated with 0.5 mM metal ions and (b) supercoiled pUC18 (14 nM) or (c) end labeled duplex 1 (10 nM) containing GGTACC sequence in buffer containing 10 mM Tris-HCl pH 7.4 at 37 °C for 1 h . Lane 1 is substrate incubated with WT (1 nM) and 0.5 mM Mg^{2+}. Lanes 2−8 are DNA incubated with H149A (100 nM) in the presence of alkaline earth and transition group cofactors as indicated. SC, L, and OC indicate the positions of the supercoiled, linear, and open circular forms of the plasmid respectively.

since each metal ion differ in their radii and co-ordination properties.[17] Further, diverse metal ions may require the same set of residues for co-ordination in the active site, or alternatively engage different residues. The Ala replacement of the H149 caused abolition of the enzyme activity with all of the metal ions tested, indicating that the cofactors employ the same catalytic mechanism (Figure 2). Substitution of Mg^{2+} with other cofactors in the mutants compromised in Mg^{2+}-mediated cleavage could reveal the differences in the metal ion co-ordination. When the DNA cleavage property of the D148A and Q175A mutants was analyzed in the presence of different metal ions, they exhibited a highly reduced activity (Supplementary Figure S3a), indicating the stringent requirement for carboxylate and carboxyamide side chains for the co-ordination with the metal ions tested. DNA binding activity of the metal ion co-ordination mutants was not affected (Supplementary Figure S3b). Therefore, the highly reduced DNA cleavage observed with the mutant enzymes is not because of the poor DNA binding ability. The indispensible role of His149 in the activation of the nucleophile and the wide range of cofactor tolerance by R.*Kpn*I is indicative of metal ions playing a significant role in electrostatic stabilization of transition state intermediates. As shown in the case of many metallonucleases and phospholipases, their role in the case of R.*Kpn*I could be to counter-balance the local negative charge formed in the active site during the course of the reaction.[20,21]

Effect of Metal Ions on DNA Cleavage Specificity. Non-canonical sequences are poorly discriminated from the canonical sequence by R.*Kpn*I in Mg^{2+}- and Mn^{2+}-catalyzed reactions but not in the presence of Ca^{2+} and Zn^{2+}.[7,8] To determine whether

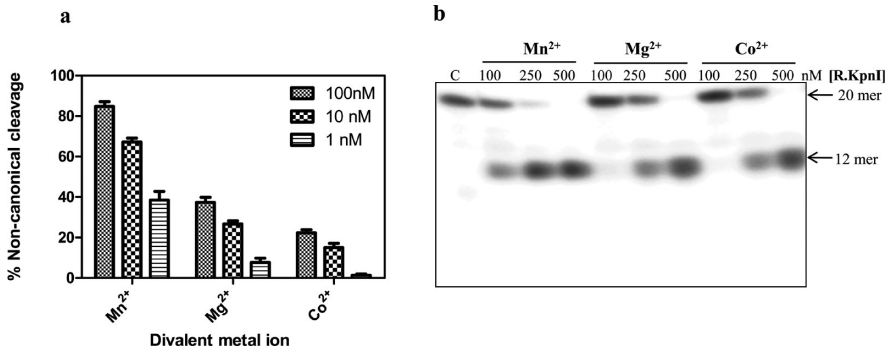

Figure 3. Effect of metal ions on the promiscuous and ssDNA cleavage activity of R.*Kpn*I. (a) The noncanonical DNA cleavage activity was assayed in the presence of different metal ions. Reactions contained 10 nM of duplex 3 (GaTACC) in 10 mM Tris-HCl pH 7.4 and 2 mM Mn^{2+}, Mg^{2+}, or Co^{2+} and incubated at 37 °C for 1 h. The percentage cleavage of the oligonucleotide was plotted against the metal ion used. Noncanonical cleavage activity at three different concentrations of the enzyme (1, 10, and 100 nM) is shown. (b) ssDNA cleavage activity of R.*Kpn*I in the presence of 2 mM Mn^{2+}, Mg^{2+}, and Co^{2+}. Different concentrations (100, 250, and 500 nM) of the enzyme was incubated with 10 nM of the ssDNA (top strand of the duplex 1) in buffer containing 10 mM Tris-HCl pH 7.4 at 37 °C for 1 h.

other cofactors induce promiscuous activity or retain specificity, the DNA cleavage was monitored using one of the most preferred noncanonical sites, GaTACC. Co^{2+} also induced a high degree of promiscuous activity of the enzyme in addition to Mg^{2+} and Mn^{2+}, whereas cleavage was not detectable with Ca^{2+}, Cd^{2+}, Ni^{2+}, or Zn^{2+} even at high enzyme concentrations and prolonged incubation periods. Promiscuous activity was most pronounced in the presence of Mn^{2+}, followed by Mg^{2+} and then Co^{2+} (Figure 3a). Mn^{2+} exhibited a 2-fold higher promiscuous activity compared to Mg^{2+}, whereas Co^{2+} exhibited 2-fold lower activity compared to the latter. Table 1 summarizes the steady-state kinetic analysis of DNA cleavage carried out in the presence of Mg^{2+}, Mn^{2+}, and Co^{2+}. The enzyme exhibited low degree of discrimination in the presence of Mg^{2+}, Mn^{2+}, and Co^{2+} (120, 20, and 280 times, respectively) for canonical versus noncanonical oligonucleotide cleavage.

Next, we investigated whether the enzyme is competent in catalyzing single stranded (ss) DNA cleavage by using the 5′-end labeled ssDNA fragment containing the recognition sequence. The enzyme exhibited endonucleolytic activity in the presence of Mg^{2+}, Mn^{2+}, and Co^{2+} (Figure 3b), but no detectable activity was observed in the presence of Ca^{2+}, Cd^{2+}, Ni^{2+}, or Zn^{2+} (data not shown). However, in contrast to dsDNA cleavage, the hydrolysis of ssDNA was observed only at higher enzyme (~250 units) and metal ion (2 mM) concentrations. DNA binding studies with ssDNA substrates revealed that the enzyme forms a weak affinity complex (Supplementary Figure S4a) that correlates with the enzyme's low activity. Cleavage of the ssDNA relies on the same HNH motif (Supplementary Figure S4b), indicating the inherent flexibility of R.*Kpn*I active site to accommodate both ssDNA and dsDNA. ssDNA cleavage observed with a few other HNH nucleases is dependent on the cofactor present in the reaction.[10,14] For example, the ColE9 nuclease prefers Mg^{2+} and Ni^{2+} for the cleavage of ds- and ssDNA, respectively.[10] Similarly, the HNH REase, R.MnlI, exhibits weak ssDNA cleavage activity in the presence of transition group metal ions.[14] Unlike the above examples, both alkaline earth (Mg^{2+}) as well as transition group (Mn^{2+} and Co^{2+}) ions induce ssDNA cleavage in the case of R.*Kpn*I. The three metal ions that induce promiscuous activity on dsDNA substrate also facilitate ssDNA cleavage indicating the role of these cofactors in relaxation of the substrate requirement.

Effect of Metal Ions on DNA Binding Specificity. Studies with various PD-(D/E)XK family of REases demonstrated that the metal ions play a major role in modulating specificity of the enzyme for DNA binding.[5,12,13] For example, the DNA binding affinity of R.EcoRV and R.PvuII at canonical sequences increased 10,000- and 6,000-fold, respectively, in the presence of Ca^{2+}.[12,13] In R.*Kpn*I, the cofactors may have a varied influence on the enzyme binding to canonical *versus* noncanonical DNA. Alternatively, the metal ions could induce discrimination at the step of phosphodiester bond cleavage. To investigate the influence of the cofactors at the DNA binding step, a catalytically inactive mutant, H149A, was used in the electrophoretic mobility shift assays (EMSA). The mutant enzyme bound to all of the cofactors tested, suggesting that the H149 is not implicated in metal ion binding (Supplementary Figure S5). DNA binding studies with canonical oligonucleotides revealed that the binding constants for the enzyme in the absence and presence of divalent metal ions did not vary significantly (Table 1), indicating that the cofactor binding may not enhance the enzyme binding to the recognition sequence. These results correlate with the Michaelis constants determined for R.*Kpn*I—canonical DNA complex in the presence of various metal cofactors (Table 1).

To examine the role of the metal ions in DNA recognition, the specificity factor (ratio of binding affinity at canonical to noncanonical sequences) was determined in the presence of each metal ion that induces DNA cleavage. The specificity factors with different metal ions were not as high compared to the other REases (Supplementary Table S1), indicating that the discrimination for cleavage of noncanonical sequences by the enzyme in the presence of Ca^{2+}, Cd^{2+}, Ni^{2+}, or Zn^{2+} occurs at a step after DNA binding. Therefore, loss of high sequence specificity observed with Mg^{2+}, Mn^{2+}, or Co^{2+} could not be solely due to the poor discriminatory capability at the step of DNA binding. For enzymes having their three-dimensional structures elucidated, it is proposed that the metal ions affect substrate positioning at the active site. In R.EcoRV, Mn^{2+} has been shown to rescue mutations that disrupt substrate positioning.[22] Similar results were observed with DNA polymerases and recombinases where substitution of Mn^{2+} for the catalytic metal ion Mg^{2+} affected specificity by altered substrate positioning.[20,21,23] Thus, it may be reasonable to assume that the different ions interact differently with the scissile phosphodiester bond at the R.*Kpn*I catalytic site.

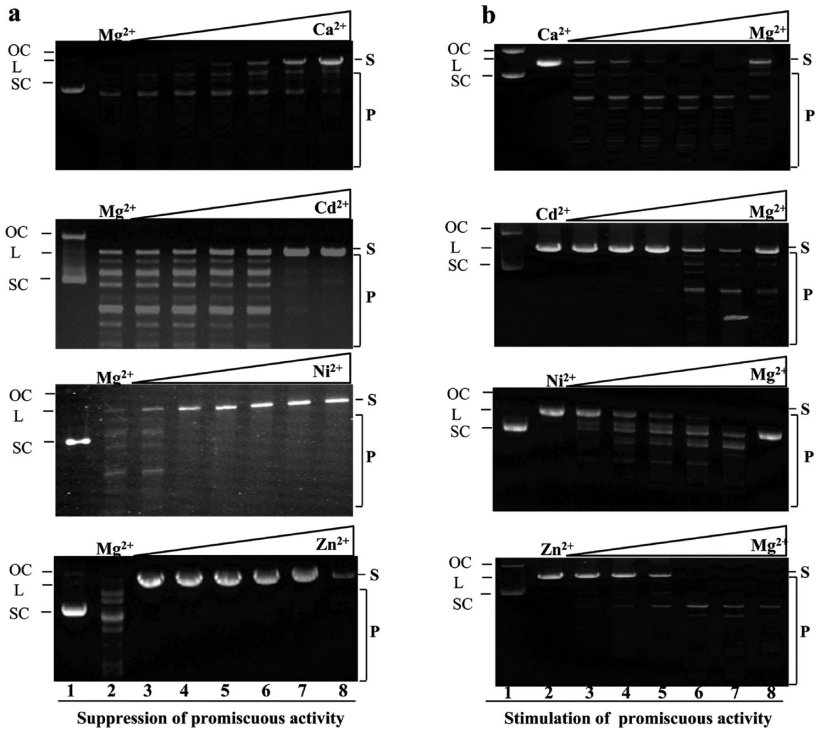

Figure 4. R.*Kpn*I has an adaptable active center. Active site flexibility was investigated by assaying DNA cleavage activity in the presence of different titrating cofactors. Reactions contained 30 nM R.*Kpn*I, and competition was performed by preincubation of the enzyme in buffer containing 10 mM Tris-HCl pH 7.4, on ice with the indicated metal ions. The reactions were initiated by addition of supercoiled pUC18 DNA (14 nM) and incubation at 37 °C for 1 h. (a) Suppression of promiscuous activity. Promiscuous DNA cleavage in the presence of 2 mM Mg^{2+} (lane 2) was titrated with increasing concentrations (0−5 mM) of Ca^{2+}, Cd^{2+}, Ni^{2+}, or Zn^{2+} (lanes 3−8). (b) Stimulation of promiscuous activity. High fidelity DNA cleavage in the presence of Ca^{2+} (2 mM), Ni^{2+}, Cd^{2+}, or Zn^{2+} (0.5 mM) was titrated with increasing concentrations (0−5 mM) of Mg^{2+}. S and P indicate specific and promiscuous DNA cleavage products, respectively. SC, L, and OC indicate the positions of the supercoiled, linear, and open circular forms of the plasmid, respectively.

Mg^{2+}, Mn^{2+}, and Co^{2+} may facilitate cleavage at noncanonical sites by relaxing the substrate positioning in the active site.

Active Site Plasticity of R.*Kpn*I. The ability to use a range of metal ions for catalysis (Figure 1) revealed the inherent flexibility of the enzyme in metal ion accommodation at the active site. Moreover, all of the cofactors tested employ the same catalytic mechanism for DNA cleavage (Figure 2). Such flexibility and utilization of a single metal ion mechanism should readily allow the exchange of the metal ions at the active site. Metal ion chase experiments were carried out on the Mg^{2+}, Mn^{2+}, or Co^{2+} bound enzyme−DNA complex by initial incubation of the enzyme with 2 mM Mg^{2+}, 2 mM Co^{2+}, or 0.5 mM Mn^{2+} followed by titration with different concentrations of Ca^{2+}, Cd^{2+}, Zn^{2+}, and Ni^{2+}. Replacement of a metal ion was monitored by the suppression of the promiscuous activity (Figure 4a). With Ca^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+}, decrease in the promiscuous activity was observed (Figure 4a, Supplementary Table S2). In converse experiments when fixed amounts of Ca^{2+} (2 mM), Ni^{2+}, Cd^{2+}, or Zn^{2+} (0.5 mM) and increasing concentrations of Mg^{2+} were used, the promiscuous activity of the enzyme was restored (Figure 4b). The altered cleavage pattern at high Mg^{2+} concentration (Figure 4b, lane 8) could be due to metal ion−DNA interactions. These observations indicate that the cofactors effectively replace each other to occupy the same active site of the enzyme.

Metal Ion Concentration Dependent DNA Cleavage Modulation. A catalytic and regulatory two-metal model has been proposed for many nucleases exhibiting either inhibition or stimulation of DNA cleavage at higher concentrations of the cofactor.[21,24,25] In general, binding of an additional Mg^{2+} or Mn^{2+} to the enzyme−DNA complex caused inhibition, whereas Ca^{2+} binding at the second site stimulated the Mg^{2+}- or Mn^{2+}-mediated DNA cleavage.[24] In order to better understand the DNA cleavage modulation of R.*Kpn*I, the metal ion activation profile was examined with both the alkaline earth and transition group ions used for the DNA cleavage studies. R.*Kpn*I was incubated with varying concentrations of the divalent metal ions, and the reactions were initiated by the addition of the substrate DNA. The enzyme exhibited robust DNA cleavage at metal ion concentrations as low as 50 μM (Figure 5 and Supplementary Figure S6). The similar activation profiles indicate plasticity in the cofactor utilization and rule out background activity from adventitious metal ion binding. At higher concentrations (>500 μM) of Cd^{2+}, Ni^{2+}, and Zn^{2+}, inhibition of DNA cleavage was evident (Figure 5b, lanes 7−9). In contrast, Mg^{2+}, Mn^{2+}, Co^{2+}, and Ca^{2+} did not inhibit the cleavage even at a concentration of 10 mM. With Mg^{2+}, Mn^{2+}, and Co^{2+} the enzyme exhibited promiscuous activity. Whereas Mg^{2+} and Co^{2+} required ≥500 μM concentrations for inducing promiscuous activity, Mn^{2+} stimulated promiscuity at ≥100 μM concentrations. However, Ca^{2+}-mediated high fidelity of the enzyme did not follow either one of the patterns.

These results imply that R.*Kpn*I exhibits two different metal activation profiles, one for the canonical DNA cleavage and another for either cleavage of the noncanonical DNA substrates

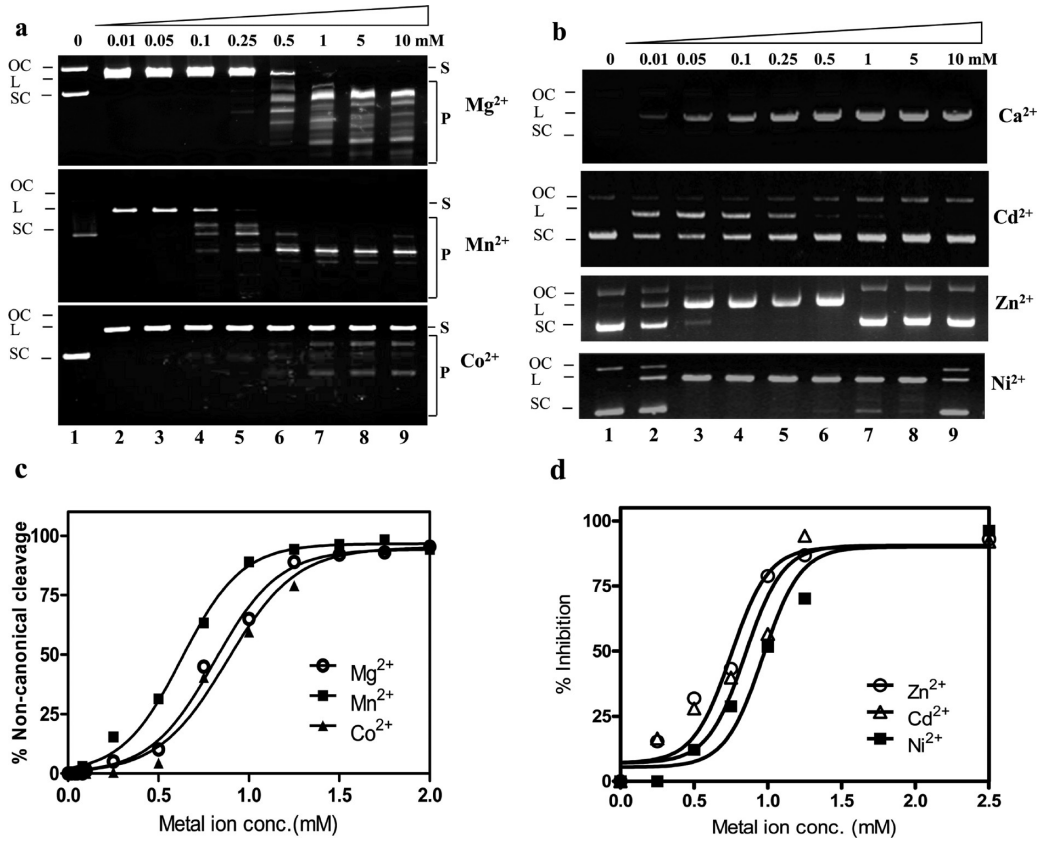

Figure 5. Concentration-dependent modulation of R.*Kpn*I activity by metal cofactors. Different concentrations of metal ions (0−10 mM) were incubated with 30 nM (a and b) or 15 nM (c and d) of R.*Kpn*I in buffer containing 10 mM Tris-HCl pH 7.4, on ice for 5 min. DNA cleavage reaction was initiated by the addition of (a and b) supercoiled pUC18 DNA (14 nM) or end labeled (c) duplex 3 (10 nM) or (d) duplex 1 (10 nM) and incubated at 37 °C for 1 h . (a) Activation profile in the presence of Mg^{2+}, Mn^{2+}, and Co^{2+}, which induce promiscuous DNA cleavage by R.*Kpn*I. (b) Activation profile in the presence of Ca^{2+}, Cd^{2+}, Ni $^{2+}$, and Zn^{2+}, which induce specific cleavage. At higher concentrations of Cd^{2+}, Ni^{2+}, and Zn^{2+}, inhibition of DNA cleavage is observed. S and P indicate specific and promiscuous DNA cleavage products respectively. SC, L, and OC indicate the positions of the supercoiled, linear, and open circular forms of the plasmid respectively. (c and d) Graphical representation of (c) noncanonical DNA cleavage with increasing concentrations (0−2.0 mM) of Mg^{2+}, Mn^{2+}, and Co^{2+}. (d) DNA cleavage inhibition with increasing concentrations (0−2.5 mM) of Ni^{2+}, Cd^{2+}, and Zn^{2+}. The dependence of DNA cleavage on [Me^{2+}] was analyzed by Hill equation using Graphpad Prism 4 as described in Methods. The maximal cleavage activity or inhibition is normalized to 100%.

or inhibition of the phosphodiester bond hydrolysis. The profile for canonical DNA cleavage suggested saturation of the HNH active site at lower concentrations (Supplementary Figure S6). At higher metal ion concentrations, the enzyme followed a sigmoidal pattern for the cleavage of noncanonical substrate or inhibition of enzyme activity (Figure 5). The sigmoidal pattern of metal activation indicated the recruitment of second metal ion (M2) to the enzyme−DNA complex in addition to the one at the active site (M1). The sharp transitions observed from high fidelity DNA cleavage to promiscuous activity (Mg^{2+}, Mn^{2+}, and Co^{2+}) or inhibition (Cd^{2+}, Ni^{2+}, and Zn^{2+}) indicate the cooperative binding behavior of the additional metal ion to the enzyme. Hill coefficients were derived to determine the number of metal ions required for the observed noncanonical DNA cleavage or inhibition of the enzyme activity (Supplementary Table S3). Hill coefficient for the promiscuous activity in the presence of Mg^{2+}, Mn^{2+}, and Co^{2+} was in the range of 3.3−3.9. Similarly, values of 3.6−4.1 were obtained for Ni^{2+}, Cd^{2+}, and Zn^{2+}, the ions that inhibit the enzyme activity at higher concentrations. Since the Hill coefficient indicates the minimal number of ligands involved in the reaction, the values represent four metal ions per dimer for promiscuous DNA cleavage or inhibition of the enzyme activity. On the basis of these results, we propose that one metal ion is essential for enzyme activation, and the binding of the second metal ion to the enzyme−DNA complex plays a role in regulating the specificity or activity. Since the DNA cleavage modulation is observed only at higher concentrations of the ions, affinity for the second metal ion should be lower and dependent on occupancy of the primary catalytic site.

Structural studies with $\beta\beta\alpha$-Me finger nucleases have provided some insights on the role of additional metal ion binding sites and the binding of second metal ion. These studies suggested that the secondary site binding could either provide structural integrity for the enzyme or modulate cleavage activity. For example, I-PpoI, R.Hpy99I, and R.PacI have two zinc binding sites that function in structural stability in addition to the active site cofactor required for catalysis.[15,26,27] The regulatory role for additional metal ion was hypothesized in the case of *Anabaena* NucA, wherein three Mg^{2+} ions were detected per monomeric enzyme.[28] Similarly, cofactor activation profiles and DNA cleavage assays suggested a modulatory role for additional metal ion in nonspecific nuclease Colicin E7.[18,29] The role for second site bound metal ion in modulation of R.*Kpn*I activity is amply evident from these studies. The data, however, do not exclude

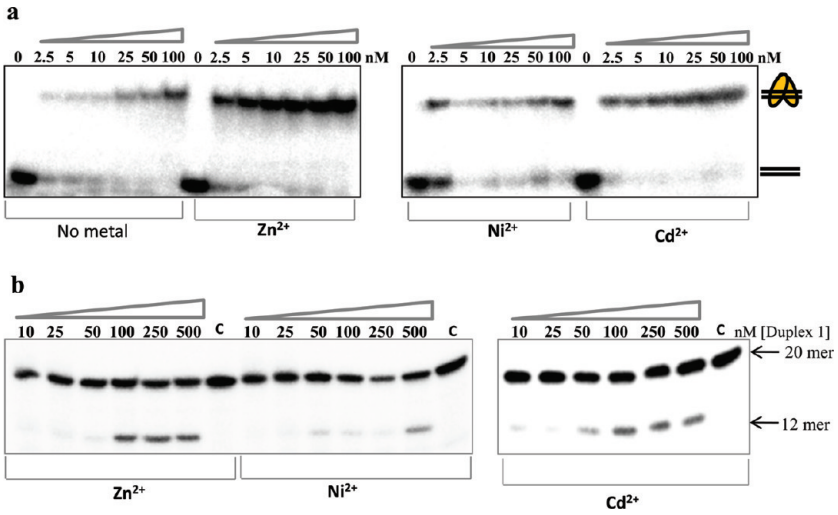

Figure 6. R.*Kpn*I forms reversible enzyme−Me^{2+}−DNA complex. (a) EMSA analyses. Different concentrations of R.*Kpn*I (0−100 nM) were incubated with 1 nM of end labeled duplex 1 and in the absence or presence of inhibitory concentrations (2 mM) of Zn^{2+}, Ni^{2+}, or Cd^{2+} as indicated in binding buffer (20 mM Tris-HCl pH 7.4, 25 mM NaCl, and 5 mM 2-mercaptoethanol) on ice for 15 min. (b) The DNA cleavage reactivation was assayed in the presence of increasing substrate concentrations. End labeled duplex 1 (10 nM) was preincubated with R.*Kpn*I (5 nM) in 10 mM Tris-HCl (pH 7.4) containing 2 mM of Ni^{2+}, Cd^{2+}, or Zn^{2+} to form the cleavage incompetent complex. The reactions were tested for reactivation by addition of increasing concentrations (0−500 nM) of unlabeled duplex 1 to the reaction mixture and incubated at 37 °C for 1 h. Lane C is DNA incubated with 2 mM of Ni^{2+}, Cd^{2+}, or Zn^{2+} as indicated.

the importance of the secondary metal ion binding for maintenance of the structure. Unaltered thermal unfolding profiles of the enzyme in the absence and presence of metal ions indicate that the structural role, if any, is not very significant (Supplementary Figure S7).

DNA Cleavage Inhibition by Cd^{2+}, Ni^{2+}, and Zn^{2+}. From the DNA cleavage studies described (Figure 5), it is evident that Ni^{2+}, Cd^{2+}, and Zn^{2+} support enzyme activity at low concentrations but inhibit at higher concentrations. Transition group metal ions are known to induce structural transitions in DNA.[17] The higher metal ion concentrations might thus affect the substrate availability to the enzyme. However, the DNA binding studies presented in Figure 6a reveal that the enzyme could bind DNA at inhibitory concentrations of Ni^{2+}, Cd^{2+}, or Zn^{2+}. Alternatively, the enzyme might form a cleavage-incompetent R.*Kpn*I−Me^{2+}−DNA complex under these conditions.

To investigate whether Ni^{2+}-, Cd^{2+}-, or Zn^{2+}-bound R.*Kpn*I-DNA complex could be reactivated by the substrate, increasing concentrations of canonical DNA was supplemented in the reaction. The enzyme regained the activity and exhibited DNA cleavage with increasing concentrations of the canonical oligonucleotide (Figure 6b), demonstrating that the inhibition exhibited by excess Ni^{2+}, Cd^{2+}, and Zn^{2+} could be relieved. The reactivation of the enzyme with substrate is indicative of the formation of a reversible cleavage-incompetent R.*Kpn*I−Me^{2+}−DNA complex. The inhibition of DNA cleavage by binding of a second Mg^{2+} is overcome by increasing the substrate concentration in case of the R.*Eco*RI family of REases.[24] Thus, we presume that binding of two Ni^{2+}, Cd^{2+}, or Zn^{2+} to the R.*Kpn*I−DNA complex leads to a catalytically inactive R.*Kpn*I−Me^{2+}−DNA ternary complex. However, under most physiological conditions, the enzyme is unlikely to be inhibited by these metal ions given their low intracellular concentration.[30]

Concentration dependent modulation of DNA cleavage specificity and activity by recruitment of additional metal ions is an intriguing feature in R.*Kpn*I. Changes in the substrate preference at greater than activating metal ion concentrations is reported for other classes of DNA transaction enzymes and attributed to binding of the cofactor at an additional weak affinity site. For example, bacteriophage T5 exonuclease exhibits endonuclease activity at low concentrations of Mg^{2+}, Mn^{2+}, and Co^{2+} and exonuclease activity at higher concentrations.[31] The DEDDh active site of 3′h exonuclease utilizes two metal ions along with a general base His.[21] The metal ion A functions in the transition state stabilization, while metal ion B has been proposed to play a role in orientation of the terminal phosphate. In contrast to the above, in the case of R.*Kpn*I, occupancy of the secondary site leads to a decrease in fidelity (Mg^{2+}, Mn^{2+}, or Co^{2+}) of the enzyme or its inhibition (Cd^{2+}, Ni^{2+}, or Zn^{2+}) depending on the cofactor (Figure 7). The larger ionic radius (1.14 Å) and flexible coordination properties of Ca^{2+} could possibly cause steric hindrance to accommodate the second atom, thus leading to high fidelity cleavage even at high concentrations. Structure of R.*Kpn*I, which has eluded so far, would be necessary to completely explain different properties induced by the metal ions. Although Zn^{2+} appears to be one of the cofactors of choice for a few HNH nucleases,[10,14,18] its concentration seems to play different roles in their activity. In ColE7, low concentrations of Zn^{2+} support DNA hydrolysis, but higher concentrations inhibits.[18] In R.*Kpn*I, we presume that at higher concentrations of the metal ion, a binuclear Zn^{2+} is preferred, rendering the enzyme not conducive for catalysis.

In summary, exquisite site specificity and dependency on Mg^{2+} for catalysis are the hallmark features of diverse classes of Type II REases. Other metal ions resembling Mg^{2+} generally do not engage in catalysis. In a few cases where they do participate, the activity is not comparable to that of Mg^{2+}.[5] R.*Kpn*I is an exception. The enzyme exhibits remarkable active site plasticity as the cofactors are readily exchanged. Both alkaline earth and transition metal ions support DNA cleavage in HNH active site catalyzed phosphodiester bond hydrolysis. However, recruitment of an additional metal ion regulates the DNA cleavage, inducing either promiscuous activity or cleavage inhibition.

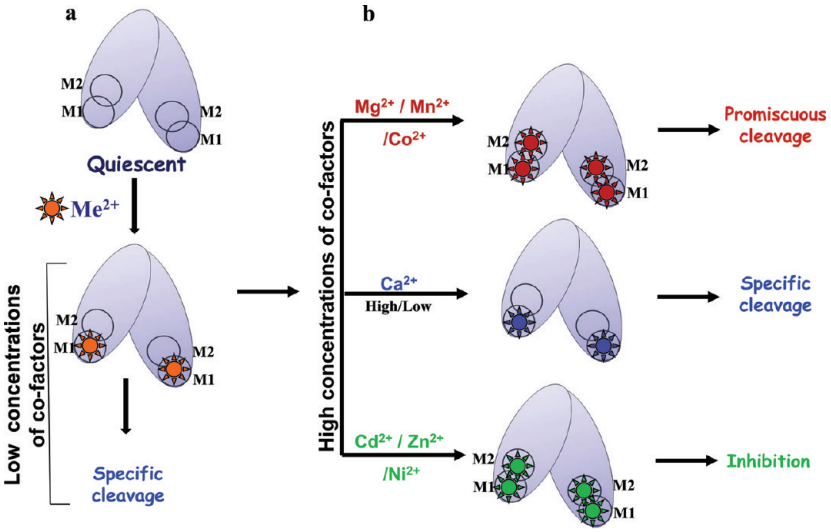

Figure 7. Model depicting the possible mechanism of metal ion-mediated reactions of R.*Kpn*I. The homodimeric model of R.*Kpn*I is shown with two metal ion binding sites (M1 and M2) per monomer. The enzyme binds to substrate in the absence of metal ion but does not cleave DNA, representing a quiescent state. The primary HNH active site is represented as M1, and the additional metal ion binding site is represented as M2. (a) Low concentrations of metal ions belonging to both alkaline earth and transition groups induce high fidelity DNA cleavage by R.*Kpn*I. (b) At high concentrations, depending on the metal ion bound at the second binding site (M2), the enzyme exhibits promiscuous activity (Mg^{2+}, Mn^{2+}, and Co^{2+}) or inhibition of DNA cleavage (Cd^{2+}, Ni^{2+}, and Zn^{2+}). High fidelity DNA cleavage is observed even at higher concentrations of Ca^{2+}, suggesting a single metal ion binding to the enzyme.

The flexibility in metal ion utilization by R.*Kpn*I could provide the organism with an evolutionary advantage since the REase can function regardless of the availability of a particular metal ion. The facile exchange of one metal ion with another at the active site may be an adaptation required to modulate the endonuclease specificity. We presume that R.*Kpn*I has the potential to exhibit promiscuous activity *in vivo*, given the free intracellular concentrations of Mg^{2+} ranging from 0.3 to 2 mM.[32,33] Retaining the promiscuity in the cleavage characteristics of the enzyme may provide certain advantage for the organism. The biological relevance of the promiscuous DNA cleavage is an important question that needs further investigation.

The ability of R.*Kpn*I and a few other HNH nucleases to utilize different metal ions opens up avenues for redesigning the cofactor specificities and generation of mutants specific to one metal ion. Such enzymes engineered for selective metal ion dependent activities could find applications in various genetic, biochemical and biophysical methods.[34] The architectural plasticity of the R.*Kpn*I active site and metal ion concentration dependent modulation of specificity and activity may reflect an emergent evolution serving yet unknown function(s) in *Klebsiella pneumoniae*.

METHODS

Bacterial Strains, Media, Enzymes, Metal Ions, and DNA Substrates. Plasmid pETRK (encoding R.*Kpn*I) was transformed into *E. coli* BL26 harboring M.*Kpn*I (pACMK), and cells were grown in Luria−Bertani broth as described.[8] The metal ion salts ($MgCl_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$, $CoCl_2$, $Cd(Ac)_2$, $CuSO_4$, $NiSO_4$, $SrCl_2$, and $BaCl_2$) were purchased from Sigma-Aldrich with ≥98% purity. Oligonucleotides used for DNA binding and cleavage assays are listed in Supplementary Table S4. Oligonucleotides were purified on 18% urea-polyacrylamide gel, end labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP (6000 Ci/mmol), and annealed to the 3-fold molar excess of the complementary strand. Cleavage assays were carried out using supercoiled pUC18 DNA (contains one canonical and multiple noncanonical sites) and 5′-[^{32} P] labeled ss or duplex oligonucleotides.

DNA Binding and Cleavage. Proteins were dialyzed against EDTA to remove the intrinsically bound metal cofactor. DNA binding and cleavage assays were carried out as described previously.[7] EMSA in the presence of different cofactors was carried out in the binding buffer containing 250 μM (Mn^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, and Ni^{2+}) or 2 mM (Mg^{2+} and Ca^{2+}) metal ions. The concentrations of metal ions used for the cleavage activity ranged from 10 μM to 10 mM. The dependence of DNA cleavage on [Me^{2+}] was analyzed with the Hill equation: $Y_{(cMe)} = (c_{Me})^n/[Me]^n_{0.5} + (c_{Me})^n$ with the maximal activity of DNA cleavage normalized to 100%; Y, the percentage of activity or inhibition observed in the presence of varying metal ion concentrations (c_{Me}); n, Hill coefficient; $[Me]_{0.5}$, the concentration of metal ion that yields 50% cleavage or inhibition. Hill coefficients were determined by fitting the data to the Hill equation using Graphpad Prism version 4.

Steady-state kinetic experiments in the presence of different cofactors were conducted as described previously.[7] The kinetic reactions were carried out under conditions of 5- to 250-fold molar excess of substrates. The kinetic parameters were determined by fitting the velocity with the substrate concentration using Graphpad Prism version 4. The turnover number (k_{cat}) was calculated as the ratio of V_{max} to the enzyme concentration.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

REase, restriction endonuclease; WT, wild type; ss, single stranded; ds, double stranded; EMSA, electrophoretic mobility shift assay

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