R.KpnI, an HNH superfamily REase, exhibits differential discrimination at non-canonical sequences in the presence of Ca$^{2+}$ and Mg$^{2+}$

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ABSTRACT

KpnI REase recognizes palindromic sequence, GGTAC$^\#C$, and forms complex in the absence of divalent metal ions, but requires the ions for DNA cleavage. Unlike most other REases, R.KpnI shows promiscuous DNA cleavage in the presence of Mg$^{2+}$. Surprisingly, Ca$^{2+}$ suppresses the Mg$^{2+}$-mediated promiscuous activity and induces high fidelity cleavage. To further analyze these unique features of the enzyme, we have carried out DNA binding and kinetic analysis. The metal ions which exhibit disparate pattern of DNA cleavage have no role in DNA recognition. The enzyme binds to both canonical and non-canonical DNA with comparable affinity irrespective of the metal ions used. Further, Ca$^{2+}$-impacted exquisite specificity of the enzyme is at the level of DNA cleavage and not at the binding step. With the canonical oligonucleotides, the cleavage rate of the enzyme was comparable for both Mg$^{2+}$- and Mn$^{2+}$-mediated reactions and was about three times slower with Ca$^{2+}$. The enzyme discriminates non-canonical sequences poorly from the canonical sequence in Mg$^{2+}$-mediated reactions unlike any other Type II REases, accounting for the promiscuous behavior. R.KpnI, thus displays properties akin to that of typical Type II REases and also endonucleases with degenerate specificity in its DNA recognition and cleavage properties.

INTRODUCTION

Type II REases are homodimers, which recognize short palindromic sequences of 4–8 bp in length and in the presence of Mg$^{2+}$, cleave the DNA within the recognition site to generate 5’-phosphate and 3’-OH ends (1). Type II REases have been broadly classified into two groups, based on structural architecture and mode of DNA recognition (2,3). Further, EcoRI and many other enzymes bind to DNA preferentially at their recognition sites in the absence of divalent metal ions (4,5). In contrast, EcoRV, TaqI and MunI bind both specific and non-specific sequences with similar affinities in the absence of divalent metal ions. With the latter set of enzymes, binding to the canonical sequence is increased by about 1000-fold in the presence of divalent metal ions (6–8). Irrespective of this difference in binding characteristics, both the groups of enzymes need divalent metal ion, generally Mg$^{2+}$, for catalysis.

Type II REases exhibit a very high degree of site specificity towards their recognition sequences over non-canonical sequences and non-specific sequences. However, many of them exhibit relaxed specificity under sub-optimal conditions (4,9). R.KpnI isolated from Klebsiella pneumoniae recognizes the palindromic DNA sequence 5’-GGTAC$^\#C$–3’ and cleaves DNA generating four base 3’ overhangs (10,11). The enzyme exhibits promiscuous DNA cleavage in the presence of Mg$^{2+}$ and attains exquisite sequence specificity in the presence of Ca$^{2+}$ (12). In addition to imparting specific DNA cleavage, Ca$^{2+}$ suppresses Mg$^{2+}$- or Mn$^{2+}$-mediated relaxed specificity of the enzyme (12).

Type II REases differ in their amino acid sequence from one another, and indeed from all other protein families (13). Recent structural and bioinformatic analysis of Type II REases revealed that they possess common structural fold around and comprising the active site (14). A vast majority of Type II REases utilize PD/EXK motif as a catalytic motif, wherein the carboxylic side chains of the acidic amino acids play a role in metal ion coordination and the bound water is involved in phosphodiester bond hydrolysis by acid–base mechanism (4). Unlike most other REases, R.KpnI utilizes HNH motif as a catalytic motif and follows the mechanism of ββα-Me finger nucleases, such as colicins E7, E9, Vvn and Serratia nuclease (15).
These enzymes recognize and cleave degenerate or non-specific DNA sequences and employ a histidine residue to activate the attacking water molecule (16–18).

Although a number of REases have been characterized, kinetic analysis has been carried out only with few of the enzymes. Amongst these, Rsrl, EcoRI, EcoRV, BamHI, MnuI and PvuII, have been subjected to detailed kinetic studies (19–26). All these extensively studied enzymes belong to PD ... D/EXK family, yet seem to have varied kinetic properties on canonical, non-canonical and non-specific sequences. Owing to its high degree of promiscuous activity, specific cleavage in the presence of Ca$^{2+}$, suppression of Mg$^{2+}$- and Mn$^{2+}$-mediated promiscuity by Ca$^{2+}$ and use of HNH motif instead of PD...D/EXK for catalysis (15), R.KpnI is markedly different from other Type II REases subjected to kinetic analysis so far. Here, we have investigated the DNA binding and catalytic behavior of R.KpnI and describe features underlying the metal ion dependence during specific and promiscuous DNA cleavage reactions.

**MATERIALS AND METHODS**

**Enzymes and chemicals**

Oligonucleotides used in the study are listed in Table 1. Oligonucleotides (Microsynth and Sigma) purified on 15% urea-polyacrylamide gel were 5’ end-labelled using T4 polynucleotide kinase and [$\gamma$-32P] ATP (6000 Ci/mmol) and further purified using G-50 spin column chromatography.

T4 polynucleotide kinase (New England Biolabs), [$\gamma$-32P] ATP, Hi-Trap Heparin column (Amersham Biochemicals), ampicillin, chloramphenicol, bovine serum albumin, polyethyleneimine, Commassie Brilliant Blue, and IPTG (Sigma). Phosphocellulose P11 (Whatman) were used in the study.

**Expression and purification of R.KpnI**

R.KpnI was expressed in E. coli BL26 [F-omp T hsdSB (r$^\text{m}$ m$^\text{n}$) gal dcm Δlac (DE3) nin5 lacUV5-T7 gene 1] cells in the presence of pACMK (plasmid-expressing kpnI methylase gene) (27). Escherichia coli BL26 cells harboring R.KpnI (pETRK) clone were grown at 37°C in LB medium containing 100 µg/ml ampicillin and 20 µg/ml chloramphenicol until the absorbance at 600 nm reached 0.6 and induced with 0.3 mM IPTG. The protein was purified to near homogeneity as described previously (27). Protein concentrations were estimated by the Bradford method using bovine serum albumin as the standard.

**Electrophoretic mobility shift assay**

Different concentrations of R.KpnI or its mutant H149A (0.5–256 nM) were incubated with 1 nM of end-labeled double-stranded oligonucleotides containing canonical (duplex I) and non-canonical sites (duplex II to VIII) in the binding buffer [20 mM Tris-HCl (pH 7.4), 35 mM NaCl and 5 mM 2-mercaptoethanol] on ice for 15 min. The gels were electrophoresed at 4°C and 100 V for 2 h. The amounts of DNA in free and bound forms were quantitated using Phosphorimagier Image Gauge software Version 2.54. The assays were repeated thrice and the dissociation constants were determined by fitting these data to a standard hyperbolic curve using Graphpad Prism software Version 4. For EMSA in the presence of metal ions, the binding buffer contained 20 mM Tris-HCl (pH 7.4), 5 mM 2-mercaptoethanol and either 5 mM CaCl$_2$ or MgCl$_2$. The gel running buffer (pH 8.2) contained 89 mM Tris-boric acid and either 2 mM CaCl$_2$ or MgCl$_2$. All other reaction conditions for electrophoresis and analysis were identical to those performed in the absence of divalent metal ions.

**Steady-state kinetic assays**

For kinetic analysis, the purified enzyme was dialyzed against 10 mM EDTA to remove any bound metal ions. Steady-state kinetic time courses with canonical and non-canonical DNA substrates were measured at DNA concentrations of 5–150-fold molar excess over dimeric enzyme (1 nM). The steady-state reactions were carried out at 37°C in an assay buffer containing 10 mM Tris-HCl, pH 7.4, 35 mM NaCl, 5 mM 2-mercaptoethanol and 5 mM Mg$^{2+}$ or Mn$^{2+}$ or Ca$^{2+}$. Aliquots were taken out at 10 different time points for the determination of initial velocity. The reactions were terminated by adding an equal volume of stop dye containing urea (4 M), 2% EDTA, 2 µl formamide, 1 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue. The samples were analyzed on 15% denaturing 8 M urea-polyacrylamide gels. Electrophoresis was carried out at 250 V with 1 x Tris-boric acid-EDTA as the running buffer and cleavage products were quantified by phosphorimaging analysis. Steady-state kinetic parameters were determined by fitting the change in the velocity with substrate concentration to the double reciprocal (1/V versus 1/[S]) Lineweaver–Burk plot using Graphpad Prism 4. The turnover number ($k_{cat}$) was calculated as the ratio of $V_{max}$ to the enzyme concentration used (28).

**Preincubation studies**

Preincubation studies were carried out by initiating the reactions with metal ion (5 mM) or oligonucleotide

**Table 1. Duplex DNA oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Duplex</th>
<th>Oligonucleotide Sequence</th>
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<tbody>
<tr>
<td>I</td>
<td>5’-ATTGCGTGTAACCCTCCTTT-3’</td>
</tr>
<tr>
<td></td>
<td>3’-TAACGCAACATGGGCGAGAA-5’</td>
</tr>
<tr>
<td>II</td>
<td>5’-ATTGCGTGTAACCCGCTCTT-3’</td>
</tr>
<tr>
<td></td>
<td>3’-TAACGCAACATGGGCGAGAA-5’</td>
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<td>III</td>
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<td>3’-TAACGCAACATGGGCGAGAA-5’</td>
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<td>IV</td>
<td>5’-ATTGCGTGTAACCCTCCTTT-3’</td>
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<td>3’-TAACGCAACATGGGCGAGAA-5’</td>
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<tr>
<td>V</td>
<td>5’-ATTGCGTGGAACCCTCCTTT-3’</td>
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<td></td>
<td>3’-TAACGCAACATGGGCGAGAA-5’</td>
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<tr>
<td>VI</td>
<td>5’-ATTGCGTGTAACCCTCCTTT-3’</td>
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<td></td>
<td>3’-TAACGCAACATGGGCGAGAA-5’</td>
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<td>VII</td>
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<td>3’-TAACGCAACATGGGCGAGAA-5’</td>
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</tr>
<tr>
<td></td>
<td>3’-TAACGCAACATGGGCGAGAA-5’</td>
</tr>
</tbody>
</table>
substrates (2.5 nM) or R.KpnI (0.5 nM) at 15°C. Samples were removed after fixed time intervals and analyzed as described for cleavage assays.

**Intrinsic fluorescence**

Fluorescence emission spectra were collected on a Jobin-Yvon fluorometer FluoroMax 3 (HORIBA, Jobin-Yvon/Spex Division, Longjumeau, France), thermostated at 25°C. EDTA-treated wt R.KpnI or H149A (2 μM concentration each) in a buffer containing 20 mM Tris-HCl pH (7.4) and 5 mM 2-mercaptoethanol were incubated with different concentrations of metal ions for 15 min at 25°C. All samples were incubated until the equilibrium was established under a particular set of conditions before measuring the fluorescence intensities. All fluorescence emission spectra and fluorescence intensities from titrations were corrected for protein tryptophan fluorescence by subtraction of control spectra and control titrations. Fluorescence intensities were plotted against the metal ion concentrations, and the data were analyzed according to the modified Stern–Volmer equation (29).

\[
\frac{F_0}{(F_0 - F)} = \frac{1}{[Q] \times f_a \times K_Q} + \frac{1}{K_d}
\]

where \(F_0\) and \(F\) are fluorescent intensities in the absence and presence of cofactor respectively, \(Q\) is the quencher (metal ion) concentration; \(f_a\) is the fractional number of fluorophores, \(K_Q\) is the quenching constant. The dissociation constants were calculated from the modified Stern–Volmer plot (a plot of \(F_0/(F_0 - F)\) versus \(1/[Q]\)), where \(K_Q = 1/K_d\) (29).

**RESULTS AND DISCUSSION**

**Steady-state kinetic analysis of R.KpnI in the presence of Mg^{2+}, Mn^{2+} and Ca^{2+}**

Previously, we demonstrated that Ca^{2+}, in addition to Mg^{2+} and Mn^{2+}, supported the R.KpnI endonuclease cleavage activity (12). Unlike many other REases, the enzyme showed high promiscuous activity in the presence of Mg^{2+} and this behavior was completely suppressed with Ca^{2+} resulting in high specificity cleavage (12). For determining the kinetic parameters of R.KpnI under steady-state reaction conditions, end-labeled 20-mer double-stranded oligonucleotides (duplex II to VIII containing substitutions at each position of the hexanucleotide recognition sequence, Table 1) were chosen as substrates. DNA cleavage was not detectable with any of the non-canonical sites when reactions were carried out with Ca^{2+} as a cofactor even at high concentrations of the enzyme and prolonged incubation periods. However, in the presence of Mg^{2+} and Mn^{2+}, all the non-canonical substrates were cleaved, although the extent of cleavage was varied. The sequences GtTACC, GaTACC, GTtACC and GGTtAC were cleaved better than the sequences GtTACC, GaTACC and GGTtAC. The kinetic parameters of the detailed analysis are summarized in Table 2. With Mg^{2+} and Mn^{2+} as cofactors, R.KpnI discriminates the non-canonical sites by 90–2500 times and 30–900 times respectively (Table 2).

In contrast, all other REases studied so far exhibit no cleavage with Ca^{2+} and reduced DNA cleavage at the canonical site with Mn^{2+} (30). For instance, EcoRV has 15-fold higher \(k_{cat}/K_m\) value for canonical duplex with Mg^{2+} when compared to that of Mn^{2+} (22). MunI and EcoRI also showed reduced DNA cleavage with Mn^{2+} as cofactor (8,31).

**Steady-state kinetic analysis at non-canonical sites**

Next, we tested the DNA cleavage of non-canonical substrates in the presence of different metal ions. For carrying out kinetic studies with DNA containing the promiscuous sites, 20-mer double-stranded oligonucleotides (duplex II to VIII containing substitutions at each position of the hexanucleotide recognition sequence, Table 1) were chosen as substrates. DNA cleavage was not detectable with any of the non-canonical sites when reactions were carried out with Ca^{2+} as a cofactor even at high concentrations of the enzyme and prolonged incubation periods. However, in the presence of Mg^{2+} and Mn^{2+}, all the non-canonical substrates were cleaved, although the extent of cleavage was varied. The sequences GtTACC, GaTACC, GTtACC and GGTtAC were cleaved better than the sequences GtTACC, GaTACC and GGTtAC. The kinetic parameters of the detailed analysis are summarized in Table 2. With Mg^{2+} and Mn^{2+} as cofactors, R.KpnI discriminates the non-canonical sites by 90–2500 times and 30–900 times respectively (Table 2).

In contrast, all other REases studied so far exhibit a larger degree of discrimination in the presence of Mg^{2+}, when recognizing the non-canonical sequences. For instance, with its natural cofactor Mg^{2+}, and under optimal reaction conditions, EcoRV and EcoRV cleave their respective recognition sites 10^5 and 10^6 times faster than sites differing by one base pair (20,32). In Mn^{2+}-catalyzed reactions, however, the discrimination of EcoRV, EcoRI and TaqI endonucleases against non-canonical sequences was greatly reduced (22,33,34). From the kinetic constants obtained for R.KpnI, it is evident that R.KpnI exhibits very low degree of discrimination during Mg^{2+}-mediated cleavage, accounting for its highly promiscuous behavior (Table 2).

**Basis of discrimination**

A high degree of sequence specificity is achieved by Type II REases as a result of their discrimination of the non-canonical sequences from the canonical sequence either during binding or cleavage or at both the steps (4,20,35). The different metal ions may have a varied influence on the enzyme binding to canonical and
non-canonical DNA sequences. Alternatively, the differences in the metal ion binding to the enzyme active site could account for the property. The results presented in Figure 2 demonstrate that all the three metal ions use the same active site characterized earlier (15). A mutant R.KpnI, in which invariant H149 of the HNH motif is replaced by alanine, did not show any cleavage irrespective of the metal ions used, indicating that the same catalytic scheme is employed in all the cases. Similar metal-ion-binding pattern by the wt and H149A mutant suggests that H149 is not implicated in metal ion binding. Fluorescence spectroscopy was used to evaluate binding of the metal ions to the enzyme, taking advantage of the presence of three tryptophans in the protein. The change in tryptophan fluorescence intensity was measured as a function of the metal ion concentration. The results presented in Figure 3A show representative spectra with Mg$^{2+}$ as cofactor. With increasing concentrations of the metal ion, fluorescence quenching was observed. Mn$^{2+}$ and Ca$^{2+}$ also showed similar concentration-dependent fluorescence quenching. However, monovalent cations such as Na$^+$ did not influence the fluorescence spectra of the enzyme (data not shown). A modified Stern–Volmer plot was used to analyze the quenching (Figure 3B). The linearity of the plot suggests that Mg$^{2+}$ binding is a dominant fluorescence-quenching phenomenon over the range of concentrations used (0.1–10 mM). The $K_D$ values calculated from modified Stern–Volmer relationship are 550 μM for Mg$^{2+}$, 200 μM for Mn$^{2+}$ and 700 μM for Ca$^{2+}$. The data presented in Figure 3C and D show comparable binding of all the three metal ions to both wt and active site mutant H149A. These results indicate that the metal binding to the enzyme by itself may not account for the lower discrimination exhibited by the enzyme.

In order to understand the role of divalent metal ions in DNA binding, we investigated the binding affinity of R.KpnI using the wt and H149A mutant. Initially, we carried out the DNA-binding analysis in the absence of any metal ion cofactors. Both the wt and the H149A mutant enzymes bound with similar affinity to the canonical site (duplex I) as well as non-canonical sites (duplex II to VIII) (Table 3). Next, we investigated the
DNA substrates & $K_M$ (M) & $k_{cat}(s^{-1})$ & $k_{cat}/K_M(M^{-1}s^{-1})$ & Discrimination factor* \\
--- & --- & --- & --- & --- \\
Duplex I (-GGTACC-) & & & & \\
Mg$^{2+}$ as cofactor & 22 (±2.6) x 10$^{-9}$ & 0.072 ± 0.002 & 3.2 (±0.3) x 10$^6$ & ndc \\
Mn$^{2+}$ as cofactor & 21 (±3.4) x 10$^{-9}$ & 0.077 ± 0.003 & 3.6 (±0.4) x 10$^6$ & ndc \\
Ca$^{2+}$ as cofactor & 36 (±2.8) x 10$^{-9}$ & 0.037 ± 0.003 & 1.0 (±0.1) x 10$^6$ & ndc \\
Duplex II (-GTAACC) & & & & \\
Mg$^{2+}$ as cofactor & 221 (±10.6) x 10$^{-9}$ & 0.00029 ± 0.00001 & 1.3 (±0.06) x 10$^3$ & ndc \\
Mn$^{2+}$ as cofactor & 198 (±3.4) x 10$^{-9}$ & 0.00003 ± 0.00001 & 4.1 (±0.50) x 10$^3$ & ndc \\
Ca$^{2+}$ as cofactor & ndc & ndc & ndc & ndc \\
Duplex III (-GGaACC) & & & & \\
Mg$^{2+}$ as cofactor & 69 (±9.3) x 10$^{-9}$ & 0.0026 ± 0.0002 & 3.76 (±0.2) x 10$^4$ & ndc \\
Mn$^{2+}$ as cofactor & 112 (±7.4) x 10$^{-9}$ & 0.013 ± 0.002 & 1.16 (±0.1) x 10$^5$ & ndc \\
Ca$^{2+}$ as cofactor & ndc & ndc & ndc & ndc \\
Duplex IV (-GGAtACC) & & & & \\
Mg$^{2+}$ as cofactor & 97 (±1.6) x 10$^{-9}$ & 0.0028 ± 0.0001 & 3.6 (±0.02) x 10$^4$ & ndc \\
Mn$^{2+}$ as cofactor & 123 (±2.7) x 10$^{-9}$ & 0.012 ± 0.0016 & 1.0 (±0.06) x 10$^5$ & ndc \\
Ca$^{2+}$ as cofactor & ndc & ndc & ndc & ndc \\
Duplex V (-GGaACC) & & & & \\
Mg$^{2+}$ as cofactor & 210 (±4.3) x 10$^{-9}$ & 0.00068 ± 0.00004 & 3.2 (±0.2) x 10$^3$ & ndc \\
Mn$^{2+}$ as cofactor & 168 (±8.4) x 10$^{-9}$ & 0.00032 ± 0.00002 & 1.9 (±0.1) x 10$^4$ & ndc \\
Ca$^{2+}$ as cofactor & ndc & ndc & ndc & ndc \\
Duplex VI (-GGTICC) & & & & \\
Mg$^{2+}$ as cofactor & 164 (±3.1) x 10$^{-9}$ & 0.0027 ± 0.0005 & 1.64 (±0.5) x 10$^4$ & ndc \\
Mn$^{2+}$ as cofactor & 103 (±4.1) x 10$^{-9}$ & 0.013 ± 0.003 & 1.26 (±0.3) x 10$^5$ & ndc \\
Ca$^{2+}$ as cofactor & ndc & ndc & ndc & ndc \\
Duplex VII (-GGAaTC) & & & & \\
Mg$^{2+}$ as cofactor & 174 (±2.8) x 10$^{-9}$ & 0.0010 ± 0.0001 & 5.7 (±0.9) x 10$^3$ & ndc \\
Mn$^{2+}$ as cofactor & 135 (±3.2) x 10$^{-9}$ & 0.0034 ± 0.00012 & 2.56 (±0.1) x 10$^4$ & ndc \\
Ca$^{2+}$ as cofactor & ndc & ndc & ndc & ndc \\
Duplex VIII (-GGTACt) & & & & \\
Mg$^{2+}$ as cofactor & 199 (±3.0) x 10$^{-9}$ & 0.00091 ± 0.00004 & 4.6 (±0.2) x 10$^3$ & ndc \\
Mn$^{2+}$ as cofactor & 156 (±2.6) x 10$^{-9}$ & 0.0043 ± 0.00014 & 2.7 (±0.2) x 10$^4$ & ndc \\
Ca$^{2+}$ as cofactor & ndc & ndc & ndc & ndc \\

*Discrimination factor = $[k_{cat}/K_M($canonical substrate$)]/[k_{cat}/K_M($non-canonical substrate$)]$. 
ndc: no detectable cleavage.

![Figure 2](https://academic.oup.com/nar/article-abstract/35/8/2777/1041786)

**Figure 2.** DNA cleavage activity of H149A mutant R.Kpnl in the presence of different metal ions. End-labeled duplex I was incubated with R.Kpnl (1 nM) and H149A (10, 100 nM) in the presence of 5 mM of Mg$^{2+}$, Mn$^{2+}$ and Ca$^{2+}$ as indicated. In all the cases, the reactions were incubated at 37°C for 30 min, and the products were analyzed on 12% urea-polyacrylamide gel.

DNA-binding properties of the mutant H149A in the presence of Mg$^{2+}$ or Mn$^{2+}$ or Ca$^{2+}$ using the canonical and non-canonical sequences. The binding affinity of the enzyme to the DNA was comparable with all the three metal ions. More importantly, the metal ions did not enhance the binding of the enzyme to the recognition sequence (Table 3).

The difference in the ability of the enzyme to cleave non-canonical sequences in Mg$^{2+}$- versus Ca$^{2+}$-mediated reactions allowed us to explore the mechanism of Ca$^{2+}$-specific discrimination of non-canonical sequence for DNA cleavage. From the DNA-binding studies presented in Figure 4A and B, it is evident that the enzyme binds to the non-canonical sites with comparable affinity to that of canonical sequence both in the presence and absence of Ca$^{2+}$ (Table 3). This was rather surprising as these sequences are refractile to Ca$^{2+}$-mediated DNA cleavage (Table 2). The mutant H149A exhibits similar affinity with both canonical and non-canonical substrates in the presence of Ca$^{2+}$ (Figure 4C, D and E). From these studies, it is clear that a Ca$^{2+}$-mediated higher degree of discrimination by the enzyme is exerted only at the step of DNA cleavage and not at the step of binding.

**Preincubation studies**

While the above experiments revealed the basis for differential discrimination imposed by the metal ions for
DNA cleavage, it is not yet clear how these differences are brought about by the enzyme using the same active site. A possible reason could be that the enzyme follows altered reaction path(s) in the presence of different metal ions. The order of binding to substrate could be varying with each metal ion. In order to understand the mechanistic details and the order of events involved in DNA cleavage reaction, we have carried out preincubation studies. DNA cleavage was initiated either by the addition of R.KpnI to preincubated DNA and Mg\(^{2+}\) or addition of DNA to R.KpnI preincubated with Mg\(^{2+}\) or by the addition of Mg\(^{2+}\) to preincubated DNA-R.KpnI complexes. Similar experiments were carried out with Mn\(^{2+}\) and Ca\(^{2+}\) as cofactors instead of Mg\(^{2+}\). The order of preincubation had significant influence on the initial rate of the product formation. Most of the DNA was cleaved within the initial time point in the case of metal-ion-initiated reactions (Figure 5). On the other hand, as can be seen from Figure 5A, at every time point between 30 and 120 s, 2-fold lower rates were observed with the reactions initiated with DNA or the enzyme, implying that the pre-formed enzyme–DNA complex could be catalytically more efficient than the pre-formed enzyme–metal ion complex. However, after 10 min, the extent of cleavage was comparable in all the three cases. From these results, it appears that R.KpnI follows a rather random order in substrate and cofactor binding, with a preference for binding the DNA substrate first.

A hallmark characteristic of Type II REases is their exquisite sequence specificity (36). R.KpnI, in contrast, exhibits high degree of promiscuous DNA cleavage in the presence of Mg\(^{2+}\) and Mn\(^{2+}\). The kinetic parameters of R.KpnI with canonical and non-canonical sequences suggest that the enzyme is not able to discriminate...
Table 3. DNA-binding affinities of wt R.KpnI and mutant H149A REases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_d$ (nM)</th>
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<tbody>
<tr>
<td><strong>R.KpnI (absence of metal ions)</strong></td>
<td></td>
</tr>
<tr>
<td>Duplex I (-GGTACC-)</td>
<td>9 ± 2.3</td>
</tr>
<tr>
<td>Duplex II (-GGACC-)</td>
<td>8 ± 2.4</td>
</tr>
<tr>
<td>Duplex III (-GaTACC-)</td>
<td>13 ± 1.7</td>
</tr>
<tr>
<td>Duplex IV (-GrTACC-)</td>
<td>12 ± 0.7</td>
</tr>
<tr>
<td>Duplex V (-GGACC-)</td>
<td>16 ± 0.3</td>
</tr>
<tr>
<td>Duplex VI (-GGTiCC-)</td>
<td>16 ± 2.1</td>
</tr>
<tr>
<td>Duplex VII (-GGTACt-)</td>
<td>29 ± 3.2</td>
</tr>
<tr>
<td>Duplex VIII (-GGTACt-)</td>
<td>28 ± 0.7</td>
</tr>
<tr>
<td><strong>H149A (absence of metal ions)</strong></td>
<td></td>
</tr>
<tr>
<td>Duplex I (-GGTACC-)</td>
<td>8 ± 1.6</td>
</tr>
<tr>
<td>Duplex II (-GGACC-)</td>
<td>18 ± 1.4</td>
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<tr>
<td>Duplex III (-GaTACC-)</td>
<td>14 ± 0.7</td>
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<td>Duplex IV (-GrTACC-)</td>
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<td>Duplex V (-GGACC-)</td>
<td>28 ± 1.8</td>
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<td>Duplex VI (-GGTiCC-)</td>
<td>23 ± 0.7</td>
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<td>Duplex VII (-GGTACt-)</td>
<td>19 ± 3.2</td>
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<tr>
<td>Duplex VIII (-GGTACt-)</td>
<td>26 ± 2.7</td>
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<tr>
<td><strong>H149A (presence of metal ions)</strong></td>
<td></td>
</tr>
<tr>
<td>Duplex I (-GGTACC-)</td>
<td>8 ± 2.4</td>
</tr>
<tr>
<td>Duplex II (-GGACC-)</td>
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<td>Duplex VIII (-GGACCt)</td>
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Figure 4. Electrophoretic mobility shift analyses for the determination of the binding affinity of the wt R.KpnI and mutant H149A in the presence of Ca$^{2+}$. (A) and (B) Gel shift showing the native gel (inset) with duplex II and III. The wt R.KpnI was titrated over the concentration range from 0 to 256 nM, and the concentration of the DNA was 25 pM. The intensity of the shifted DNA band are expressed as% bound. (C), (D) and (E) are the gel shifts showing the native gels (inset) with duplex I, II and III respectively using mutant H149A. The average values of the three different experiments were plotted in the graph.
non-canonical sequences effectively from the canonical sequence. In Mg\(^{2+}\)-catalyzed reactions, R.KpnI exhibits comparable \( k_{cat} \) value (4 min\(^{-1}\)) for canonical substrate as that of other REases, such as RsrI, EcoRV, EcoRI and BamHI (4.2, 8.4, 2.4 and 20.8 min\(^{-1}\) respectively) (19,22,31,37). However, in contrast to those enzymes, it exhibits very low degree of discrimination at non-canonical sites (Table 2). Our earlier analysis showed that R.KpnI belongs to the HNH endonuclease superfamily (15) and follows the mechanism similar to that of I-PpoI and I-CmeoI in that all of them utilize an invariant histidine residue as a general base during catalysis (38,39). I-PpoI is capable of cleaving sequences with single, double or multiple substitutions within the homing site (40,41). Kinetic analysis with I-CmeoI and I-PpoI showed that these enzymes exhibit \( k_{cat} \) values of 0.26 and 0.046 min\(^{-1}\) respectively. These values are 20–100 times lower than that of R.KpnI (4 min\(^{-1}\)). The specificity constant value of R.KpnI (3.1 \( \times \) 10\(^6\) M\(^{-1}\) s\(^{-1}\)) is 14 and 24 times lower than that of I-CmeoI (4.3 \( \times \) 10\(^7\) M\(^{-1}\) s\(^{-1}\)) and I-PpoI (7.5 \( \times \) 10\(^7\) M\(^{-1}\) s\(^{-1}\)) respectively. The higher values with the latter enzymes could be due to their binding to the long, extended DNA substrate with high affinity (39,42). Thus, as in the case of PD...D/EXK family enzymes, the members of HNH superfamily exhibit variations in kinetic properties. The present studies reveal that the lower degree of discrimination between canonical and non-canonical sequence for DNA binding by R.KpnI largely accounts for its higher promiscuity in Mg\(^{2+}\)-catalyzed reactions.

From the vast body of literature and this article, it is apparent that the nucleases exhibit a wide range in discrimination of the non-canonical sequences, manifested at the level of DNA binding and/or catalysis. Non-specific nucleases would, by and large, be non-discriminatory at the level of binding and catalysis.
In contrast, highly specific REases and enzymes with moderate sequence specificity may discriminate at the level of binding or during catalysis or at both the steps. Kinetically well-characterized REases such as EcoRV and PvuII discriminate at the level of DNA cleavage and not at the level of binding (23,26). In a recent structural and kinetic study, binding of EcoRV to GAATTC (EcoRI recognition site) was about two orders of magnitude lower compared to GATATC (EcoRV recognition site). The enzyme, however, was shown to discriminate GAATTC site for cleavage. The cleavage at this non-canonical sequence was reduced five orders of magnitude (43). EcoRI, on the other hand, discriminates non-canonical sequences at the level of both binding and catalysis (20). In contrast, I-PpoI, a member of HNH superfamily, fails to show appreciable discrimination while binding to homing sites and its variant sequences (40,41). Studies described here reveal that R.KpnI is a poor discriminator while binding to the non-canonical sequences. However, during catalysis, it exhibits remarkably varied property. Although bound to non-canonical sequences, DNA cleavage is not observed in the presence of Ca\(^{2+}\). Further, the extent of sequence discrimination in Mg\(^{2+}\)-catalyzed reactions is very low compared to any other site-specific, high-fidelity REases. Whether the low degree of discrimination and high promiscuity of R.KpnI is an intrinsic design of the organism is not yet known, and is an important question that needs to be addressed.

In conclusion, we have carried out analysis of DNA binding and cleavage by R.KpnI, a ββz-Me finger nuclease containing HNH active site. We have demonstrated that the enzyme has comparable specificity constant with Mg\(^{2+}\), Mn\(^{2+}\) and Ca\(^{2+}\), a unique property not seen with other REases. In addition, our study has established an unusually low discriminatory property of R.KpnI in Mg\(^{2+}\)-catalyzed reactions, atypical to other REases. Further, the Ca\(^{2+}\)-mediated discrimination towards non-canonical sequences is exerted only at the step of cleavage. The presence of HNH catalytic motif and the associated unusual properties of R.KpnI suggest its closer link to the enzymes which recognize degenerate recognition sequences and a distinct evolutionary origin from that of typical Type II REases.

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endonuclease modulated by a distal manganese ion binding site. 


