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Generation of a Manganese Specific Restriction Endonuclease with Nicking Activity†

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ABSTRACT: A typical feature of type II restriction endonucleases (REases) is their obligate sequence specificity and requirement for Mg^{2+} during catalysis. R.KpnI is an exception. Unlike most other type II REases, the active site of this enzyme can accommodate Mg^{2+}, Mn^{2+}, Ca^{2+}, or Zn^{2+} and cleave DNA. The enzyme belongs to the HNH superfamily of nucleases and is characterized by the presence of a $\beta\beta\alpha$-Me finger motif. Residues D148, H149, and Q175 together form the HNH active site and are essential for Mg^{2+} binding and catalysis. The unique ability of the enzyme to cleave DNA in the presence of different metal ions is exploited to generate mutants that are specific to one particular metal ion. We describe the generation of a Mn^{2+}-dependent sequence specific endonuclease, defective in DNA cleavage with Mg^{2+} and other divalent metal ions. In the engineered mutant, only Mn^{2+} is selectively bound at the active site, imparting Mn^{2+}-mediated cleavage. The mutant is impaired in concerted double-stranded DNA cleavage, leading to accumulation of nicked intermediates. The nicking activity of the mutant enzyme is further enhanced by altered reaction conditions. The active site fluidity of REases allowing flexible accommodation of catalytic cofactors thus forms a basis for engineering selective metal ion-dependent REase additionally possessing nicking activity.

Type II REases[1] typically cleave DNA at or near its recognition sequence to introduce double-strand breaks. More than 3900 REases have been characterized to date (*1*). Almost all these enzymes, except the phospholipase D family of REases, require a divalent cation for catalysis. Exquisite site specificity and a dependence on Mg^{2+} for catalysis are the hallmark characteristics of these diverse enzymes (*2*). Phosphodiester bond cleavage by metallonucleases is of central importance in many DNA transaction processes and involves several important mechanistic steps that include (a) activation of a nucleophile by a general base, (b) stabilization of the phosphoanion transition state, and (c) protonation of the leaving group (*3*). Although Mg^{2+} is the preferred cofactor for a vast majority of enzymes, Mn^{2+} can support DNA cleavage to varying degrees in several cases. Most often, Mn^{2+} replacement at the active site leads to low activity and fidelity in DNA cleavage in a variety of enzymes (*3*−*7*). A notable exception for this paradigm is R.KpnI, an enzyme discovered in 1978 that is a typical type IIP REase recognizing the palindromic sequence GGTACC (*8*). The enzyme shows comparable activity and catalytic efficiency in the presence of both Mg^{2+} and Mn^{2+} (*9*). In addition, the enzyme exhibits robust DNA cleavage in the presence of Ca^{2+} and Zn^{2+} (*10*, *11*).

R.KpnI was also the first REase identified to belong to the HNH superfamily having a $\beta\beta\alpha$-Me motif (*12*). From the studies on structures of several HNH enzymes, the $\beta\beta\alpha$ supersecondary structure has been implicated in providing a structural scaffold for the active sites. Residues present in the motifs determine metal ion selectivity. Homology modeling and its validation by site-directed mutagenesis showed that D148, H149, and Q175 residues of R.KpnI correspond to the D, H, and N or H residues of the HNH nucleases (Figure 1). Residues D148 and Q175 coordinated with Mg^{2+}, whereas H149 was shown to be required for activation of the nucleophile in the active site (Figure 1B).

Growing applications of REases in various in vitro and in vivo DNA manipulations provided the impetus for engineering hybrid enzymes' recognition of long DNA targets, or enzymes with nicking activities and altered specificities. Generation of hybrid REases that recognize long recognition sequences was achieved by fusion of the DNA cleavage domain of the type IIS REase FokI to modular zinc finger domains or using cleavage deficient mutants of the homing endonucleases (*13*−*15*). Type IIS REases have also been used to create nicking endonucleases (NEases) by inactivating or replacing one of the two subunits, or by changing the enzyme's oligomerization status (*16*−*19*). The majority of NEases are engineered by exploiting the asymmetric recognition pattern of type IIS REases. However, rational engineering of REases to possess altered specificities has met with limited success, most likely because of their high degree of DNA sequence specificity attained through multiple contacts with various structural elements of the enzyme (*20*−*22*). Type IIP enzymes cleave within their target sequence (*23*), and thus, nicking variants would be useful for generation of a nick within the palindromic recognition sequence. Although the role of metal ions in REases has been well-established by biochemical, kinetic, and structural studies, rational engineering to switch metal ion specificity has not been explored (*2*, *24*, *25*). The unique ability of R.KpnI to utilize different metal ions provides an opportunity for generation of mutants that are specific to one particular metal

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[1]Abbreviations: REase, restriction endonuclease; WT, wild type; K_d, dissociation constant; K_m, Michaelis constant; k_{cat}, rate constant; ds, double-stranded; NEase, nicking endonuclease.

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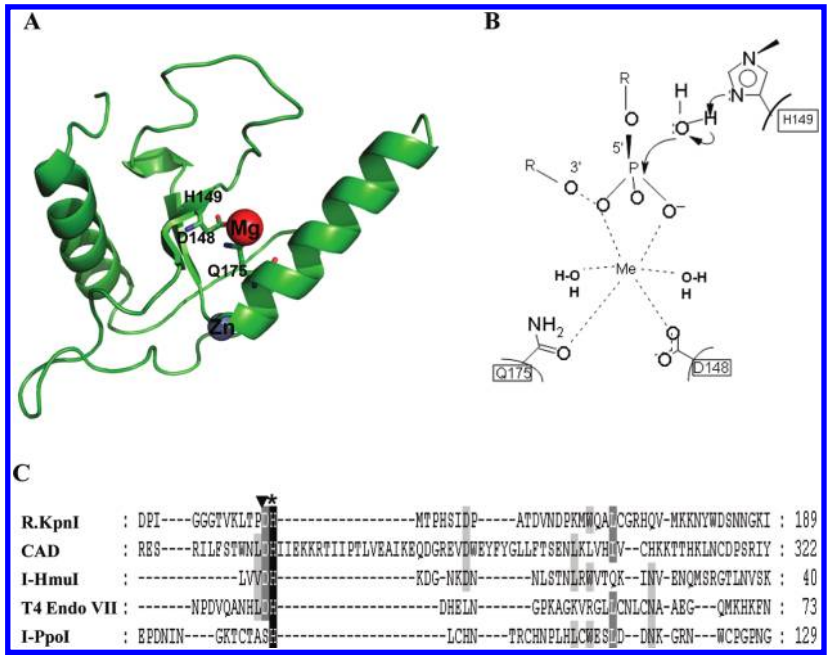

FIGURE 1: HNH motif and catalytic mechanism of R.KpnI. (A) The R.*Kpn*I monomer model is shown as a ribbon (with helices as spirals and strands as arrows). The Mg^{2+} and Zn^{2+} ions are shown as red and gray spheres, respectively. The side chains of active site residues are shown as sticks and labeled. (B) Catalytic mechanism of R.KpnI. Residues D148 and Q175 are shown in this scheme to be involved in metal coordination with H149 acting as a general base. H149 activates the nucleophilic water molecule to facilitate phosphodiester bond cleavage. (C) Multiple-sequence alignment of R.KpnI with other HNH enzymes was conducted to illustrate the conserved regions. The conserved regions are displayed as shaded boxes. The asterisk denotes the invariant histidine residue of HNH nucleases, and the arrowhead indicates the residue targeted for mutagenesis. Alignment was generated using ClustalW version 2.0 and analyzed using GeneDoc.

ion. We describe here the generation of a Mn^{2+}-dependent REase and sequence specific NEase by mutation of a single residue of the active site of R.KpnI.

EXPERIMENTAL PROCEDURES

Enzymes, Chemicals, and DNA. R.KpnI and its mutants were purified using the method described previously (*26*). The enzymes were diluted in buffer containing 20 mM Tris-HCl (pH 7.4), 25 mM NaCl, and 5 mM 2-mercaptoethanol for all the studies. The concentration of the proteins was estimated by the Coomassie Brilliant Blue dye binding procedure of Bradford using bovine serum albumin as a standard. The purity of the proteins was assessed by both sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and mass spectrometry.

Oligonucleotides (Microsynth and Sigma) purified on a 15% urea−polyacrylamide gel were labeled at the 5′ end using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (6000 Ci/mmol, Perkin-Elmer Life Sciences) and purified using G-50 spin column chromatography.

The Hi-Trap heparin column (GE Healthcare), ampicillin, chloramphenicol, bovine serum albumin, polyethyleneimine, Coomassie Brilliant Blue, IPTG (Sigma), and phosphocellulose P11 (Whatman) were used.

Site-directed mutants of R.KpnI were generated by the mega-primer inverse PCR method (*27*). Expression plasmid pETRK encoding the *kpnI*R gene (GenBank accession number P25237) was used as a template. Oligonucleotide primers carrying the respective mutant amino acid codon substitutions were used as forward primers, and the T7 terminator sequence was used as a reverse primer (Table S1 of the Supporting Information). The megaprimers generated served as complementary primers for the second round of PCR amplification. After the mutations had been confirmed by sequencing, the mutant REases were expressed in *Escherichia coli* BL26 [F^- *omp T hsd*SB (rB^- mB^-) gal *dcm* lac (DE3) *nin*5 *lac* UV5-T7 gene 1] containing the M.KpnI plasmid (*26*).

Electrophoretic Mobility Shift Assay. Different concentrations of the enzymes (0 to 256 nM) were incubated with 0.2 pmol of 5′ end-labeled double-stranded oligonucleotides (20 mer) containing the recognition site in binding buffer [20 mM Tris-HCl (pH 7.4) and 5 mM 2-mercaptoethanol] for 15 min on ice. Increasing concentrations of R.KpnI and its mutants were used to calculate the apparent dissociation constant (K_d) for DNA binding. The free DNA and the enzyme-bound complexes were resolved by 8% native PAGE in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 1 mM EDTA). The mobility shift assay in the presence of Mg^{2+} was conducted in the binding buffer containing 2 mM $MgCl_2$. The gel running buffer contained 89 mM Tris-boric acid and 2 mM $MgCl_2$. Gels were visualized with a phosphorimager (Fujifilm, FLA 5100) and quantified with Multi Gauge version 2.3.

In Vitro DNA Cleavage and Steady-State Kinetic Analysis. Digestions were conducted by incubating different units of R.KpnI and its mutants with 500 ng of pUC18 DNA (contains a single site for R.KpnI) or 0.2 pmol of labeled oligonucleotides in assay buffer containing 10 mM Tris-HCl (pH 7.4) and 10 μM to 10 mM Mg^{2+} or Mn^{2+} for 1 h at 37 °C. The reactions were terminated by adding stop dye (20% glycerol, 50 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol), and the cleavage products of plasmid DNA and oligonucleotides were analyzed on a 1% agarose and 12% urea−polyacrylamide gels, respectively. For the metal ion dependence assays, we initiated the reactions by adding substrate DNA and heating the reaction mixture to 37 °C after incubating the enzyme with different concentrations of metal ion on ice for 5 min. Steady-state kinetic experiments in the presence of 2 mM Mg^{2+} or 2 mM Mn^{2+} were conducted as described

previously (*28*). The kinetic parameters were determined by fitting the change in the velocity with substrate concentration to the double-reciprocal ($1/v$ vs $1/[S]$) Lineweaver−Burk plot by using GraphPad Prism version 4.

Circular Dichroism (CD) Measurements. Far-UV (250−205 nm) CD spectra of R.KpnI and its mutants were recorded at 20 °C using a Jasco J-720 spectropolarimeter with a 0.2 cm path length quartz cuvette. The protein concentration in the samples was 4 μM in 20 mM Tris-HCl (pH 7.4). The CD spectral data represent the average of three scans after correction for the buffer baseline and were reported as mean residue ellipticity [θ]. CD wavelength spectra were smoothed as described by Savitsky and Golay (*29*). CD spectra for metal ion-dependent structural changes in R.KpnI were recorded after incubation of the enzyme with different concentrations of metal ion on ice for 5 min. Binding constants were determined from the plot of ellipticity changes at a wavelength of 222 nm with an increasing metal ion concentration. Apparent dissociation constants were determined by fitting the curve to Hill analysis by GraphPad Prism version 4.

Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) Analysis. Metal ion stoichiometry was determined by using ICP-AES (PerkinElmer). Wavelengths used were 279.077 and 285.213 nm for Mg and 257.610 and 259.372 nm for Mn. EDTA-treated R.KpnI D148G (10 mg) was incubated with either 2 mM Mg^{2+} or 2 mM Mn^{2+}, and unbound metal ion was removed by dialysis overnight against 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl at 4 °C with buffer changes. Dialyzed proteins were quantified and digested with 1 N HNO_3. Digested samples were resuspended in 5 mL of doubly distilled water and subjected to ICP-AES analysis. The dialyzed buffer was used as a blank, and the residual metal ion background was subtracted from the measurement of protein samples.

RESULTS

Manganese Specific DNA Cleavage by the D148 Mutant. R.KpnI is the prototype of the HNH REases (*12*). In the enzyme, H149 represents the invariant first histidine of the HNH motif that activates a water molecule for an in-line nucleophilic attack on the scissile phosphate (Figure 1B). D148 and Q175 constitute the other two residues in the enzyme active site for Mg^{2+}-mediated catalysis (Figure 1). Q175 in R.KpnI substitutes for the last histidine of the HNH motif and plays a role both in Mg^{2+} coordination and in DNA binding (*12*). D148 is homologous to catalytically relevant residues found in other HNH nucleases (Figure 1C), and mutation of the residue results in impaired DNA cleavage by the enzyme in the presence of Mg^{2+} (*12*). The ability of the enzyme to cleave DNA in the presence of four metal ions, viz., Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+}, could imply utilization of different catalytic site(s) as each metal ion has a different coordination geometry (*30*). However, mutation of the invariant H149 residue of the HNH motif caused abolition of enzyme activity with Mg^{2+} as well as with three other metal ions, indicating that the cofactors employ the same catalytic mechanism for DNA cleavage (Figure S1 of the Supporting Information). The varied atomic radii of these metal ions and the differences in their coordination properties (*30*, *31*) warrant their binding to different sets of residues at the active site. Substitution of Mg^{2+} with other cofactors, viz., Mn^{2+}, Ca^{2+}, and Zn^{2+}, in the mutants compromised in Mg^{2+}-mediated cleavage could reveal differences in the metal ion coordination and might lead to altered cofactor specificity. This hypothesis was tested by generating mutants as described in Experimental Procedures. The CD studies showed no major structural perturbations with the purified mutant proteins (Figure S2 of the Supporting Information). When the DNA cleavage property of the mutants was analyzed in the presence of 2 mM Mg^{2+}, the D148E mutant exhibited activity comparable to that of R.KpnI. Because the Asp to Glu replacement caused no alteration in the side chain charge, coordination of Mg^{2+} and the DNA cleavage activity is retained. In contrast, alanine and glycine substitutions at position 148 showed a greatly reduced level of DNA cleavage (Table 1), indicating the stringent catalytic requirement for carboxylate side chains for Mg^{2+}-mediated cleavage. Next, the ability of the D148 variants to cleave DNA in the presence of Mn^{2+} was examined. Although the D148E mutant showed cleavage activity similar to that of Mg^{2+}-catalyzed reactions in the presence of Mn^{2+}, the D148A mutant did not exhibit efficient DNA cleavage (Table 1). However, substitution of D148 with Gly resulted in a high Mn^{2+}:Mg^{2+}cofactor specificity (determined as the ratio of specific activity in the presence of Mn^{2+} and Mg^{2+}). The mutant enzyme exhibited ~250-fold higher activity in the presence of Mn^{2+} versus Mg^{2+} (Figure 2 and Table 1). Significantly, replacement of Mn^{2+} with other divalent metal ions, viz., Ca^{2+} or Zn^{2+}, did not yield appreciable DNA cleavage, indicating that Mn^{2+} specifically rescues the loss of function of the D148G mutation (Figure S3 of the Supporting Information).

Table 1: Specific Activities of the Wild Type and D148 Variants

enzyne	specific activity (units/mg) Mg^{2+}	specific activity (units/mg) Mn^{2+}	cofactor specificity (Mn^{2+}:Mg^{2+})
WT	700000	850000	1.2
D148E	400000	600000	1.5
D148A	500	650	1.3
D148G	200	50000	250

To improve our understanding of the preferential Mn^{2+}-dependent activity in the D148G mutant, DNA cleavage was conducted as a function of divalent metal ion concentration. R.KpnI exhibits Mg^{2+}- and Mn^{2+}-mediated canonical DNA cleavage with 50% activity at metal ion concentrations of 40 and 35 μM, respectively, and reaches a plateau at 100 μM (Figure 2C). For the D148G mutant, Mn^{2+}-dependent specific cleavage shows a plateau at 1 mM with 50% activity at a metal ion concentration of 400 μM (Figure 2D). However, in the presence of Mg^{2+}, the mutant did not exhibit appreciable DNA cleavage even at high metal ion concentrations, indicating a possible impairment of Mg^{2+} binding ability in the mutant.

The D148G Mutant Is Quiescent in Vivo. Next, we investigated whether the D148G mutant that shows efficient DNA cleavage in vitro is also competent in conducting DNA cleavage in vivo. The intracellular cleavage activity was analyzed using plasmids harboring either R.KpnI or a mutant gene, by transforming the *E. coli* BL26 strain with or without the KpnI methyltransferase gene. Cells transformed with R.KpnI failed to survive in the absence of MTase, while cells expressing D148G did not exhibit such a growth defect (Figure 3). The mutant enzyme purified from cells lacking cognate MTase exhibited cleavage in the presence of Mn^{2+}. The absence of a growth defect could be an indication of reduced intracellular activity of the mutant.

Kinetic Analysis and Cleavage Specificity of the Mn^{2+} Rescue Mutant. To understand the characteristics of the

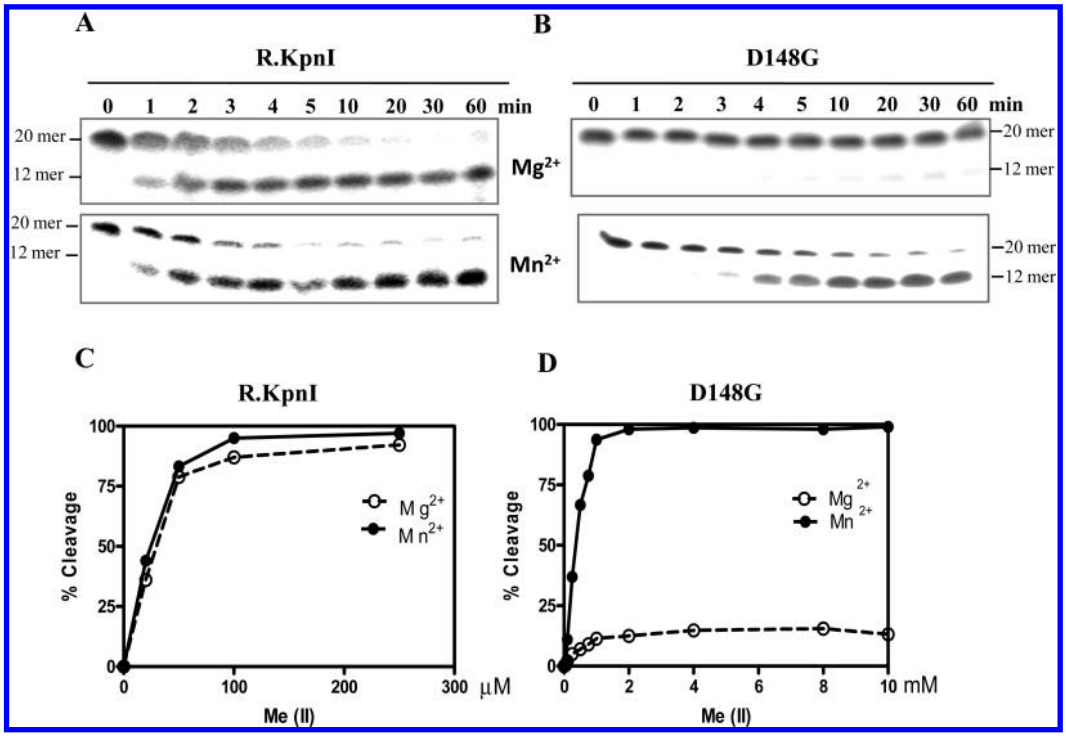

FIGURE 2: Cofactor preference of the D148G mutant. A 20 bp canonical oligonucleotide harboring a single R.KpnI site (GGTACC) was used for the assay that upon cleavage generates a labeled 12 mer fragment. (A and B) DNA cleavage activity of WT (1 nM) and D148G (10 nM) in the presence of Mg^{2+} and Mn^{2+}. Reactions were conducted with 0.2 pmol of oligonucleotide using 2 mM Mg^{2+} or 2 mM Mn^{2+}. The reaction mixtures were incubated at 37 °C. Aliquots were removed at different time intervals, and reactions were terminated by addition of 50 mM EDTA containing stop dye. (C and D) Metal ion-dependent DNA cleavage by WT and D148G. Reactions were conducted at 37 °C with varying concentrations of divalent metal ion (0−10 mM). The percentage of product formed was plotted versus metal ion concentration. (C) Metal activation profile of WT in the presence of Mg^{2+} and Mn^{2+}. (D) Metal activation profile of the D148G mutant enzyme in the presence of Mg^{2+} and Mn^{2+}.

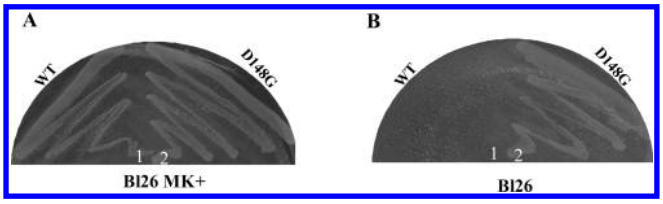

FIGURE 3: In vivo activity of the R.KpnI mutant. Growth patterns of *E. coli* BL26(DE3) cells overexpressing R.KpnI and its D148G mutant. The cytotoxicity assay was conducted in the (A) presence or (B) absence of the constitutively expressing KpnI methyltransferase gene in *E. coli* BL26(DE3) cells. The respective competent cells were individually transformed with the plasmid DNA, pETRK encoding either wild-type or D148G enzyme. After revival, the cells were streaked on an LB agar plate and incubated at 37 °C overnight to show their respective pattern. (A) Sector 1, WT in *E. coli* $BL26MK^+$; sector 2, D148G in *E. coli* $BL26MK^+$. (B) Sector 1, WT in *E. coli* BL26; sector 2, D148G in *E. coli* BL26.

D148G Mn^{2+} enzyme, catalytic parameters were determined by measuring the initial rate of substrate cleavage as a function of substrate concentration. Kinetic constants (K_m and k_{cat}) derived from Lineweaver−Burk plots indicated that the mutant exhibits robust DNA cleavage with Mn^{2+} (Table 2). Kinetic parameters could not be determined for the D148G Mg^{2+} enzyme even at high enzyme concentrations. The D148G mutant had a K_m of 14 nM for the canonical sequence, which is comparable to that of WT (21 nM). However, the mutant enzyme had a lower k_{cat} (0.5 min^{-1}). Thus, the comparable K_m of the mutant is an indication of a similar substrate affinity, while the lower k_{cat} suggests slower rates of subsequent cleavage steps.

Noncanonical sequences are poorly discriminated from the canonical sequence in Mn^{2+}-catalyzed reactions by R.KpnI (*9*, *10*).

Table 2: Kinetic Parameters

substrate	K_M (nM)	k_{cat} (min^{-1})	k_{cat}/K_M (s^{-1} M^{-1})	
		-GGTACC		
R.KpnI				
Mg^{2+a}	23 ± 2.6	4.32 ± 0.12	3.2×10^6	1[b]
Mn^{2+a}	21 ± 3.4	4.62 ± 0.18	3.6×10^6	1.12[b]
D148G				
Mn^{2+}	14 ± 2.1	0.50 ± 0.04	6.0×10^5	0.18[b]
		-GaTACC		
R.KpnI				
Mg^{2+a}	77 ± 1.6	0.17 ± 0.01	3.6×10^4	0.011[b]
Mn^{2+a}	123 ± 2.7	0.72 ± 0.12	1.0×10^5	0.031[b]
D148G				
Mn^{2+}	70 ± 1.7	0.01 ± 0.001	2.4×10^3	0.001[b]

[a]Kinetic constants obtained from Saravanan et al. (*9*). [b]Relative values with k_{cat}/K_M set to 1 for Mg^{2+}-mediated canonical DNA cleavage exhibited by WT.

To understand whether the preference for Mn^{2+} over Mg^{2+} affects the promiscuous behavior of the enzyme, a cleavage assay was conducted using one of the noncanonical oligonucleotides (-GaTACC-) as a substrate in the presence of 2 mM Mn^{2+} (Figure S4 of the Supporting Information). Steady-state kinetic analysis of cleavage at a noncanonical site revealed that the activity of the mutant is ~40-fold lower than that of WT (Table 2). The D148G mutant shows a higher degree of discrimination (discrimination factor of ~180) for canonical versus noncanonical oligonucleotide cleavage.

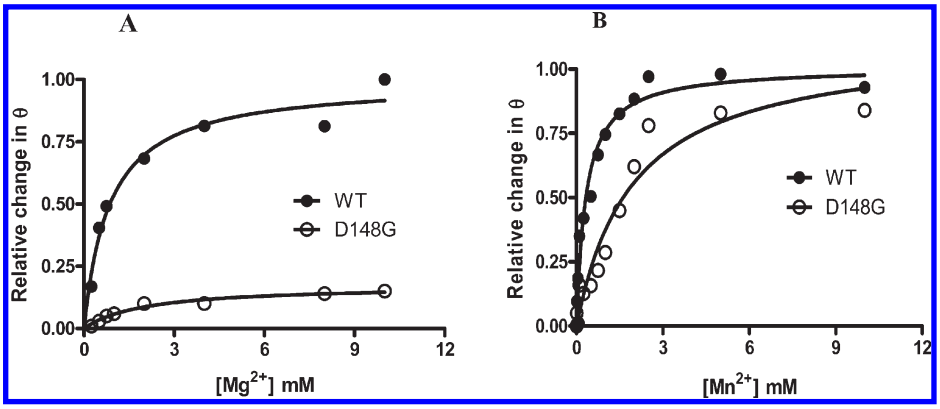

FIGURE 4: Metal ion binding analysis of the D148G mutant. Far-UV CD was used to assess the conformational changes in WT and D148G upon metal ion binding. The reaction mixtures contained 4 μM protein in Tris-HCl buffer (pH 7.4) at 20 °C. Binding isotherms were generated by fitting the change in ellipticity (θ) at 222 nm to a hyperbolic curve using GraphPad Prism version 4. Binding isotherms of WT and D148G in the presence of (A) Mg^{2+} and (B) Mn^{2+} cofactors.

Mn^{2+} Binding to the Active Site of D148G. Further, we investigated the basis of Mn^{2+} versus Mg^{2+} discrimination in DNA cleavage observed with the mutant. Different metal ions may have a diverse influence on the enzyme binding to the recognition sequence. Alternatively, the differences in Mn^{2+} versus Mg^{2+} binding to the enzyme active site could account for the reduced level of Mg^{2+}-mediated cleavage. To determine whether the impaired DNA cleavage is caused by poor DNA binding in the presence of Mg^{2+}, DNA binding analyses were conducted using oligonucleotides containing the R.KpnI recognition site. In the absence of metal ion, R.KpnI binds to cognate DNA with a K_d of 9 nM. D148G had a K_d of 3.7 nM for the cognate DNA sequence (Figure S5 of the Supporting Information). In the presence of Mg^{2+}, the affinity remained unaltered, refuting the idea that defective cleavage may occur because of impairment of DNA binding (Figure S5 of the Supporting Information).

In the case of the D148G mutant, increasing the Mn^{2+} concentration in the reaction mixture increased the level of DNA cleavage, while no significant cleavage was observed even at high concentrations of Mg^{2+}, suggesting impaired Mg^{2+} binding. This is tested further by analyzing the metal ion-mediated conformational changes. CD experiments showed significant changes in the far-UV CD spectrum of R.KpnI on binding of metal cofactors. The enzyme exhibited a hyperbolic binding isotherm with K_d values of 0.64 ± 0.01 and 0.63 ± 0.07 mM for Mg^{2+} and Mn^{2+}, respectively. In contrast, the D148G mutant exhibited conformational changes only in the presence of Mn^{2+}, with a K_d of 1.75 ± 0.2 mM (Figure 4). Further, ICP-AES analysis was conducted to estimate the direct binding of metal cofactors to the active site of the D148G mutant. When the mutant was incubated with 2 mM $MgCl_2$, no bound Mg^{2+} was detected in the mutant enzyme. In contrast, when the enzyme was incubated with 2 mM $MnCl_2$, ICP-AES analysis revealed binding of two ions of Mn^{2+} per dimer, confirming the cofactor binding in the D148G variant of R.KpnI (Table 3). From these studies, it is apparent that swapping Asp with Gly at position 148 of R.KpnI results in the reduced flexibility of the active site, thus barring Mg^{2+} and accommodating Mn^{2+}.

Nicking Activity of D148G. In most type IIP REases, the enzyme binds to its target site in a dimeric fashion, and each monomer nicks one DNA strand in a concerted manner, resulting in an effective double-stranded break. Unlike R.KpnI, the Mn^{2+} specific D148G mutant cleaved DNA in a stepwise manner (Figure S3 of the Supporting Information). Thus, the D148G mutation not only altered the metal ion accommodation at the active site of the enzyme but also affected the ability to cause concerted DNA cleavage characteristic of type IIP REases. The nicking activity of the mutant was subjected to further analysis.

Table 3: ICP-AES Analysis of the D148G Enzyme[a]

sample preparation of the D148G enzyme	protein concentration (μM)	molar ratio	
		Mg^{2+}	Mn^{2+}
EDTA treatment	4	0.01	0.01
EDTA treatment followed by 2 mM Mg^{2+}	4	0.03	0.01
EDTA treatment followed by 2 mM Mn^{2+}	4	0.02	1.6

[a]Values reported are averages of two individual experiments.

Plasmid DNA cleavage characteristics of R.KpnI and the D148G mutant were compared at different time intervals in the presence of Mn^{2+}. R.KpnI generated double-stranded breaks through concerted DNA cleavage (Figure 5A). In contrast, the D148G variant cleaved DNA sequentially, resulting in the accumulation of ~50% nicked intermediates (Figure 5B). It is evident that the mutation at the D148 position resulted in impaired concerted DNA cleavage. One explanation for this behavior is a change in the oligomeric status of the mutant. However, gel filtration studies reveal a dimeric form of the mutant enzyme similar to WT (data not shown). Another possibility could be dissociation of the DNA-bound enzyme before the cleavage of two strands. but the DNA binding studies demonstrate that the D148G mutant forms a high-affinity complex. Thus, it appears that the lower affinity of Mn^{2+} for individual subunits results in accumulation of nicked intermediates. The influence of the bound metal ion on the nicking activity of the enzyme was investigated further by variation of Mn^{2+} concentration and pH.

The concerted cleavage of R.KpnI is affected at 0.5 mM Mn^{2+}, pH 7.0, and 37 °C, resulting in accumulation of ~10% nicked intermediates (Figure 6). Similar observations were made with R.EcoRV, where a decrease in pH resulted in a loss of concerted DNA cleavage (*32*). Under the altered conditions, viz., 0.5 mM Mn^{2+} and pH 7.0, the nicking activity of the R.KpnI mutant was further enhanced, resulting in the accumulation of ~60% nicked intermediates (Figure S6 of the Supporting Information). It is noteworthy that D148G exhibits nicking activity comparable to that of other engineered NEases, such as variants of monomeric homing endonucleases, I-SceI and I-AniI (*33*, *34*). While I-SceI and I-AniI variants exhibited nicking at 100 nM and 1 μM enzyme, respectively, the R.KpnI mutant nicked 60% of the plasmid substrate within 1 h at an enzyme concentration of 10 nM.

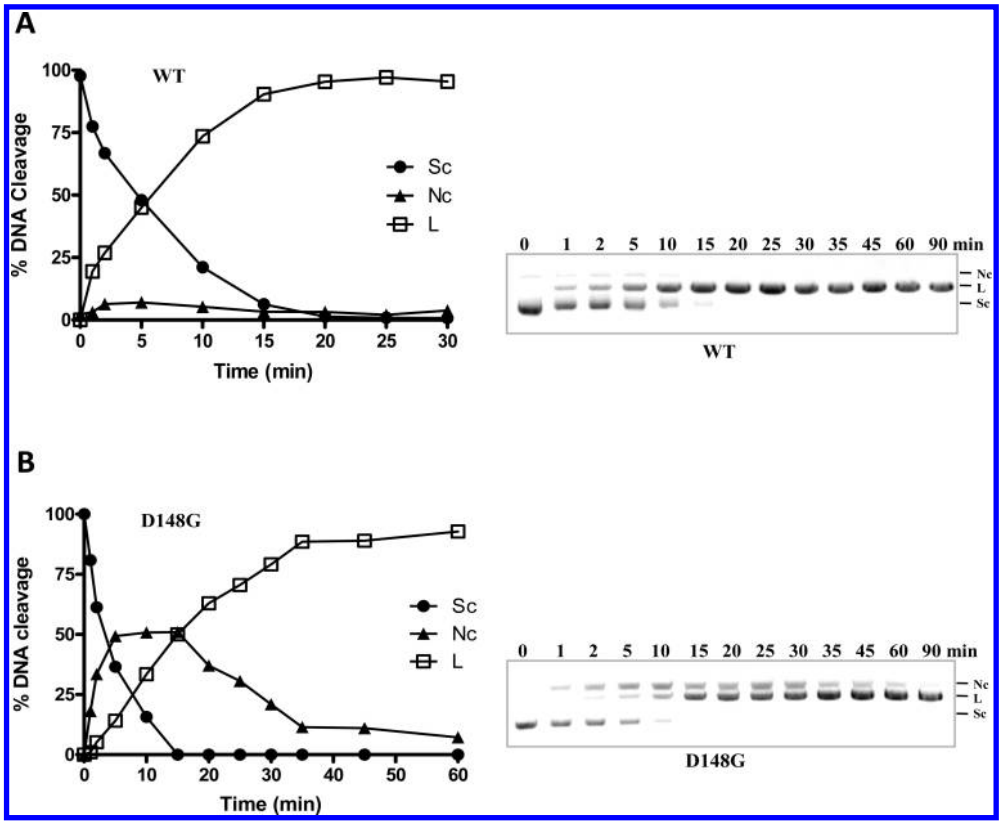

FIGURE 5: Loss of the concerted cleavage ability of the R.KpnI mutant. Time course of cleavage of the pUC18 plasmid substrate containing the R.KpnI recognition sequence (GGTACC) by (A) WT (1 nM) and (B) D148G (10 nM). DNA cleavage reactions were initiated by addition of 2 mM Mn^{2+} to the plasmid enzyme mixture. The intensities of supercoiled (Sc), nicked circular (Nc), and linear (L) bands were plotted at different time points (left) after separation on a 1.2% agarose gel (right).

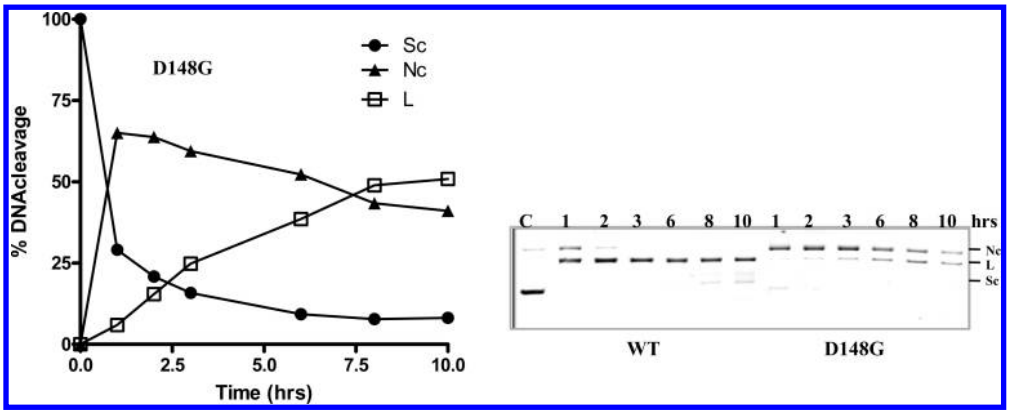

FIGURE 6: R.KpnI mutant that generates a specific nicked intermediate under optimal nicking conditions. Time course of cleavage of the pUC18 plasmid substrate by WT and the D148G mutant. Reactions were conducted with WT (1 nM) and D148G (10 nM) enzymes in the presence of 0.5 mM Mn^{2+} in Tris-HCl buffer (pH 7.0). The reactions were conducted for longer incubation periods (1−10 h) and terminated via addition of 50 mM EDTA containing stop dye. For the D148G mutant, the intensities of supercoiled (Sc), nicked circular (Nc), and linear (L) bands were plotted at different time points (left) after separation on a 1.2% agarose gel (right).

The nicked intermediate generated in the latter case was stable over a range of 3 h but displayed double-strand cleavage upon extended incubation (10 h) (Figure 6). With the altered reaction conditions, the D148G mutant introduced nicks within the recognition sequence and also generated double-stranded breaks by sequential cleavage of both DNA strands during extended incubations.

DISCUSSION

Type II REases are typically characterized by their sequence specificity and Mg^{2+}-dependent accurate DNA cleavage. Because of to their high specificity and diversity, REases are excellent model systems for studying protein−DNA interactions and serve as good starting materials for protein engineering. Rational protein design and directed evolution approaches have been used to generate REases with altered substrate specificity, for recognition of long DNA targets and nicking activities. The unique ability of R.KpnI to cleave DNA in the presence of different metal ions offered a rationale for generating mutants that are specific to one particular metal ion. The replacement of Asp with Gly at position 148 in R.KpnI converted the enzyme into an exclusively Mn^{2+} specific REase. This study thus constitutes an example for

alteration of metal ion specificity of an enzyme with a single point mutation.

The D148G mutation in R.KpnI induced variations in the metal ion coordination affecting the architectural plasticity of the active site, causing exclusion of Mg^{2+}, Ca^{2+}, and Zn^{2+} while allowing Mn^{2+} occupancy, albeit at higher concentrations. One explanation for the selective change is that, because the coordination complexes of Mn^{2+} are usually more flexible and stable, the metal ion may also interact with a water molecule or other residues in the active site. Additionally, the absence of a side chain in the Gly residue could allow a flexible coordination sphere with other neighboring residues necessary for retention of Mn^{2+} binding at the active site. Rescue of activity by Mn^{2+} substitution was also observed when active site residues were replaced with Cys in Tn10 transposase, RAG1 V(D)J recombinase, and HIV integrase (*35*−*38*). In these cases, the change in metal ion specificity is due to preferential coordination of Mn^{2+} to Cys. The specificity ratios (Mn^{2+}:Mg^{2+}) determined with the Cys-substituted enzymes were found to be in the range of 5−160 (Table S2 of the Supporting Information), whereas in the case of the R.KpnI D148G mutant, the specificity ratio was 250. Mutants of R.EcoRI and R.EcoRV that show an altered preference for metal ion cofactors have been described previously (*39*−*42*). In R.EcoRV, the change in metal ion binding specificity from Mg^{2+} to Mn^{2+} is due to a single Ile to Leu mutation. I91 of R.EcoRV does not directly interact with the metal ion but points away from it into the hydrophobic core. The I91L mutation thus alters the coordination sphere, providing better coordination of Mn^{2+}.

Mn^{2+} rescue of catalytic activity for the D148G mutant of R.KpnI required a higher concentration of the metal cofactor. Because Gly is not a steric equivalent to the Asp residue, it is likely that the position of the metal binding atom and consequently orientation of the Mn^{2+} is not optimal. This would result in reduced Mn^{2+} binding affinity and a shift in the concentration optimum toward maximal activity. Similar observations were made with phage T7 RNA polymerase, where mutation of metal binding residue D812 decreased the affinity, resulting in an increased metal ion concentration for optimal catalytic activity (*43*). In R.EcoRV, lowering the pH of the reaction mixture to decrease the affinity of the active site metal ion resulted in an impaired concerted cleavage (*32*). The reduced affinity of Mn^{2+} in the D148G mutant also might account for the impaired concerted DNA cleavage. Further, decreasing the metal ion concentration or lowering the pH presumably alters the binding of Mn^{2+} to the active site and therefore would increase the nicking activity of the mutant. Concerted cleavage of both strands of the duplex requires intersubunit communication to synchronize the two catalytic centers (*44*). Thus, an alternate possibility would be that the D148G mutation could have impaired intersubunit conformational behavior leading to the accumulation of nicked intermediates.

Nicking variants of REases are present in nature, viz., Nt.CviPII, Nt.CviQII, Nt.BstNBI, Nb.BsrDI, and Nb.BtsI (*45*−*48*). Most of these naturally occurring NEases exhibit homology to known type IIS REases. Hence, type IIS REases are an obvious choice for engineering enzymes that nick DNA near their short recognition sequences by inactivating or replacing one of the two subunits or by changing their oligomeric status (*17*−*19*). Another strategy that has been employed for monomeric type IIS REases containing two active sites involves inactivation of one of the active sites individually to create strand specific NEases (*16*). In type IIS REase R.Eco31I, mutations that affect the sequence-dependent DNA interaction or cofactor binding also influenced the sequential cleavage of two strands (*49*). Nicking variants have also been constructed from homing endonucleases with large recognition sequences (*33*, *34*). However, the target specificity of homing endonucleases limits their application to in vivo methods. Because type IIS REases recognize asymmetric nucleotide sequences and cut outside of their recognition sites, they generate strand specific nicking activity. However, because of their palindromic recognition and obligate requirement for dimerization, type IIP REases have not been considered for engineering nicking activities, with the exception of R.EcoRV (*50*). Introduction of mutations at the cofactor binding site could be used as a strategy to generate nicking variants of type IIP REases.

The D148G mutant of R.KpnI retains its high Mn^{2+}-dependent DNA cleavage activity in vitro, while being quiescent in vivo. The low concentration of free Mn^{2+} in *E. coli* could account for the enzyme's greatly reduced in vivo activity (*51*). Such in vivo dormancy could be effectively utilized in engineering REases with altered specificities. Catalysis deficient mutants have been employed to alter the DNA recognition specificity (*22*, *52*, *53*). The mutants exhibiting altered DNA specificity were selected on the basis of the DNA binding, and later the cleavage center was reconstituted as that of WT. Engineering selective metal ion-dependent REases, such as R.KpnI D148G, could circumvent the use of catalysis deficient mutants for screening type II REases with altered specificities. REases engineered for metal ion specificities, which exhibit high activity under in vitro conditions, could be employed as a general strategy for obtaining REases with novel specificities.

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SUPPORTING INFORMATION AVAILABLE

Oligonucleotides used for the study, secondary structure analysis, and DNA binding and cleavage properties of the point mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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