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# Structural integrity of the Beta Beta Alpha-Metal finger motif is required for DNA binding and stable protein–DNA complex formation in R.*Kpn*I

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**Abstract**

Restriction endonuclease (REase) R.*Kpn*I from *Klebsiella pneumoniae* is a homodimeric enzyme, which recognizes palindromic sequence GGTAC|C and cleaves generating 4 base 3′ end overhangs. R.*Kpn*I belongs to the HNH superfamily of nucleases, which are characterized by the presence of the ββα-Me finger motif. Structurally, this motif consists of a twisted β-hairpin followed by an α-helix, and serves as a scaffold for side chains of residues involved in co-ordination of a divalent metal ion that is required for catalysis. Homology modeling studies of R.*Kpn*I suggested a crossover structure for the α-helix, which could possibly form dimeric interface and/or structural scaffold for the active site. We have evaluated the role of the residues present in this α-helix in intersubunit interactions and/or stabilization of the active site. We show here that mutations of residues in the α-helix lead to a loss of the enzyme activity, but not dimerization ability. Intrinsic fluorescence and circular dichroism studies revealed that the loss of function phenotype was due to the structural perturbation of the ββα-Me finger motif. The results of mutational analysis suggest that the α-helix of the ββα-Me finger of R.*Kpn*I plays an important role for the stability of the protein–DNA complex.


*Keywords:* Restriction endonuclease; R.*Kpn*I; ββα-Me finger; HNH Motiff; Site directed mutagenesis; Protein DNA complex

## 1. Introduction

Type II restriction endonucleases (Type II REases) are homodimeric enzymes, which recognize and cleave DNA sequences of 4–8 base pairs in length [1]. Most Type II REases are homodimeric, with one active site per monomer. In these enzymes, the protomers contact each other via various secondary structural elements, resulting in effective dimerization. Different modes of dimerization serve to place the two active sites at positions specific for different cleavage patterns, from 5′ overhangs, to blunt ends, to 3′ overhangs. REases with structurally similar protomers typically retain similar dimerization mode and cleavage patterns as well, however sometimes a dramatic rearrangement of the dimerization mode can occur, leading to complete rearrangement of a cleavage pattern, such as that reported in the case of the R.*Bgl*I enzyme, when compared to R.*Eco*RV and R.*Pvu*II [2].

Most Type II REases studied so far have a catalytic domain from the PD-(D/E) XK superfamily of nucleases. However, a growing number of REases was shown to possess unrelated nuclease domains, e.g. from the PLD, GIY-YIG and HNH families [3–7]. The HNH nuclease domain is widely distributed in all three domains of life. It is present in homing endonucleases, non-specific bacterial and fungal nucleases [8,9]. It is also present in a broad group of enzymes acting on DNA including transposases, restriction endonucleases, DNA packaging factors, and a bacterial factor involved in a developmentally controlled DNA rearrangement [10,11]. The HNH domain contains the ββα-Me finger structural motif, which serves as a scaffold for a catalytic center involved in the phosphodiester bond cleavage [8,9]. Crystallographic studies carried out on non-specific nucleases and homing endonucleases of this protein superfamily showed that the ββα-Me motif interacts mostly with the minor groove of the

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DNA substrate, primarily contacting DNA phosphate groups near the 3′ hydroxyl of the scissile phosphate. The specific interactions of homing endonucleases with DNA are brought about by additional DNA-contacting elements [12]. Also, from these studies it has been observed that the non-catalytic residues in the active site might help in stabilizing protein–DNA complexes. However, very little experimental evidence is available to support this hypothesis.

R.*Kpn*I is one of the most commonly used Type II REases, which recognizes and cleaves palindromic DNA sequence 5′ GGTAC|C3′, generating four base 3′-overhangs [13]. Unlike most other REases, the active site of R.*Kpn*I contains the HNH motif and follows the mechanism of ββα-Me finger class of nucleases, such as I-*Ppo*I, T4 Endo VII, Colicin E7 and *Serratia* nuclease [7]. Previously, we have generated a homology model of the R.*Kpn*I C-terminal catalytic domain (residues spanning from 97 to 190) dimer, based on the I-*Ppo*I crystal structure [14]. Although R.*Kpn*I monomer exhibits more similarity with T4 Endo VII monomer, the cleavage pattern of the two enzymes is very different to construct a dimeric homology model. Also, generation of R.*Kpn*I dimer model based on T4 Endo VII results in formation of repulsive charge–charge interactions. Further, the R.*Kpn*I does not show significant sequence similarity with any other restriction endonuclease whose structure has been determined. The R.*Kpn*I model built based on I-*Ppo*I structure was validated by sequence-directed mutagenesis, which suggested that D148, H149 and Q175 residues of R.*Kpn*I correspond to the critical D, H and N or H residues of the HNH nucleases. Substitutions of these three conserved residues led to the loss of the DNA cleavage activity by R.*Kpn*I, revealing their importance in catalysis [7]. Since R.*Kpn*I and I-*Ppo*I belong to the same HNH superfamily of nucleases and exhibit a similar cleavage pattern (i.e., generation of 3′ overhangs), it is likely that the dimeric interface of both the enzymes are arranged in a similar manner. Thus, both in the I-*Ppo*I protein–DNA complex and in our model of the R.*Kpn*I-CTD dimer, the dimerization of HNH domains is mediated by the α-helix of the ββα-Me motif.

From the structures of the several ββα-Me finger motif containing enzymes, the α helix has been implicated in providing a structural scaffold for the correct juxtapositioning of the catalytic residues. However, no mutagenesis data exists to delineate its role so far. Since in our model the two α helices cross over each other, we asked two important questions. Does the crossover of the two α helices contribute to the dimer formation? And/or does it provide support for structural integrity? In this manuscript, we have investigated the role of residues of the α-helix crossover structure.

Table 1
DNA oligonucleotides used in this study

| Primers used for mutagenesis | |
|---|---|
| T7 terminator sequence | 5′-GCTAGTTATTGTTCAGCGGTGGG -3′ |
| V176D | 5′-ATCAA**GAT**ATGAAAAAAAATTAT TG-3′ |
| N180A | 5′-TATGAAAAAA**GCT**TATTGG-3′ |
| Y181A | 5′-G AAAAAAAAT**GCT**TGG GAT TC-3′ |
| Duplex DNA used for EMSA | 5′-ATTGCGTGGTACCCGCTCTT-3′ |
| | 3′-TAACGCACCATGGGCGAGAA-5′ |

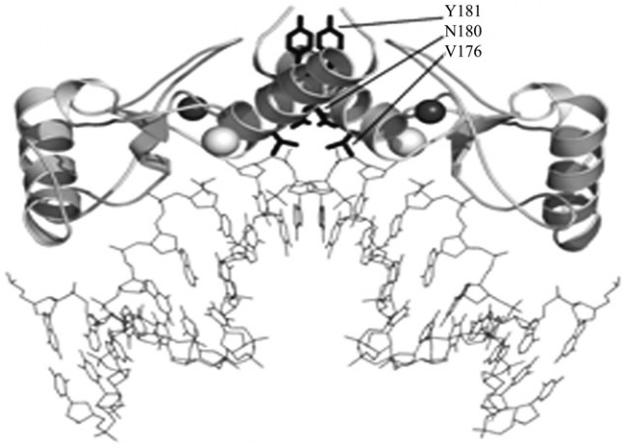


Fig. 1. Ribbon diagram of R.*Kpn*I modeled structure. The R.*Kpn*I dimer is shown as a ribbon (with helices as spirals and strands as arrows). The $Mg^{2+}$ and $Zn^{2+}$ ions are shown as white and dark balls, respectively. The sidechains of mutated residues are shown in the stick representation and labeled.

## 2. Materials and methods

### 2.1. Enzymes and DNA

T4 polynucleotide kinase, Pfu DNA polymerase and R.*Dpn*I were purchased from New England Biolabs, USA. Plasmid pUC18 DNA was prepared by alkaline lysis method. Oligonucleotides (Sigma Aldrich) were purified on 18% urea-polyacrylamide gel [15]. The purified oligonucleotides were end labeled with T4 polynucleotide kinase and $\gamma$-$^{32}$[P] ATP (6000 Ci/mmol).

### 2.2. Site-directed mutagenesis

Mutants of R.*Kpn*I were generated by site-directed mutagenesis using the megaprimer inverse PCR method [16]. Expression plasmid pETRK encoding the wild type (wt) *kpnI*R gene was used as a template. The GenBank accession no. of the *kpnI*R gene product is P25237. Oligonucleotide primers carrying the respective mutant amino acid codon substitutions (Table 1) were used as forward primers and T7 terminator sequence was used as a reverse primer (Table 1). The mega primers generated were used as complementary primers for the second round of PCR amplification. After confirming the mutation by sequencing, the mutant REases were expressed in *Escherichia coli* BL26 [F$^-$ *omp T hsd*S$_B$ (r$_B$-m$_B$-) gal *dcm* lac (DE3) *nin*5 *lac* UV5-T7 gene 1] containing M.*Kpn*I plasmid [17].

### 2.3. Purification of R.KpnI and its mutants

R.*Kpn*I and its mutants were purified using the method described previously [17]. 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 [18] using bovine serum albumin as standard. The purity

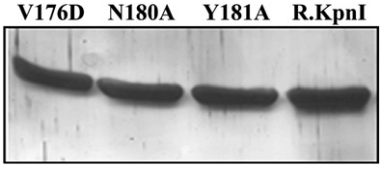


Fig. 2. SDS-PAGE analysis of the purified R.*Kpn*I and its variants. Recombinant proteins were expressed in *E. coli* and purified using protocol described under Materials and methods. Analysis by SDS-PAGE of the purified proteins illustrates that all the mutant variants are co-migrating with wild type enzyme.