SYNOPSIS

Restriction modification (R-M) systems are important components of the prokaryotic arsenal against invading genomes. R-M systems directly target the foreign DNA and are often considered as primitive immune systems in bacteria. The defense system comprises of two contrasting enzymatic activities – a restriction endonuclease (REase) and a methyltransferase (MTase). Functionally, REases cleave a specific DNA sequence endonucleolytically at the phosphodiester bonds generating 5' or 3' overhangs or blunt ends. MTases catalyze the transfer of a methyl group from S-adenosyl-L-methionine to adenine or cytosine. Four types of R–M systems are found in bacteria, viz., Types I, II, III and IV. Type II R-M systems, comprising of a separate REase and MTase, are the most abundant and well-studied enzymes. Type II REases recognize and cleave DNA within or near their recognition sequences. Surprisingly, these enzymes share little or no sequence homology amongst them. All the enzymes identified so far can be grouped into conventional PD-(D/E)XK, ββα-Me, GIY-YIG, phospholipase-derived and half-pipe endonucleases according to their folds and active site structures. Owing to their high specificity and defined cleavage pattern, they have become indispensable tools in molecular biology and have been widely exploited for studying protein–DNA interactions.

The work presented in this thesis deals with R.KpnI, which belongs to the HNH superfamily of nucleases and is characterized by the presence of a ββα-Me finger motif. The REase isolated from Klebsiella pneumoniae recognizes the palindromic DNA sequence GGTAC/C and cleaves DNA as indicated. The enzyme is unique in exhibiting promiscuous DNA cleavage in the presence of Mg²⁺, a natural co-factor for a vast majority of REases. Surprisingly, Ca²⁺ and Zn²⁺ completely suppress the Mg²⁺ mediated promiscuous activity and induce high fidelity cleavage. These unusual features of R.KpnI led to the functional characterization of the ββα-Me finger active site motif. In addition, the studies were aimed at understanding the mechanism and the biological significance of substrate and co-factor promiscuity exhibited by the enzyme. The salient aspects of the thesis are summarized below.

A general introduction and overview of the literature on structure-function studies, mechanism of recognition and catalysis by REases with special emphasis on Type II enzymes is presented in the Chapter 1. An account of co-factor specificity in REases, role of metal ions in DNA binding as well as in phosphodiester bond hydrolysis is
The various aspects of R-M systems that target the invading DNA elements and counter strategies employed by the foreign genomes to evade the restriction are also covered. The new developments that provide insights in understanding the diversity of R-M systems and additional biological roles that could increase the fitness of the host organism harboring them are described. The features of substrate and metal ion specificity in REases and the efforts undertaken to alter the specificity have been dealt at the end of the chapter.

From the structures of the several ββα-Me finger nucleases, 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. Homology modeling studies of R.KpnI suggested a crossover structure for the α-helix of the ββα-Me finger active site motif, which could possibly form dimeric interface and/or structural scaffold for the active site. Chapter 2 describes the computational modeling and mutational analysis performed to understand the role of the residues present in this α-helix in intersubunit interactions and/or stabilization of the active site. Mutation of the residues present in the α-helix lead to the loss of the enzyme activity, but not dimerization ability. Subsequent biophysical experiments showed that the α-helix of the ββα-Me finger of R.KpnI plays an important role for the stability of the protein–DNA complex needed for its function.

In Chapter 3, unusual co-factor flexibility for R.KpnI is shown by using a battery of divalent metal co-factors differing in ionic radii and coordination geometries. A number of alkaline earth and transition group metal ions function as co-factors for DNA cleavage. The metal ions replaced each other readily from the enzyme’s active site revealing the active site plasticity. Mutation of the invariant His residue of the HNH motif caused abolition of the enzyme activity with all the co-factors indicating that the enzyme follows single metal ion mechanism for DNA cleavage. The indispensability of the invariant His in nucleophile activation together with the broad co-factor tolerance of the enzyme indicated the role of metal ions in electrostatic stabilization during catalysis. At higher concentrations, Mg$^{2+}$, Mn$^{2+}$ or Co$^{2+}$ stimulate promiscuous cleavage while Cd$^{2+}$, Ni$^{2+}$ or Zn$^{2+}$ inhibit phosphodiester bond hydrolysis. The underlying molecular mechanisms for the modulation of the enzyme activity by the metal ion binding to the second site are presented. Regulation of the endonuclease activity and fidelity by a second metal ion binding is a unique feature of R.KpnI among
REases and HNH nucleases. The identification of additional metal ion binding residues would help in engineering REase variants with enhanced activity and/or specificity.

Chapter 4 describes the generation of an R.KpnI variant with altered co-factor specificity by exploiting the active site plasticity of the enzyme. The mutant enzyme is a Mn$^{2+}$ -dependent 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 \textit{in vitro} activity while being dormant \textit{in vivo}. In addition to the Mn$^{2+}$ selectivity, the mutant is impaired in concerted double-stranded DNA cleavage leading to the accumulation of nicked intermediates. The nicking activity of the mutant enzyme is further enhanced by altering the reaction conditions. Thus, a single point mutation in the active site of R.KpnI generates a Mn$^{2+}$ -dependent REase and a sequence specific nicking endonuclease. The potential applications of such enzymes engineered for selective metal ion dependent activities have been discussed.

R.KpnI is peculiar in retaining robust promiscuous cleavage despite being a typical Type II REase in all other characteristics. Chapter 5 presents results of the growth properties and phage titer analysis carried out with R.KpnI and its high fidelity variant to understand the biological significance of promiscuous activity. The enzyme isolated from the \textit{K. pneumoniae} exhibited biochemical properties similar to that of R.KpnI overexpressed in \textit{E.coli}. It was observed that the wild type but not the high fidelity variant could effectively restrict bacteriophages methylated at GGTACC. These results show that the REase exhibits promiscuous activity \textit{in vivo}, which would be advantageous for the organism to better target the incoming foreign DNA. The promiscuous behavior of the R.KpnI could be one of the counter strategies employed by the bacteria against the constantly evolving phages in the co-evolutionary arms race.

In conclusion, the work described in this thesis provides new insights about structure, function and biology of REases in general and R.KpnI in particular. The co-factor and substrate promiscuity of R.KpnI may indicate its evolutionarily intermediate form that is yet to attain a high degree of specificity. Alternatively, it is possible that this unique feature is retained during the evolution of the HNH REases serving some unknown function(s) in the cell, in addition to having an edge in countering the phage infections.