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Reduced Activity of *Bam*HI Variants C54I, C64W, and C54D/C64R Is Consistent with the Substrate-Assisted Catalysis Model

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Three specific mutants, C54I, C54W, and a double-mutant C54D:C64R of restriction endonuclease *Bam*HI, were generated and studied to investigate the role, if any, of the 54th and 64th cysteine residues in the catalysis of *Bam*HI. The mutation was achieved using the megaprimer approach for PCR. The mutant genes were cloned and characterized by sequencing. The mutant and the wild-type proteins were expressed and purified and their kinetic parameters were determined using short synthetic oligonucleotides as substrates. All mutants had higher K_m values than that of the wild-type enzyme suggesting a decrease in the affinity of the enzyme for its substrate. The mutant protein C54W showed significant changes in the CD spectra vis-a-vis wild-type enzyme and had the lowest K_m/K_{cat} value among the mutants indicative of changes in the secondary structure of the protein. The melting curves of the mutant proteins overlapped that of the wild-type enzyme. Analysis of the K_{cat} values in the context of cocrystal structure suggests that the effect of Cys54 mutation is probably through the perturbation of the local structure whereas reduced activity of the double mutant is consistent with the substrate-assisted catalysis mechanism.

***Key Words:* *Bam*HI variants; site-specific mutagenesis; Cys54 mutation.**

*Bam*HI restriction endonuclease specifically recognizes and cleaves in the presence of Mg^{2+}, the palindromic sequence 5′-GGATCC-3′ between the two guanines generating a four nucleotide 5′ overhang (1). In addition to Mg^{2+}, *Bam*HI also requires DTT or 2-mercaptoethanol for its DNA cleavage. The DNA cleavage is inhibited by sulfhydryl modifying agents, which can be reversed by 2-mercaptoethanol. This inhibition was studied in detail by Nath (2), who inferred that cysteines in *Bam*HI must play a catalytic role. The *Bam*HI protein has three cysteines, Cys34, Cys54, and Cys64. The cocrystal structure of *Bam*HI–DNA complex shows that Cys54 and Cys64 are at the protein DNA interface but not involved in binding to the DNA (3). Mutational studies (4) have identified three catalytic amino acid residues as Asp94, Glu111, and Glu113 in *Bam*HI, positionally equivalent to catalytic residues Asp91, Glu111, and Lys113, respectively, in *Eco*RI. Based on the structural and biochemical knowledge, two catalytic mechanisms have been considered for *Bam*HI. A substrate assisted catalytic model, a kind of general mechanism for type II endonuclease having similar arrangement of catalytic residues at the active sites has been proposed by Jeltsch *et al.* (5, 6) and a two-metal-ion catalysis model has been proposed by Viadiu *et al.* (7). The basic difference between these two mechanisms is the residue which is believed to activate a suitably placed water molecule to mount nucleophilic attack on the scissle phosphate; it is Glu113 in the two metal ion model whereas it is the next phosphate 3′ to the scissle phosphate in the substrate-assisted model. The debate in case of *Bam*HI is compounded by the fact that unlike *Eco*RI, *Eco*RV, and *Pvu*II, *Bam*HI requires DTT for catalysis and has a glutamic acid in place of a semiconserved lysine found in the catalytic centers of the other three enzymes. It may be noted that mechanism of phosphodiester cleavage by restriction endonucleases in no case has been elucidated unequivocally although the structures of the active sites and the catalytic amino acid residues have been known in several cases (8).

Recently a *Bam*HI variant, C54A, has been reported to have enhanced catalytic activity compared to that of the native enzyme (9) and C64A mutant has considerably reduced activity shown by both *in vitro* and *in vivo*

Abbreviations used: DTT, dithiothreitol; dinD, DNA damage-inducible promoter; X-gal, 5-bromo-4-chloro-3-indolyl-β-galactopyranoside; IPTG, β-D-isopropyl-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin.

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assays (unpublished observation). In view of all these, we replaced Cys54 with few other amino acids to better understand it's role, if any. We report here biophysical and biochemical characteristics of the variants, C54I, C54W, and a double-mutant C54D:C64R and show that substrate assisted catalysis model is more plausible to explain our mutational data.

MATERIALS AND METHODS

Bacterial strains, plasmid, and media. The following bacterial strains and plasmids were used: DH10B (mcr^-) harboring the plasmid pMAP6 a derivative of pACYC184 (tet^r)containing the *Bam*HI methylase gene cloned (earlier in our laboratory) under its own promoter at the *Eco*RI site. BL21 (DE3) was used for the expression of *Bam*HIR gene under the T7 promoter. JH139 which carry dinD1::lacZ fusion was used to detect *in vivo* SOS induction. pRSET A (InVitrogen) was used for cloning the *Bam*HIR gene from the recombinant clone pPMPBamR.0 (9).

Luria–Bertani (LB) medium and Luria agar were supplemented with antibiotics (Sigma); ampicillin (Amp) at 100 μg/ml, tetracycline (Tet) at 10 μg/ml, or kanamycin (Kan) at 50 μg/ml as and when required.

Enzymes and plasmid DNA preparation. All restriction enzymes were from New England Biolabs and were used as recommended. T4 DNA ligase and *Taq* polymerase were from Bangalore Genei.

Medium scale isolation and purification of plasmid DNA was done using alkaline lysis method followed by phenol chloroform extraction. For sequencing, DNA was prepared using Qiagen plasmid mini kit and sequenced by the dideoxy chain termination method using the T7 Sequenase Version 2.0 DNA sequencing kit (Amersham Life Science).

Mutagenesis, cloning, and characterization of recombinant BamHI endonucleases. The megaprimer method (10) was used to create mutation at cysteine 54 of *Bam*HI endonuclease. This method needs just three primers, a 5′ primer, a mutagenic primer containing the desired mutation and a 3′ primer and involves two successive PCR steps. In the first PCR step, the 5′ primer and the mutagenic primer generate a double stranded mutated fragment, the megaprimer. The megaprimer, which contains the mutation toward its 3′ end is then used as 5′ primer in the second PCR step together with the 3′ primer to amplify the rest of the gene.

The 5′ and the 3′ primers purchased from Yale University, has the sequence

5′-dGCGAATTG**CATATG**GAAGTAGAAAAAGAG-3′
*Nde*I

5′-dCCG**GAATTC**CGAAGCCTCTTTACC-3′
*Eco*RI

To generate a number of mutants in a single PCR step a mixture of mutagenic primers was synthesized by incorporating NNS at codon 54, where N stands for equimolar mixture of A, T, G and C nucleotides and S stands for equimolar mixture of G and C nucleotides. The sequence of the synthetic mutagenic primer is 5′-GGTACTACCGTT**SNN**ATTCTTTTTCGG-3′.

The recombinant construct, pPMBamR.0 was used as the template for amplifying the *Bam*HIR gene. The first PCR was carried out with the 5′ primer and the mutagenic primer using *Taq* polymerase. A single amplified band of 194 bp was eluted out from the gel and used as a primer (the megaprimer) along with the 3′ primer in the second PCR step to amplify the rest of the *Bam*HIR gene. A single band of 681 bp was amplified.

The amplified product was purified and digested with *Nde*I and *Eco*RI. The double digested PCR fragment was then ligated to *Nde*I–*Eco*RI digested vector, pRSET A and transformed into competent cells of *Escherichia coli* DH10B which already harbored *Bam*HI methylase gene in pMAP6. Wild-type *Bam*HIR gene was identically cloned in pRSET A vector earlier for overexpression giving the construct pAABRw, details of which has been reported elsewhere (11). Recombinants which showed increase in size were characterized by restriction analysis. Mutations in the positive clones were further confirmed by sequencing.

In vivo SOS induction assay. The SOS strain, JH139 (12) was transformed with the recombinant plasmids containing the endonuclease gene or its variants in the absence of pMAP6. The transformants were plated on LB agar plates containing the antibiotics ampicillin (100 μg/ml) and kanamycin (50 μg/ml) which were preplated with 40 μl of X-gal (Bangalore Genei) (20 mg/ml in dimethyl formamide, SRL). The plates were incubated at 37°C overnight.

Expression and purification of recombinant BamHI protein. For expression of the recombinant *Bam*HI variants the corresponding recombinant plasmid construct was cotransformed along with pMAP6 into BL21(DE3) cells. The transformant (single colony) was inoculated into 3 ml LB containing antibiotics ampicillin (100 μg/ml) and tetracycline (10 μg/ml) and grown overnight at 37°C in the shaker. The overnight grown culture was used to inoculate 10 × 100 ml media containing appropriate amounts of the two antibiotics. The cultures were grown till OD_{600} reached 0.6 and induced with IPTG (Sigma) at a final concentration of 0.4 mM. The cells were allowed to grow for 3 h and then harvested at 4°C and sonicated in lysis buffer [10 mM potassium phosphate (pH 7.0), 1 mM DTT, 1 mM EDTA, 200 mM NaCl, 1 mM PMSF and 100 μg/ml lysozyme] using Ultrasonics (Model W385) sonicator. The sonicated cell suspension was centrifuged to pellet down the cell debris and the supernatant was loaded onto the phosphocellulose column, already activated and equilibrated with the equilibration buffer [10 mM potassium phosphate (pH 7.0), 200 mM NaCl, 1 mM EDTA, 1 mM DTT and 10% glycerol]. The bound proteins were eluted with a 200-ml linear gradient of NaCl (0.2–1 M). The eluate was collected in 2-ml fractions and 1 μl of each fraction was checked for *Bam*HI activity using plasmid DNA, pRSET A. *Bam*HI endonuclease activity eluted between 0.35 and 0.55 M NaCl concentration. The active fractions were pooled, the protein concentration was measured by micro Bradford method using BSA as standard (13) and the enzyme assay was done to find the specific activity after phosphocellulose column purification. The rest of the pooled fraction was loaded onto hydroxyapatite column equilibrated with the equilibration buffer. The column was then washed and eluted with a 100-ml gradient of potassium phosphate (pH 7.0) from 0.01 to 0.5 M. The fractions showing activity were pooled and the protein content quantified using micro Bradford method as well as the specific activity was determined. The pooled fraction after hydroxyapatite step was concentrated and the buffer content was changed to storage buffer [300 mM KCl, 10 mM Tris–HCl (pH 8.0), 1 mM DTT, 1 mM EDTA and 10% glycerol] using Centricon YM-10 assembly (Millipore) (10 kDa). The purified enzyme was stored at 4°C. Protein sample collected after each chromatography step were also analyzed on 12% SDS–PAGE.

BamHI endonuclease assay. One microliter of each chromatography fraction was added to 10 μl digestion mixture containing 100 ng pRSET A, in *Bam*HI buffer [10 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT] and incubated at 37°C for 1 h and checked for linearization of pRSET A, which has a single *Bam*HI site.

Determination of BamHI enzyme units. Lambda DNA (Promega) was used as substrate for determining units of *Bam*HI endonuclease activity present in the pooled fraction and the specificity of the mutant endonucleases. One microgram of lambda DNA was digested with decreasing amounts of pooled fraction in 20 μl reaction mix containing *Bam*HI buffer at 37°C for 1 h. The minimum amount of fraction capable of complete digestion of 1 μg lambda DNA in 1 h was defined as one enzyme unit.

Catalysis in the presence and absence of reducing agent. DTT was first dialyzed out of the purified enzymes and then used to digest one μg of lambda DNA in 1× *Bam*HI buffer [10 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl) with and without 1 mM DTT for 16 h (overnight) at 37°C.

In vitro cleavage kinetics. Continuous spectrophotometric assay, based on the hyperchromic effect upon DNA cleavage was followed as per the published procedure (14) to determine the K_m and K_{cat} values of the purified *Bam*HI endonuclease and its variants. For this purpose a 13-mer self complementary oligonucleotide, purchased from Yale University (0.2 μM scale), of the sequence 5′-AGTGCG-GATCCGC-3′ was used as substrate.

Oligonucleotide solution was diluted to a concentration of 4 μM in 1× *Bam*HI buffer, without DTT, in a stoppered cuvette having path length of 1 cm. The solution was heated to 80°C for 2 min using a heating block and allowed to cool slowly. When the temperature came down to 25°C, the absorbance at 260 nm was recorded to calculate the concentration of the substrate using the extinction coefficient ($17.58 \times 10^3\ M^{-1}\ cm^{-1}$) of double stranded oligonucleotide substrate. The temperature of the cuvette holder was held at 25°C. The assay was started by adding DTT (final concentration 1 mM) and an excess of *Bam*HI endonuclease (final concentration 150 nM) and immediately started monitoring the increase in absorbance at 254 nm with time. The final absorbance after the increase had ceased was noted. The difference in absorbance just after adding the enzyme and after complete digestion at 25°C was used to calculate the change in absorbance per nanomole of substrate.

To determine the K_m and K_{cat} values the same procedure as above was followed except that the amount of enzyme was kept low, approximately 15 nM under various oligomer concentrations in the range of 2–30 μM. Assays were carried out at 25°C and each reaction was monitored for about 1 h. The initial rate of cleavage was determined by the rate of increase in absorbance and the total absorbance increase per nanomole of product formed. K_m and V_{max} values were determined from Lineweaver–Burk plot and the K_{cat} values were calculated using the formula $K_{cat} = V_{max}/[E]$ where [E] is the enzyme concentration.

Circular dichroism (CD) spectroscopy. CD spectra of the purified proteins were recorded on Jasco J720 A spectropolarometer calibrated with D-camphorsulfonic acid and interfaced with a IBM PC-compatible computer. Spectra was measured from 210 nm to 240 nm, at 25°C.

Melting profiles of the purified proteins. The melting profiles of *Bam*HI and its variants were determined in UV-160A spectrophotometer (Shimadzu) connected to a linear temperature program controller CE 247 (Cecil Instruments). The purified proteins were diluted in 1× *Bam*HI buffer to about OD_{280} = 0.3–0.4. The protein solutions were heated at the rate of 1°C per minute in a stoppered cuvette and OD_{280} were recorded every minute. The relative absorbance value was calculated and plotted against temperature.

RESULTS

Cloning, Characterization, and Expression of Recombinant BamHI Endonucleases

The mutants of *Bam*HI endonuclease generated by the megaprimer approach were screened and the recombinants were characterized by restriction analysis and sequencing. Three specific mutants selected for detail study are C54W, C54I, and a double-mutant C54D:C64R, the corresponding plasmid constructs were designated as pAABR23, pAABR37, and pAABR47, respectively.

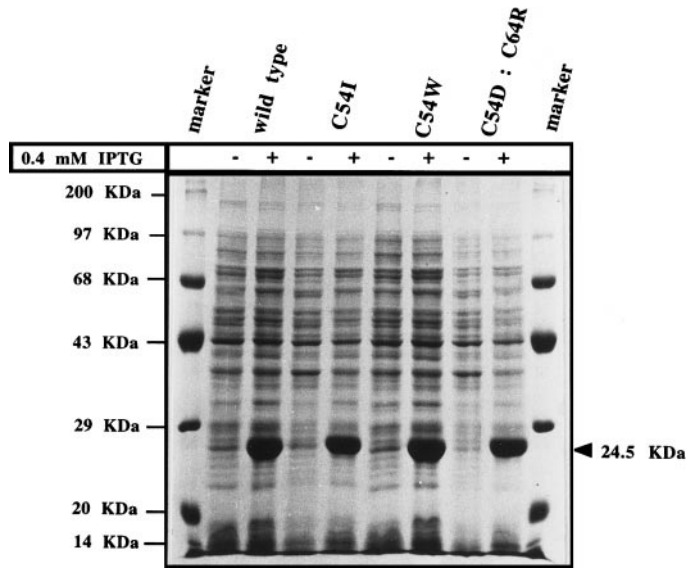

FIG. 1. Overexpression of the wild type and the three variants of *Bam*HI endonuclease. Crude extracts from BL21(DE3) cells transformed with the constructs pAABRw, pAABR37, pAABR23, and pAABR47 were analyzed on SDS–PAGE. Lanes 1 and 10 show protein molecular weight marker. Lanes 2, 4, 6, and 8 show uninduced and lanes 3, 5, 7, and 9 IPTG-induced level of expression. *Bam*HI endonuclease is marked with an arrowhead.

The *E. coli* host strain DH10B lacks T7 polymerase, the *Bam*HI gene which is under the T7 promoter is not expected to express in this host strain. For expression, the *Bam*HI recombinant plasmid was cotransformed along with pMAP6 into BL21(DE3) cells. The transformants were found to be slow growing. Even in liquid media these transformants were found to grow slowly compared to the cells that were transformed with only the vector pRSETA and pMAP6. Strangely, the transformants harboring the recombinant *Bam*HI clone could not grow beyond 0.6 OD. The problems with the wild-type *Bam*HI overexpressing clone has already been described (12).

The BL21(DE3) cells were always freshly transformed with any of the three mutant constructs each time to isolate the respective proteins. All the three mutant clones expressed to the same extent as the wild-type clone as shown in Fig. 1.

In Vivo SOS Induction Assay

In the absence of cognate methylase, *Bam*HI endonuclease would cleave at the *Bam*HI sites on the host DNA. Such an action in *E. coli* induces SOS response when a set of genes are triggered on to repair the damage. SOS response is monitored using strains which carry the lactose operon fused to the damage inducible (din) promoter (15). When the *Bam*HI expressing clones are transformed into JH139 cells (16), small amounts of *Bam*HI endonuclease is produced

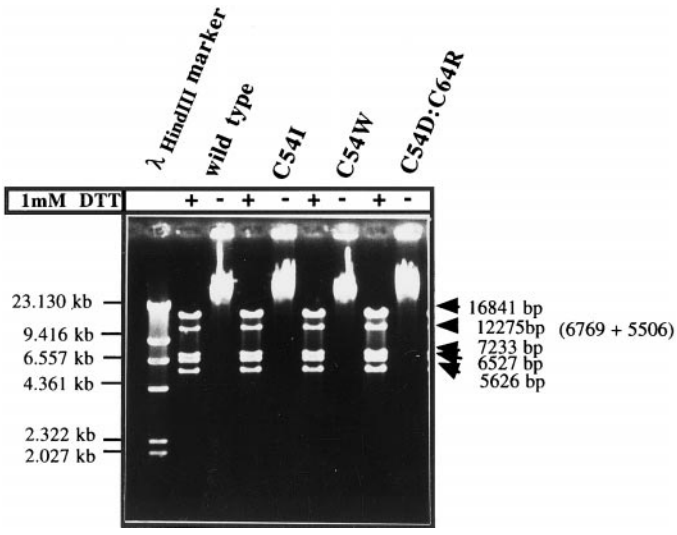

FIG. 2. Cleavage activity and specificity of the *Bam*HI endonucleases in the presence and absence of DTT. One microgram of λ-DNA was incubated with purified *Bam*HI protein in 1× *Bam*HI digestion buffer (10 mM Tris–HCl; pH 8.0; 10 mM $MgCl_2$ and 100 mM NaCl) with and without 1 mM DTT for 16 h at 37°C. The digestion mixtures were loaded on 0.7% agarose gel and electrophoresed in TBE buffer.

due to leaky expression of the T7 promoter which induce SOS response measurable by β-galactosidase activity (9). All the three mutant proteins induced SOS response. It was observed that after transformation the cells were unable to grow and gave a blue colored stain on the plate due to cell lysis caused by DNA damage beyond the repairing capacity of the cells. No colony formation was found with the wild type *Bam*HI endonuclease clone also (data not shown).

Purification and in Vitro Cleavage Kinetics

The *Bam*HI endonuclease and its variants were purified using phosphocellulose and hydroxyapatite chromatography. The purified variant proteins could not cleave λ DNA in the absence of DTT and showed that mutations did not affect the specificity of the endonuclease (Fig. 2).

A short synthetic oligonucleotide substrate which is double stranded under buffer conditions as shown by melting curve (T_m = 56.5°C) measurements, becomes single stranded after cleavage leading to an increase in the absorbance at 254 nm. The rate of substrate cleavage can then be spectrophotometrically monitored and the kinetic parameters determined as described (17). The kinetic assay performed at 25°C resulted in Lineweaver–Burk plots ($1/v$ versus $1/[S]$) for each of the three different *Bam*HI variants (Fig. 3). Table 1 shows the K_m, V_{max}, K_{cat} and the specificity constant (K_{cat}/K_m) values of the wild-type and the mutant *Bam*HI enzymes. The values of the kinetic parameters (K_m, K_{cat}) determined for the wild-type enzyme agreed well with those reported in the literature (17). All the three variants exhibit increased K_m values indicating a decrease in the affinity of the mutant enzyme for its substrate. The increase in the K_m values is more pronounced in the C54D:C64R double mutant and the C54W mutant and less in case of C54I variant. The C54W variant has the highest K_m of 5.1 μM. The activity of all the mutants is less than that of the wild type, but the K_{cat} of C54W and C54D:C64R is drastically less.

Structural Changes

To check for any major secondary or tertiary structural changes due to mutation, CD spectra and melting curves of the purified proteins were measured. The melting curves of the wild type and the mutants proteins (Fig. 4) are overlapping suggesting that mutations have not greatly affected the tertiary structure of the proteins. The changes in secondary structural elements are reflected in the CD spectra of *Bam*HI variants (Fig. 5).

DISSCUSSION

The substrate affinity of the *Bam*HI enzyme has decreased as a result of Cys54 mutation, although the X-ray cocrystal structure does not reveal any contact of Cys54 with the DNA. The neighboring Val57 makes an important contact to the sugar phosphate backbone of DNA at the central part of the recognition site (3). It is possible that Cys54 mutation perturbs the local conformation and compromises the backbone Val57 contact resulting in a decrease in binding. The observed increase in K_m of the variants roughly correlate with the size of the side chain of the amino acid substituted in place of Cys, which is consistent with the above view. The severity of local perturbation is evident in CD spectra of C54W, which has the highest K_m and lowest K_{cat} among the four variants. The bulky aromatic side chain of tryptophan can severely perturb the backbone contacts, raising the K_m but also can compromise the transition state complex geometry by introducing strong aminoaromatic interaction (17) between tryptophan and the flanking two asparagines. Such interactions are known to stabilize the tertiary structure. The amino acids 50–57 are in a loop in the native enzyme, which makes the Val57–DNA backbone contact. When Trp is substituted for Cys54 in this loop region an extended β strand structure is predicted by the Chou Fasman analysis of the stretch of amino-acids 45-73 covering the β2, the loop region and α3. The CD spectra of C54W mutant is very different from that of the wild type protein and the other variants suggests a large local change in the secondary structure in C54W variant. Such secondary structure alteration would affect not only the Val57 but the Lys61 contact

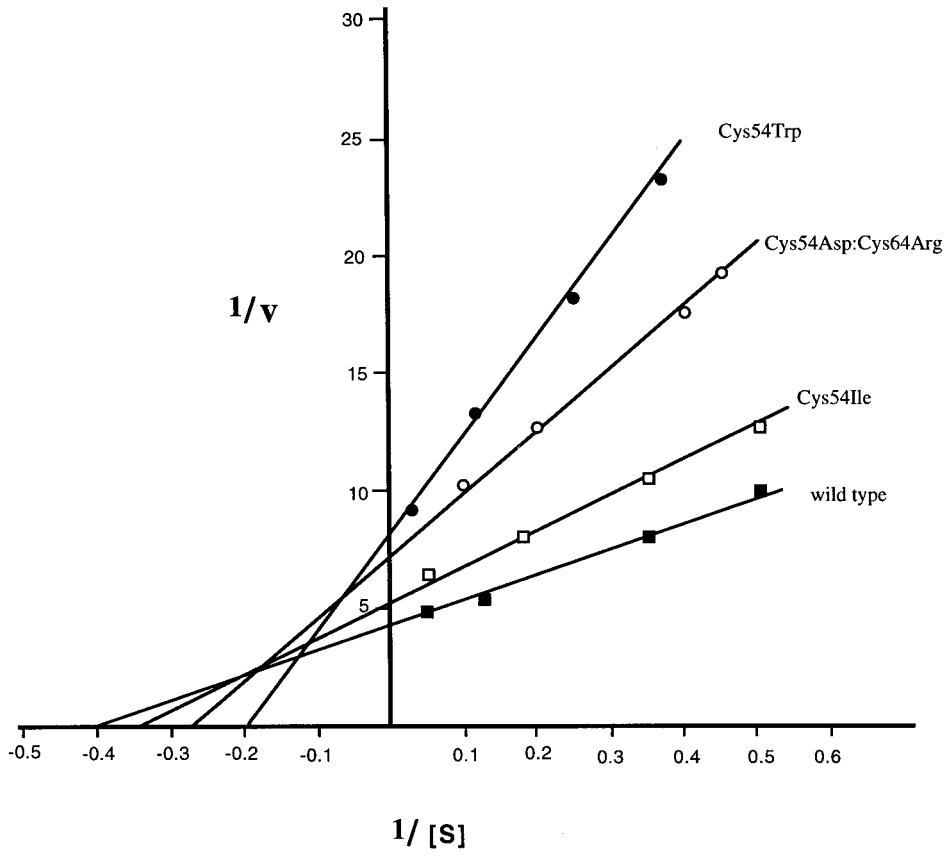

FIG. 3. Lineweaver–Burk plot to determine the kinetic parameters of the purified wild-type and variant *Bam*HI endonucleases. See text for details.

also. Modification of Lysine residues with pyridoxal phosphate inhibits *Bam*HI activity (17). The Lys61 is actually hydrogen bonded to the phosphate group 3′ to the scissle bond and hence can participate in substrate assisted catalysis (6). The CD spectra of C54D:C64R double mutant is similar to those of the C54I variant or the wild-type protein but has very different K_m and K_{cat} values. The specificity constant (K_{cat}/K_m) of this double mutant is less than half of the wild-type enzyme; whereas C54I variant has K_m and K_{cat} values only slightly different from that of native protein. It was observed earlier that C64A mutation drastically reduced DNA cleavage activity of *Bam*HI suggesting a role of Cys64 in catalysis. Thus, if we assume the mutation C54D would not alter the kinetic parameters much, like the C54I variant, the major effect must have resulted from the C64R mutation. The recently published crystal structures of a pre reactive *Bam*HI–DNA complex (7) with two Ca^{2+} ions bound in the active site and a post reactive complex with two Mn^{2+} ions bound at the active site do not show Cys64 involvement in binding to any of these metal ions. It may be noted that in presence of Ca^{2+} *Bam*HI does not cleave the DNA and so it is not the real transition state complex, and the Mg^{2+} coordination can be different from that of Ca^{2+}. In the presence of Mn^{2+} although the DNA is cleaved, the stereochemistry of the post reactive complex may not be relevant for inferring about the transition state productive complex. There can be several possibilities, which can explain the effect of Cys64 mutation.

One possibility is that if a salt bridge forms between Asp54 and Arg64 in this double mutant, it would con-

TABLE 1

Kinetic Parameters of the Wild-Type and the Variant *Bam*HI Endonucleases

Endonuclease type	K_m (μM)	V_{max} (nmol min^{-1})	K_{cat} (min^{-1})	K_{cat}/K_m (μM^{-1} min^{-1})
Wild	2.47	0.238	39.3	15.9
Cys54Ile mutant	2.87	0.191	30.5	10.6
Cys54Trp mutant	5.1	0.123	19.4	3.8
Cys54Asp:Cys64Arg mutant	3.7	0.139	22.5	6.08

Note. Kinetics of cleavage of a 13-mer synthetic deoxyoligonucleotide substrate was followed spectrophotometrically by noting the increase in absorbance over a period of 1 h. The assay was done as per procedure described in Ref. (14). K_m and V_{max} were obtained from Lineweaver–Burk plot.

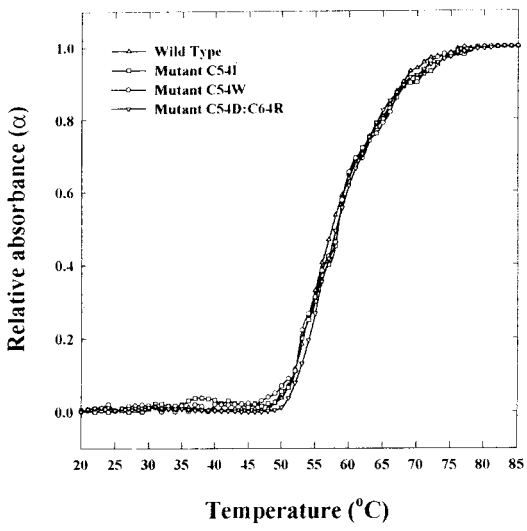

FIG. 4. UV melting profiles of the purified wild-type and variant *Bam*HI endonucleases.

straint and perturb the loop structure in amino acid 45–73 region which makes important contacts with the DNA backbone. That could change both the K_m and K_{cat} severely as explained above in case of C54W. However, this possibility is discounted by the CD spectra of this variant which is very similar to that of the C54I and the wild-type enzyme and not like that of C54W.

The second possibility is that if Cys64 is pulled in (like Glu77 in the Ca^{2+} bound monomer) upon Mg^{2+} binding and participate in the Mg^{2+} coordination sphere shaping a Mg^{2+} binding pocket quite different from that of the Ca^{2+} binding pocket shown in the ground state complex (7). The prereactive Ca^{2+} coordinated complex although ideally suited to follow the two metal ion mechanism proposed for *Bam*HI, yet the DNA is not cleaved indicates that the stereochemistry is different from the Mg^{2+} coordinated transition state complex. However, a direct participation of Cys64 in catalysis is probably ruled out as Cys64 mutation does not abolish catalysis.

A third possibility is that the Cys64 mutation can abolish the Lys61 contact to the next phosphate 3′ to the scissle phosphate. That would affect catalysis according to the substrate assisted catalysis model (6). Essential features of the model are (a) the attacking water molecule is oriented and deprotonated by the 3′-phosphate, (b) the metal ion(s) is bound by two conserved acidic amino acid, (c) the 3′-O leaving group is protonated by Mg^{2+} bound water and d) the extra negative charge on the pentacovalent transition state is stabilized by Mg^{2+} and a nearby lysine which is present in *Eco*RI, *Eco*RV, and *Pvu*II but not in *Bam*HI. In *Bam*HI the equivalent position is occupied by Glu113. It has been argued that pK_a of phosphodiester is too low ($pK_a \leq 2$) to deprotonate a water molecule at neutral pH. Pingoud *et al.* (8) points out that proton abstraction could be a rare event as the restriction enzymes are slow. We think rather, lowering the pK_a of the bound water molecule would serve the same purpose of providing the nucleophile. If the attacking water molecule is hydrogen bonded to the 3′ phosphate, the surrounding electrostatic field due to nearby positive charge of lysine and Mg^{2+} would stabilize the dipolar negative charge on the water oxygen effectively lowering the pK_a of water substantially. In *Bam*HI, the 3′ phosphate is not only hydrogen bonded to the attacking water molecule, it is contacted by Lys61 which may play a similar role like the lysine in the other three enzymes. A K61A mutant in the *in vivo* SOS assay shows much reduced activity is consistent with this view. In *Bam*HI a second metal ion is bound to Asp94 and Glu77 and a water molecule from the inner hydration sphere of this metal ion is supposed to protonate the 3′-O leaving group (7). Mn^{2+} bound water (pK_a = 10.6) is more acidic than Mg^{2+} bound water (pK_a = 11.4) and should be more efficient in protonation yet *Bam*HI is 10-fold less active with Mn^{2+} than with Mg^{2+} as cofactor. A E77K variant, on the other hand, displays increased activity when Mn^{2+} is substituted for Mg^{2+} in the reaction buffer (4). Here also the protonating water molecule is hydrogen bonded to 3′-oxygen of the scissle phosphate and hence placed between the negatively charged transition state and positive charges of the Mn^{2+} and Lys77 effectively lowering the pK_a of the bound water further below 10.6, which would explain Mn^{2+} stimulation of E77K endonuclease activity.

Biochemical evidence of Nath and our mutational data do not fit the two metal ion mechanism proposed from the crystal structure data. It is always difficult to arrive at catalysis mechanism from static data of crystal structure, more so in case of endonucleases as the Mg^{2+} binding geometry is not known, except perhaps in

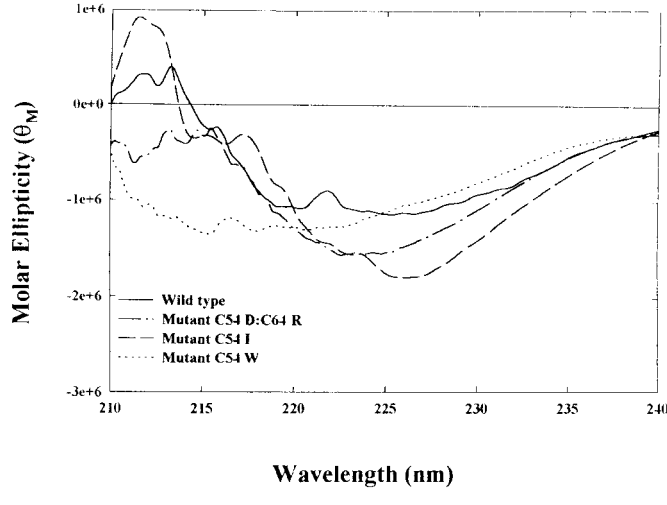

FIG. 5. CD spectra of the purified wild-type and variant *Bam*HI endonucleases.

case of *Eco*RV. All known structures of type II endonucleases, Mut H, ^ exonuclease and *Fok*I are seen to have a similar catalytic core and, with exception of *Bam*HI, to have two acidic residues and a lysine that seem to be critical for catalysis (18). The role of Lysine is adequately accounted for only in the substrate assisted catalysis model. Our data is consistent with this model, which has the additional merit of being plausible for *Eco*RI, *Bam*HI, and *Fok*I, which is very likely as the active site architectures and cleavage patterns of these enzymes are remarkably similar.

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