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Properties and Secondary Structure Analysis of *Ban*I Endonuclease: Identification of Putative Active Site

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Biochemical properties of Type II restriction enzyme *Ban*I were characterized. Kinetic parameters were evaluated and an enhancement of rate was observed when the recognition site was located in a more central position in the substrate, suggesting that *Ban*I locates its recognition site by a sliding mechanism. As *Ban*I has three cysteine residues in its primary sequence, the effect of thiol inhibitors on *Ban*I activity was also studied. Partial inhibition was observed only at a very high concentration of the inhibitor indicating that cysteine residues are not directly involved in catalysis. The gel electrophoretic mobility shift assay demonstrated specific complex formation between *Ban*I and the DNA substrate in the presence of poly dI-dC and Mg^{2+}. A secondary structure analysis and comparison with *Eco*RI and *Bam*HI crystal structure revealed a putative active site similar to that seen in *Bam*HI but different in the order in which the catalytic domain (central β-sheet) and recognition domain (adjacent α-helix) were arranged in the protein.

***Key Words:* *Ban*I; thiol inhibitors; mismatch substrate; secondary structure prediction.**

*Ban*I is a Type II restriction enzyme from Bacillus aneurolyticus, which has been cloned and sequenced (1), yet not much is described about its biochemical and catalytic characteristics in the literature. The crystal structure of R.*Ban*I is also not yet known. *Ban*I yields a staggered cut like *Bam*HI and *Eco*RI whereas *Pvu*II and *Eco*RV cut across the double strand of DNA to give blunt end fragments. *Ban*I and *Bam*HI have almost the same recognition sequence, GGTACC and GGATCC respectively, which differ only in the reversal of the central two A.T basepairs yet have the same cleavage site viz. between the two guanines. Previous work on R.*Bam*HI action on mismatch oligodeoxynucleotide substrates led to interesting insights regarding the mode of *Bam*HI action (2, 3). Recently using similar approach we have shown that *Ban*I-DNA interaction must take place through the major groove like *Bam*HI, and identified important protein contact site (4).

*Ban*I protein like *Bam*HI, contains three cysteine residues although it is not known if sulfhydryl modifying reagents affects its DNA cleavage activity as in *Bam*HI (5). In the present report we describe certain biochemical and catalytic characteristics of *Ban*I. In view of excellent similarity between *Bam*HI and *Ban*I we compare the predicted secondary structure of the *Ban*I protein with the known crystal structure of *Bam*HI and identify the putative active site.

MATERIALS AND METHODS

Restriction enzyme *Ban*I was purchased from New England Biolabs. The sulfhydryl inhibitors, DTNB and iodoacetic acid, were from Sigma. The substrate λ-DNA was obtained from Promega.

Reaction kinetics with plasmid-derived substrates. Kinetic constants were determined following a published procedure (2). 20 μg pUC-19 was digested with *Pvu*II (50 units) and *Hin*dIII (40 units) to generate two fragments of length 141- and 181-bp, respectively, each containing a single *Ban*I site. 0.5 pmoles of each fragment substrate was labeled at 3′-end using Klenow enzyme (0.5 units) and ^{32}P α-dATP (15 μCi) and each of the other three cold dNTPs (1 mM) in a total reaction volume of 10 μl. The labeled fragments were purified from free label and other contaminants of the extension reaction by 8% polyacrylamide gel electrophoresis (6). Varying concentrations of substrate (141- and 181-bp fragments) in the range of 9–90 nM were incubated with fixed amount of enzyme (3 nM) at 37°C. Each reaction mix contained a fixed amount of 3′-radiolabeled substrate (0.1 nM) and different concentrations of unlabeled substrate in a total reaction volume of 30 μl in standard *Ban*I digestion buffer. After initiating the reaction by adding *Ban*I enzyme, six aliquots of 5 μl each were withdrawn at different time-points of 1, 2, 3, 4, and 5 min respectively and the reaction was terminated by mixing with a 5 μl solution containing 80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10% glycerol, and 50 mM EDTA. These samples were heated to 90°C for 2 min after which they were chilled on ice and quickly loaded on a 5% denaturing 7M urea-polyacrylamide gel. Electrophoresis was done at a constant current of 30 mA until the bromophenol dye front had migrated two-third of the gel. The gel was transferred to a Whatman 3M sheet, covered with Saran wrap, and exposed to X-ray film. The autoradiogram was scanned densitometrically (Ultroscan software) to determine the cleavage percentage. The

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velocity curves were obtained by plotting the percent cleavage versus time for different substrate concentrations and the initial velocities determined (Fig. 1).

Effect of thiol inhibitors. For preincubation studies, 1 μl of an inhibitor or 2-mercaptoethanol solution was added to 5 μl of the double strength reaction buffer containing 5 units of the restriction endonuclease and incubated at 37°C for 1 h. The reaction was initiated by the addition of 5 μl of a solution that contained 0.33 μg λ-DNA in 10 mM Tris–HCl (pH 8.0). Alternatively, 1 μl of the inhibitor or 2-mercaptoethanol was added to 10 μl of a reaction mixture (on ice) that contained the enzyme and λ-DNA, for the reaction to proceed without any preincubation. After incubation at 37°C for 60 min the reaction was terminated by the addition of 2.5 μl of a stop solution containing 170 mM EDTA (pH 8.0), 10% glycerol, and 0.02% bromophenol blue. The double-strength restriction buffer for *Ban*I contained 20 mM Tris–HCl (pH 7.9), 20 mM $MgCl_2$, and 100 mM potassium acetate and that for *Bam*HI contained 125 mM Tris–HCl (pH 7.5), 125 mM NaCl, and 25 mM $MgCl_2$. The stock solutions of DTNB (100 mM) and iodoacetate (100 mM) were prepared fresh in 10 mM glycine (pH 9.0) and appropriate amounts of 1M NaOH to achieve a final pH of 8.5. Further dilution was carried out in 10 mM Tris–HCl (pH 8.0). Stock solutions of 2-mercaptoethanol (0.1, 1, and 5 M) were prepared fresh in sterile water.

The restricted samples (about 10–11 μl) were incubated at 65°C for 5 min followed by 5 min on ice and were placed in slots of 0.6% agarose horizontal slab gel. The gel and the electrophoresis buffer (40 mM Tris-acetate, 5 mM EDTA, pH 8.0) contained 0.5 μg/ml of ethidium bromide. Electrophoresis was performed at 60 V for 1 h and recorded on a gel documentation system.

Isolation and purification of BanI endonuclease. About 10 g cells of the strain *B.aneurinolyticus* IAM1077 were processed for *Ban*I isolation using the purification method developed by Sugisaki *et al.* (7) which involved phosphocellulose and DEAE-52 cellulose column chromatographies. The dialysed preparation was used for gel shift assay.

Gel electrophoretic mobility shift assay. Initially about 5 μg of the phosphocellulose purified *Ban*I preparation was incubated separately with 1 ng of 5′-end radiolabeled oligodeoxynucleotides TA-13 (5′-AGTGCGGTACCGC-3′) and TG-13 (5′-AGTGCGGTGCCGC-3′) (T.G mismatch containing sequence) in presence and absence of 1 μg calf thymus DNA as a nonspecific competitor. Binding reaction was carried out at room temperature in standard *Ban*I digestion buffer (pH 7.9) containing no Mg^{2+} but 50 mM EDTA for 2 h and analysed on 8% native PAGE. In a subsequent experiment, about 1 μg of the DE-52 purified *Ban*I preparation was incubated with 0.1 ng of radiolabelled TA-13 or TG-13 for 2 h at 30°C in standard *Ban*I digestion buffer (pH 7.9) containing either (a) 10 mM magnesium acetate and no EDTA or (b) in presence of 50 mM EDTA but no Mg^{2+}. To prevent nonspecific binding, 1 μg poly dI-dC or calf thymus DNA was also included in the reaction. The mixture was carefully loaded on a 8% native polyacrylamide (30:1 acrylamide–bisacrylamide ratio) in 0.5X TBE buffer and electrophoresed at 100 V constant voltage at room temperature till the bromophenol blue dye had migrated halfway down the gel. The gel was dried between Whatman 3M sheets, covered with Saran wrap and exposed to X-ray film.

Programs used for secondary structure prediction. The sequence analysis package by Genetic Computer Inc. (the GCG package) has been used for the analyses. The Chou-Fasman, "nnpredict" and "SSP" programs that assign a secondary structure (α-helix, β-sheet, turn, coil) to each amino-acid in the protein sequence were used. The SSP program uses linear discriminant analysis to assign segments of a given amino-acid sequence to a particular type of secondary structure, by taking into account the amino-acid composition of internal parts of the segments as well as their terminal and adjacent regions.

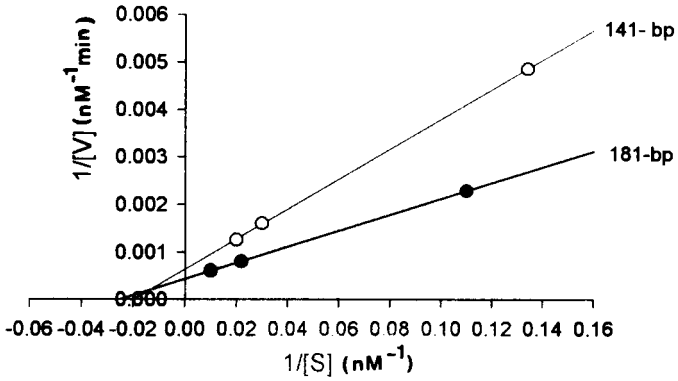

FIG. 1. Lineweaver-Burk plot of the cleavage kinetics for the 141- and 181-bp fragments.

RESULTS AND DISCUSSION

Kinetic Properties of BanI Endonuclease with Linear DNA Substrates

The 141- and 181-bp substrates used for kinetic analysis, contained a single *Ban*I site each. The 141-bp fragment had the *Ban*I site asymmetrically positioned near the 3′-end, and produced the 39- and 102-bp fragments upon *Ban*I cleavage. Kinetic parameters from Lineweaver-Burk plot (Fig. 1) were calculated for both the substrates. The K_m values for the 181- and 141-bp fragments were found to be 45.45 and 66.7 nM respectively. The corresponding k_{cat} values were 0.505 and 0.35 min^{-1}. Thus the endonuclease exhibited faster reaction rate with the longer (181-bp) substrate having the recognition site in a more central location. This suggests that *Ban*I can locate its recognition site by a sliding or hopping mechanism when an enhancement of rate would occur if the recognition site is located in a more central position. In the case of *Bam*HI a similar observation had been made (8) and our results are consistent with those observed for other Type II restriction enzymes (9, 10).

Effect of Thiol Inhibitors

Both *Ban*I and *Bam*HI proteins are known to have three cysteine residues each. Nath (5) studied the effect of sulfhydryl group inhibitors, 5,5-dithiobis (2-nitrobenzoic acid (DTNB), iodoacetate (IAA), *p*-mercuribenzoate (PMB) and *N*-ethylmaleimide (NEM) on the activity of eleven restriction endonucleases excluding *Ban*I. He reported that *Bam*HI endonuclease activity was totally inhibited by DTNB which could be rescued when 2-mercaptoethanol was added in the reaction mixture. It is known that *Bam*HI requires DTT for its cleavage reaction. It is imperative, therefore, to compare the effect of thiol inhibitors on the activity of R.*Ban*I and R.*Bam*HI when the two enzymes have so much similarity. Our results with DTNB and iodoacetic acid on the activity of commercially purchased *Ban*I are as follows.

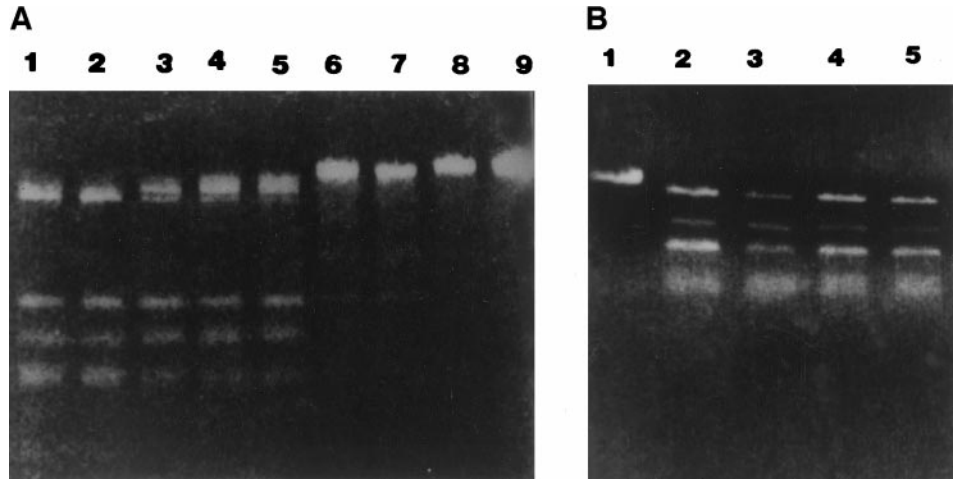

FIG. 2. (A) Effect of DTNB on *Ban*I activity. Varying concentrations of DTNB were preincubated with *Ban*I and the activity assayed using λ-DNA as a substrate. DTNB concentrations in lanes 1–8: 0, 2.5, 5, 10, 20, 30, 50, and 100 mM. Lane 9: undigested λ-DNA. (B) Effect of iodoacetic acid on *Ban*I activity. Varying concentrations of iodoacetic acid were added to the reaction mix and incubated for 1 h at 37°C. λ-DNA was used as substrate. Concentrations of iodoacetic acid used: lane 1, 100 mM IAA; lane 2, 30 mM IAA; lane 3, 16.7 mM IAA; lane 4, 1.67 mM IAA; lane 5, 0 mM IAA.

Effect of DTNB

When preincubated at 37°C for 5 min, the endonuclease *Ban*I was affected very little by DTNB in the concentration range of 0–20 mM DTNB (Fig. 2A) and without preincubation the result remains the same. At DTNB stock concentrations of 30 mM and higher, however inhibition was observed, which could not be rescued by the addition of 83 mM 2-mercaptoethanol.

Nath reported that the inhibition of *Bam*HI activity became evident above 0.45 mM DTNB in the formation of partial digest and preincubation with 16.7 mM DTNB abolished the *Bam*HI activity.

Effect of Iodoacetic Acid

Preincubation of *Ban*I with iodoacetic acid (0–30 mM) for 10 min, did not affect *Ban*I activity (Fig. 2B). The results obtained without preincubation were similar.

Nath had found that preincubation with 16.7 mM iodoacetate caused slight inhibition in *BamH*I activity but had no effect on the activities of the other restriction endonucleases. It is evident that the inhibitor iodoacetic acid is less effective than DTNB.

Nath classified the effects of sulfhydryl inhibitors into three categories. The first category includes those restriction endonucleases which are not affected and probably does not involve participation of thiol groups in catalysis, e.g., *Eco*RI, *Hind*III, *Sal*I, *Bgl*II, *Hpa*I, and *Sst*II. Among these, *Hind*III and *Sst*II activities were only partly inhibited by high concentrations of DTNB but appeared insensitive to the addition of 2-mercaptoethanol. This could imply that DTNB perhaps reacted with groups other than thiols in these two restriction endonucleases. We observed *Ban*I to behave like this group of enzymes.

The second category includes the restriction endonucleases, whose activity can be totally and reversibly inhibited by DTNB hence possibly depended on participation of reduced thiol groups as in *Bam*HI. Importantly, the DTNB inhibition when rescued by sulfhydryl reducing agents imply that the availability of the reactive thiol group is rate-limiting.

The third category includes those endonucleases whose reactivity probably depended upon the active participation of oxidised thiol groups (S-S). These enzymes normally do not react with PMB but upon preincubation, a partial inhibition can be observed.

Perturbation of Sequence Specificity by Organic Solvents (Star Activity)

Effect of glycerol. Increasing the concentration of glycerol in the reaction mixture containing *Ban*I under standard digestion conditions caused inhibition of the enzyme activity. When pUC-19 was used as substrate and glycerol concentration in the reaction mixture was varied from 0–70%, partial inhibition of activity became evident at glycerol concentrations of 30%. Inhibition became more apparent as the glycerol concentration in the reaction mixture was increased (Fig. 3A). Thus, no star activity was observed in case of *Ban*I.

Effect of dimethyl sulfoxide. A similar cleavage pattern with pUC-19 as seen above with glycerol was also observed with DMSO. Inhibition of *Ban*I activity became evident at concentrations above 10% DMSO (Fig. 3B). These observations may be explained in the context of an enhanced selectivity for the outer bases by the enzyme since the central basepair is degenerate in the *Ban*I recognition sequence **GGPyPuCC.** When organic perturbants are added, conformational perturba-

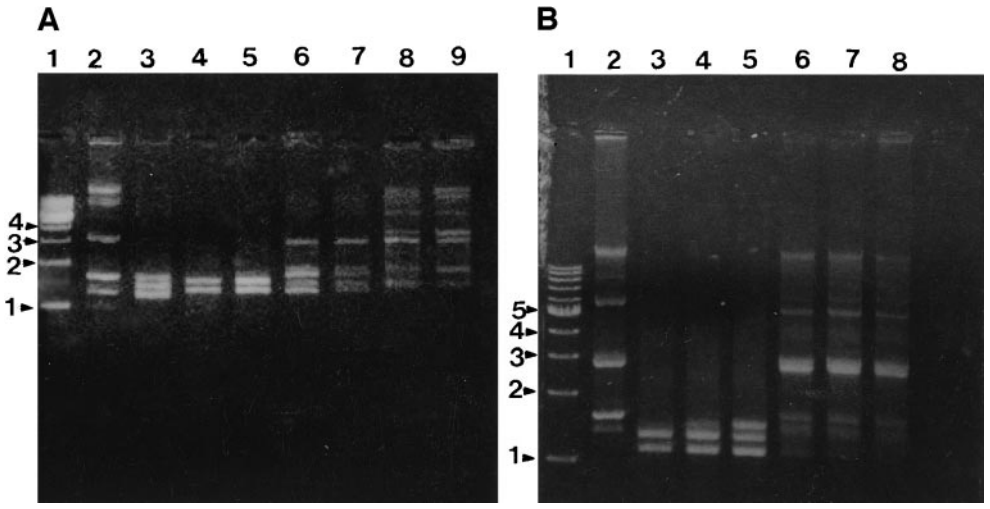

FIG. 3. (A) Effect of perturbation on *Ban*I activity by glycerol. Lane 1, 1-kb step ladder (Promega); lane 2, undigested pUC-19; lanes 3–9, reaction mixtures containing pUC-19 and *Ban*I under standard digestion conditions to which 0, 10, 20, 30, 50, 60, and 70% glycerol, respectively, was added. (B) Effect of perturbation on *Ban*I activity by dimethylsulfoxide. Lane 1, 1 kb step ladder (Promega); lane 2, undigested pUC-19; lanes 3–8, reaction mixtures containing pUC-19 and *Ban*I in standard digestion conditions to which DMSO was added at concentrations of 0, 2, 5, 10, 17, and 20%, respectively.

tion in the DNA and/or the protein may induce new contacts at the cost of essential contacts with the outer bases leading to an inhibition of activity. However, at high nuclease concentrations during modified reactions, digestion of pBR-322 DNA or lambda DNA in the presence of high glycerol and dimethyl sulfoxide were reported to have produced additional fragments pertaining to star activity (11).

Binding Properties (Gel Retardation Assay)

The results of our binding experiments are interesting. When partially (phosphocellulose column) purified *Ban*I protein was incubated with TA-13 or TG-13 oligodeoxynucleotide substrates, a retarded band was seen in the gel mobility shift assay only in the absence of Mg^{2+} and calf thymus DNA (data not shown). Addition of nonspecific DNA, in absence of Mg^{2+}, titrated out the retarded band. This suggest that the retarded band is not due to specific binding. *Ban*I possibly binds to non-specific and specific sequences with equal affinity in the absence of Mg^{2+}. Similar results had been reported in the case of *Eco*RV, where specific binding required Mg^{2+} (12). In presence of Mg^{2+}, however, the substrate might be cleaved and one may not see the gel retardation of the specific complex. As TG-13 oligomer is cleaved by *Ban*I very slowly (4), we incubated this mismatch substrate with *Ban*I preparation, which was already further purified on DE-52 ion-exchanger. The results of this assay is shown in Fig. 4. Clearly a faint retarded band is seen in presence of Mg^{2+} which persists even when the incubation mixture contained poly dI-dC. Surprisingly, no retarded band due to nonspecific complex in the absence of Mg^{2+} (Fig. 4, lanes 1 and 2) is seen as was seen previously. This implies that the retarded band seen earlier was probably due to some other associated protein and not *Ban*I, since the phosphocellulose purified *Ban*I preparation had several major protein contaminants.

FIG. 4. Gel electrophoretic mobility shift assay. TG-13 was incubated with DE-52 purified *Ban*I extract under the following conditions: lane 1, absence of Mg^{2+}, absence of poly dI-dC; lane 2, absence of Mg^{2+}, presence of poly dI-dC; lane 3, presence of Mg^{2+}, absence of poly dI–dC; lane 4, presence of Mg^{2+}, presence of poly dI–dC.

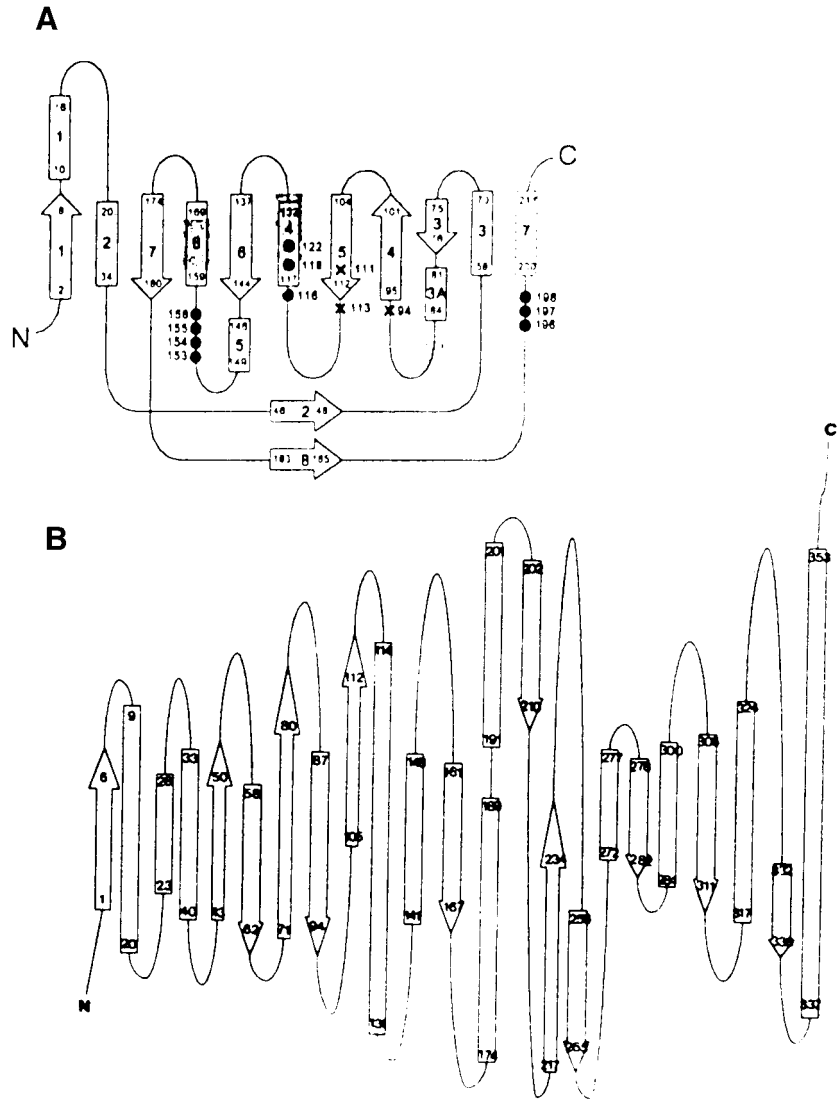

FIG. 5. Predicted organization of the secondary structure of R. *Ban*I (this work) compared with known structure of *BamH*I. Cylinders represent the α-helices and arrows indicate the β-sheets. (A) Secondary structure of R. *Bam*HI (reproduced from Ref. 13 and reprinted with permission from *Nature* **368,** 660–664, copyright 1994, Macmillan Magazines Ltd.). (B) Predicted secondary structure of *Ban*I.

Secondary Structure Prediction

The known X-ray structures of *Bam*HI and *Eco*RI enzymes show that the important secondary structure elements bearing the catalytic residues and amino-acids that make DNA contacts lie in the 100 to –150-aa region (13). When we compared the crystal structures with the predicted secondary structures by various programs, we found that Chou-Fasman plot had a better predictability in this important "common core motif" region (data not shown). We therefore considered Chou-Fasman predictions for the secondary structure of *Ban*I. This structure (Fig. 5) when compared with the crystal structure of *Bam*HI, a very striking structural homology is observed in the amino-acid region 100–200 having the same number of α-helices and β-sheets. This 100-amino-acid stretch is the functionally important region as seen in the crystal structures of *Bam*HI and *Eco*RI. The active site residues for both *Bam*HI and *Eco*RI identified by elegant mutation studies fall in this region (14–16), comprising of a central beta sheet followed by an α-helix. In *Ban*I also, we can identify an α-helix (aa 175–190) followed by a β-sheet (aa 203–211), which bear the base binding residues Glu 203, Arg 204, and Arg 210. The intervening region (aa 191–200) is interesting in the sense that it bears Asp 195, Glu 196, Lys 198, and Lys 199, which are known to take part in catalysis (D/E) and binding (K) and seem to be the functionally most important. We therefore identify this α-helix loop-β sheet as the putative active site of *Ban*I. The reverse order of arrangement of

the α-helix and the β-sheet in *Ban*I compared to that observed in *Bam*HI, is possibly related to the reversal of the two central base pairs in the *Ban*I site (4).

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