Chapter 5

Interaction of BanI With Canonical And Mismatch Substrates
The interaction of Ban I with native and altered oligodeoxynucleotide substrates with mismatch basepairs was studied with a view to define the role the functional groups on base edges play in the process of enzyme binding. Substrates that bind yet may not be cleaved, or not bind at all or substrates that bind and get cleaved at very different rates compared to the ideal substrate, would give appropriate insight in the recognition process. To discriminate between these alternatives, cleavage analysis as well as cleavage kinetic studies and finally inhibition kinetic studies with the mismatch substrates were done. The rationale for the design of the substrates has been previously discussed (Chapter 2). Here we discuss the interaction of these substrates with the native enzyme.

Cleavage Analysis Of The Oligodeoxyribonucleotide Substrates

5.1 12-mer Studies

The oligodeoxyribonucleotide dodecamer substrates were 5'-endlabeled using \( \gamma^{32}\text{P}-\text{ATP} \) and T4-polynucleotide kinase and incubated with BanI in standard digestion conditions. The cleavage products were then analysed on 30% denaturing polyacrylamide gels.

5.1.1 Endlabeling Oligonucleotide substrates:

The dodecamers were radiolabeled at 5'-end to high specific activity as described by Newman et al., (1990), with minor modifications. Briefly, 20 pmoles of the dodecamer, 20 \( \mu\text{Ci} \) \( \gamma^{32}\text{P}-\text{ATP} \) and 10 units of T4 polynucleotide kinase in 30\( \mu\text{l} \) buffer
was incubated at 30°C for 48 hours. Then, a further 10 units of T4 polynucleotide kinase was added and the reaction was allowed to continue for another 24 hours. The labeling was about 90% as estimated by spotting 1 µl of the reaction mixture on DE-81 Whatman paper, which was then washed successively once with 0.5M sodium phosphate buffer (pH 7.) and twice with TE buffer. The radioactive counts were measured in a beta counter before and after the wash to determine the level of $^{32}$P incorporation. The labeled oligonucleotides were purified from free $\gamma^{32}$P-ATP by polyacrylamide gel electrophoresis. When run on a 30% denaturing urea polyacrylamide gel, all the five oligonucleotides showed a single band indicating the purity (Figure 33).

5.1.2 BanI Digestion

5 pmoles of 5'-end-labeled dodecamers was digested in a microfuge tube with varying amounts (10-50 units) of BanI (New England Biolabs) in a 30 µl reaction mix containing 10 mM Tris- acetate (pH 7.9), 10 mM MgCl₂, 50 mM potassium acetate, 1mM dithiothreitol, at 37°C for two hours. For the altered substrates the time of incubation was varied from 2 hours to 16 hours to determine whether cleavage took place. The reaction was stopped by immersing the reaction tubes in a boiling water bath for 2 minutes. The digested products were separated from the uncleaved substrates on 30% denaturing polyacrylamide gels and visualised after autoradiography. As the dodecamers were endlabeled at the 5'-end only, one of the cleavage product was visible on autoradiography. Hence to determine the direction and site of cleavage, it was
Fig. 33: Denaturing 7 M urea-polyacrylamide gel for 5’-end-labeled dodecamers showing the purity. Lanes 1-5 show the kinased 12-mer substrates, AT-12, TA-12, TG-12, AG-12 and AC-12 consecutively.

Fig. 34: Cleavage analysis of BanI digestion products of the dodecamers on 7M urea-PAGE. Lane 1: Undigested TA-12. Lane 2: TA-12 after BanI digestion. Lane 3: TG-12 after overnight BanI digestion. Lane 4: Undigested TG-12. Lane 5: AT-12 after BanI digestion. Lane 6: AG-12 after BanI digestion.
necessary to know the sizes of both the products. We therefore carried out a rekination reaction just after the BanI digestion in the same buffer. The cleaved product will have a 5'-phosphoryl group and therefore an exchange reaction should have been used. However to avoid further repurification which might lead to a considerable loss of the short oligomer we chose to continue with the same reaction conditions. T4-polynucleotide kinase being able to catalyse a reversible reaction, an exchange of free label does take place with the 5'-phosphoryl group with a lower efficiency (35% as compared to 65% for the exchange reaction) of transfer to the oligonucleotide (Van de Sande et al., 1973, Berkner and Folk 1977.)

Typically the oligonucleotide products formed by BanI cleavage were again kinased by further addition of 10 pmoles of γ-32P ATP, 10 units of T4 polynucleotide kinase and carrying out incubation for 24 hours at 30°C, to determine the site and directionality of cleavage.

5.1.3 Results

When the dodecamers were digested with Ban I in standard digestion condition and analysed on denaturing polyacrylamide gels, it was observed that apart from the native T.A sequence, only the sequence containing T.G mismatch in the central region was cleaved at the same site but at a much slower rate. No other sequence was detectably cleaved even after digestion for 6 hours or even overnight at 37°C (Figure 34), and despite repeated attempts.
The double kination experiments with the 12-mers were not successful owing to the inability to optimise the conditions of this reaction which is already reported to have low efficiency (Van de Sande et al., 1973, Berkner and Folk, 1977). Hence we decided to alter our strategy by using 13-mers as described below.

5.2 13-mer Studies

(Determination of the site and directionality of cleavage in 13-mers)

Self-complementary 13-mer oligodeoxynucleotides of the design, 5'-AGTGCGGTACCGC-3' were synthesised which forms a double-stranded structure with 3-base overhangs and could be extended by Klenow enzyme and dNTP's to a double stranded 16-mer oligodeoxynucleotide with radiolabel at 3'end.

5.2.1 Endfilling reaction: 3'-label

The endfilling reaction was carried out according to Maniatis et al (1989). Briefly, to a 10 μl reaction mix containing 100 ng oligodeoxynucleotide 13-mer (20 pmole 5'ends) in 10mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 mM each of dCTP, dTTP and 12.5μCi (approximately 20 pmole) of α-³² P-dATP, 0.5 unit of Klenow fragment of DNA polymerase I was added and incubated at 37°C for 2 hours. To generate flush ends, further 0.5 units of Klenow fragment and 1 mM each of the three dNTPs (cold) were added and the incubation continued for one more hour. This is called as the “cold chase reaction”. The extent of incorporation was estimated by spotting 1 μl of the reaction on DE-81 paper, which was then successively washed with 0.5M sodium phosphate buffer
and twice with TE buffer, and the radioactive counts were measured in a beta counter both before and after the wash. This procedure gave high specific activity of about 90%.

5.2.2 *The same 13-mers could also be 5'end-labeled.*

The TG–13 mismatch substrate was 5'-end-labeled using γ²²P-ATP and T4 polynucleotide kinase and subsequently PAGE purified. Upon BanI digestion, this yielded the 5'-labeled product. Hence both the products of cleavage could be visualised (and the cleavage site could be determined) using denaturing polyacrylamide gel electrophoresis. The native TA-13 sequence on 5'end labeling and subsequent cleavage should yield a 6-mer fragment (5'-AGTGCG-3') but when 3' labeled by extension with Klenow, a 10-mer fragment (5'-GTACCGCAct-3') should be visible. The 6-mer from 5'-end and a 9-mer fragment from 3'-end were observed in the case of TA-13 (Figure 35). When TG-13 (Figure 36) was 3'-end-labeled and digested with BanI, a 9-mer instead of expected 10-mer fragment was observed. This occurred because even after the "cold chase reaction", a 15-mer in place of a 16-mer was obtained (Fig.38) which upon BanI cleavage would produce the 9-mer fragment (Fig.36). No other sequence was detectably cleaved as had been observed in the case of the 12-mers (Figure 37) substrates even when they were extended to 15-mer (Fig. 38).

5.3 Cleavage analysis of the 15-mers:

Considering that the length of the oligomer may be a factor for efficient binding and hence cleavage we tried also to see the rate of cleavage on 15-mers of the design
Fig. 35: Cleavage analysis of TA-13. Lane 1: Oligonucleotide marker (4-22 basepair oligo dT ladder)(Gibco-BRL). Lanes 2 & 4: 5'-end labeled TA-13 with and without *BanI* digestion respectively. Lanes 3 and 5: 3'-end labeled TA-16, digested and undigested respectively, with *BanI*.

Fig. 36: Cleavage Analysis of TG-15 and TG-16: Lane 1: Oligonucleotide molecular weight marker (Gibco-BRL). Lanes 2 & 3: 5'-end labeled TG-15 with and without *BanI* digestion respectively. Lanes 4 & 5: 3'-end labeled TG-16, with and without *BanI* digestion respectively.
Fig. 37: Cleavage analysis of other 13-mer substrates and AG-17.
Lane 1: Oligonucleotide marker (Gibco-BRL);
Lane 2: Undigested AT-13; Lane 3: AT-13 after Ban I digestion;
Lane 4: Undigested AG-13; Lane 5: AG-13 after Ban I digestion;
Lane 6: Undigested AC-13; Lane 7: AC-13 after Ban I digestion;
Lane 8: Undigested AG-17; Lane 9: AG-17 after Ban I digestion.

Fig. 38: Cleavage analysis of remaining 13-mers extended to 16-mers.
Lane 1: Oligonucleotide marker (Gibco-BRL); Lane 2: Undigested AC-16;
Lane 3: AC-16 after Ban I digestion; Lane 4: AT-16 after Ban I digestion;
Lane 5: TC-16 after Ban I digestion; Lane 6: AG-16 after Ban I digestion;
Lane 7: Undigested AT-16.
5'-TCTAGAGGTGCCTCT-3' (denoted as TG-15) and 5'-TCTAGAGGCACCTCT-3' (denoted as CA-15). These could be 3' labeled by Klenow enzyme to form blunt-ended 18-mers and thus the site and directionality of cleavage could be easily ascertained as with 13-mers. Both TG-15 and CA-15 were also 5'-end labeled with $\gamma$-32P ATP and polynucleotide kinase and then gel-purified. The TG-15 sequence was cut by BanI and yielded the expected 7-mer fragment (5’-TCTAGAG-3’) (Figs. 36 and 39) whereas the CA-15 sequence was not cleaved despite overnight digestion at 37°C (Fig. 39). The efficiency of cutting of the TG-15 sequence was not much different from that observed with the 13-mers described above.

Even when these 15-mers were extended to 18-mer double stranded deoxyoligonucleotide substrates with Klenow polymerase and $\alpha$-32P-dATP and dGTP, gel-purified and then subjected to BanI cleavage under standard conditions, the results were no different.

5.4 Cleavage Analysis of the 17-mer AG Mismatch

The efficiency of extension by Klenow of the AG-13 sequence was found to be very poor. With a view to create a better double stranded sequence which would not pose the problem of self structure as had been observed with the self-complementary 18-mers, two complementary 17-mer oligonucleotides containing –AG– mismatch in the central basepair of the BanI recognition sequence were synthesised. These could be 5’end labeled with T4 Polynucleotide kinase. After 5’end labeling each of the deoxyoligonucleotides separately they were column-purified via the Qiagen nucleotide removal kit to remove the free label and eluted in 5 mM Tris-HCl (pH 8.0), 0.5M EDTA
Fig 39: Cleavage analysis of 15-mers, TG-15 and CA-15: Lanes 1-4 represent cleavage kinetics of TG-15 where products were analysed at time-points of 0, 60, 120 and 180 minutes respectively. Product formation was very slow. Lane 5-8 represent the status of CA-15 upon treatment with BanI. The samples were analysed at time-points of 0, 60, 120 and 180 minutes respectively and no cleavage products could be seen.

Fig. 40: Cleavage analysis of 18-mers. Lane 1: AT-18 after BanI digestion; Lane 2: TC-18 after BanI digestion; Lane 3: Undigested TA-18; Lane 4: TA-18 after BanI digestion; Lane 5: Undigested TG-18; Lane 6: TG-18 after BanI digestion.
Equimolar concentrations of the above oligonucleotides were mixed, heated to 90°C and slowly annealed overnight to 4°C. To 5 pmoles of the annealed oligonucleotides was added 50 units of BanI in standard digestion buffer and incubated at 37°C for six hours. No detectable cleavage was observed upon denaturing polyacrylamide gel electrophoresis. We were thus able to verify the result of AG-12 using this strategy of a longer substrate which proved that AG mismatch does not get cleaved by BanI whether the substrate is a 12-mer or a 17-mer (Fig. 37).

5.5 Cleavage analysis of the 18-mers

The 13-mer mismatch sequences displayed low efficiency of extension by Klenow enzyme. This implied that the presence of the mismatch might be hampering the Klenow reaction. To avoid this deficiency, blunt ended 18-mer self complementary oligonucleotides were also used. They were denoted by AT-18, TA-18, TG-18 and TC-18 and were of the general design:

5'-TAGCGCGG**CCGCGCTACG-3'

where * represent the bases where alteration was done except in the case of the TC sequence where the recognition sequence is 5'-GGTATC-3'. These were 5'-end labeled with T4 polynucleotide kinase and γ²³³P-ATP followed by purification on Qiagen nucleotide removal column to remove the free ATP and then eluted in 5 mM Tris-Cl, 0.5 mM EDTA (pH 8.0). The oligodeoxynucleotides were then subjected to BanI digestion. The cleavage results supported our previous data namely that only the –TA-
and the -TG sequences were cleaved (Figure 40). The -AT-and -TC substrates were not
detectably cleaved even after overnight digestion with BanI at 37\(^\circ\) C.

However the efficiency of cutting of the 18-mer native sequence by BanI was
observed to be poorer than that seen with the 13-mers. This could be explained if there
was self structure formation in the 18-mers. This is a problem we frequently encountered
that as the length of the self-complementary oligonucleotide is increased, the probability
of self structure formation also increased. It is apparent that these 18-mers exist in two
different forms in solution; a mixture of a major form (hairpin) which migrated faster
than the minor form (duplex) in a 20\% native polyacrylamide gel (Fig.41). This
contention is also supported by uv – melting curve data (Fig. 27) and CD spectra
(Fig. 32).

5.6 Discussion

The data of this section is summarised in Table 5. All the substrates, except the
18-mer oligodeoxynucleotide substrates exist in double-stranded B-DNA conformation
as indicated from the T\(_m\) and C.D measurements. The 18-mers, however, exist in solution
as two conformers, the lower melting double-stranded form and the higher melting
hairpin form. The T\(_m\) values for the altered substrates are not significantly lower than the
cognate substrate in all cases. This implies that under cleavage conditions, the double-
stranded B-DNA conformation is still maintained even for the altered substrates.
Fig. 41: Migration of 18-mers in 20% native polyacrylamide gel.
Lane 1: TA-18  Lane 2: TG-18
## Table 5

<table>
<thead>
<tr>
<th>Sequence notation</th>
<th>Sequence</th>
<th>Conformation as from UV and CD measurements</th>
<th>Cleavage and fragment sizes</th>
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<tr>
<td>TA-12</td>
<td>5'-CGCGGTAACCGC-3'</td>
<td>Double-stranded, B-form</td>
<td>++, 4-mer, 8-mer</td>
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<td>AT-12</td>
<td>5'-CGCGGATCCGC-3'</td>
<td>Double-stranded, B-form</td>
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<tr>
<td>TG-12</td>
<td>5'-CGCGGTGCCGC-3'</td>
<td>Double-stranded, B-form</td>
<td>+, 4-mer, 8-mer</td>
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<td>Double-stranded, B-form</td>
<td></td>
</tr>
<tr>
<td>AC-12</td>
<td>5'-CGCGTTAACC-3'</td>
<td>Double-stranded, B-form</td>
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</tr>
<tr>
<td>TA-13</td>
<td>5'-ATGCGTACCACCG-3'</td>
<td>Double-stranded, B-form</td>
<td>++, 6-mer, 7-mer</td>
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<td>5'-ATGGCGTGACCGC-3'</td>
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<td>++, 6-mer, 9-mer</td>
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<td>5'-ATGCGGATCCGC-3'</td>
<td>Double-stranded, B-form</td>
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</tr>
<tr>
<td>AT-16</td>
<td>5'-ATGGCGGATCCGCAT-3'</td>
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</tr>
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<td>TG-13</td>
<td>5'-ATGGCGTGACCGC-3'</td>
<td>Double-stranded, B-form</td>
<td>+, 6-mer, 7-mer, 10-mer</td>
</tr>
<tr>
<td>TG-16</td>
<td>5'-ATGGCGTGACCGC-3'</td>
<td>Double-stranded, B-form</td>
<td>+, 6-mer, 7-mer, 10-mer</td>
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<tr>
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<td>5'-ATGGCGGAGCGCG-3'</td>
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<tr>
<td>AG-16</td>
<td>5'-ATGGCGGAGCGCGCAT-3'</td>
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<tr>
<td>AC-13</td>
<td>5'-ATGGCGTACCACCGC-3'</td>
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<tr>
<td>AC-16</td>
<td>5'-ATGGCGTACCACCGCAT-3'</td>
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</tr>
<tr>
<td></td>
<td>Sequence</td>
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<td>TC-16</td>
<td>5'-ATGGCGGTATCGCCAT-3'</td>
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<td>TG-15</td>
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<td>CA-15 (pH 5.5)</td>
<td>5'-TCTAGAGGCACCTCT-3'</td>
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<td>CA-15 (pH 7.9)</td>
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<td>Double-stranded, B-form</td>
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<tr>
<td>AG-17</td>
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<td>Double-stranded, B-form</td>
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<td>+, 7-mer, 11-mer</td>
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<td>5'-TAGCGCGGATCCGCGCTA-3'</td>
<td>Double-stranded, hairpin?</td>
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<td>TG-18</td>
<td>5'-TAGCGCGGTGCCGCGCTA-3'</td>
<td>Double-stranded, hairpin?</td>
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<tr>
<td>TC-18</td>
<td>5'-TAGCGCGGTATCGCGCTA-3'</td>
<td>Double-stranded, hairpin?</td>
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</table>
However, under cleavage conditions, only the sequences containing the T.A and T.G central basepair are cleaved. Inability of BanI to cleave the other sequences could stem from local distortions in DNA backbone introduced by mismatches. These distortions can compromise proper alignment of the scissile bond with respect to the catalytic moieties in the active site, thus preventing hydrolysis.