2′-*O*-methyl nucleotide modified DNA substrates influence the cleavage efficiencies of *Bam*HI and *Bgl*II

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Induction of endonucleolytic DNA cleavage is an essential event that links the initiating stimuli to the final effects of cells. The cleavage efficiency and thus the final yield could be affected by many factors, including structures of DNA substrates, composite structures of enzymes–substrates or enzymes–nucleic analogs and so on. However, it is not clear whether a nucleotide derivative-substituted in DNA substrates can influence the efficiency of enzymatic cleavage. To investigate the effect of sugar pucker conformation on DNA–protein interactions, we used 2′-*O*-methyl modified nucleotides (OMeN) to modify DNA substrates of isocaudemers *Bam*HI and *Bgl*II in this study, and used FRET assay as an efficient method for analysis of enzyme cleavage. Experimental results demonstrated that OMeN-substituted recognition sequences influenced the cleavage rates significantly in a position-dependent manner. OMeN substitutions can reduce the cleavage as expected. Surprisingly, OMeN substitutions can also enhance the cleavage rates. The kinetics parameters of V_{max} and K_m have been obtained by fitting the Michaelis-Menten kinetic equation. These 2'-OMe nucleotides could behave as a regulatory element to modulate the enzymatic activity *in vitro*, and this property could enrich our understanding about the endonuclease cleavage mechanism and enhance our ability to regulate the enzymatic cleavage efficiency for applications in synthetic biology.

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1. Introduction

Due to the development of novel nucleotide derivatives, nucleic acid chemistry has had remarkable progress (Kurreck 2003). Nucleotide derivatives are a group of nucleotides modified chemically either on the nucleobases, the phosphodiester backbone or the sugar moiety (Lee *et al.* 1994). The chemically modified nucleotides demonstrate many unique physicochemical properties different from the ordinary ones. Previous studies have shown that nucleotide derivatives with modified sugar moiety have been much less explored. This class of nucleotide derivatives includes 2′-*O*-methyl nucleotide (OMeN), 2′-fluore nucleotide (FNA), locked nucleotide (LNA) and many others. Nucleotide derivatives could affect sugar pucker conformation, binding affinity, hydrophobicity, hydrogen bond formation, biocompatiblility and ability to resist nuclease degradation. Consequently, they have been considered as the promising candidates in the anti-sense therapy. In the case of 2′-OMeN, the –OH group on the C2′ atom of the native nucleotide is replaced by a $–OCH_3$ group, generating a larger steric hindrance C3′-endo pucker conformation is the most distinctive feature of 2′-OMeN and that is dominant

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in A-form DNA and RNA. When incorporated into oligonucleotides, 2′-OMeN could enhance binding affinity toward their complementary DNA targets, (Freier and Altmann 1997) Thus, 2′-OMeN exhibits a faster hybridization dynamics than usual nucleotides (Majlessi *et al.* 1998). Furthermore, 2′-OMeN demonstrated the capability of modulating structure transitions of the G-quadruplexes between the parallel and the anti-parallel folding topologies (Dominick and Jarstfer 2004).

These researches come to our notice that how the nucleotide derivatives affect the DNA–protein interactions, particularly interactions with type II restriction endonucleases. Type II restriction endonucleases (REs) play extremely important roles in life science. Among thousands of type II REs discovered to date, a group of REs called isocaudamers are of particular interest. In our study, we take one pair of isocaudamers as an example. *Bam*HI and *Bgl*II recognize the sequence of GGATCC and AGATCT, respectively. Enzymatic cleavage of these two enzymes generate the same GATC sticky ends. The sticky ends can be rejoined to link two DNA duplex fragments. The approach has been widely used to connect BioBricks in synthetic biology.

In our research, we investigated the enzymatic cleavage process of *Bam*HI and *Bgl*II. We used 2′-OMeN to substitute the recognition sequences of different substrates, and employed FRET-based assay to monitor and determine the initial reaction velocities and kinetic parameters of each endonuclease, respectively. We proposed that the site-specific 2′-OMeN substitutions of their recognition sequences affected the relevant ions between the DNA substrates and enzymes.

2. Materials and methods

2.1 *Preparation of oligonucleotides and endonucleases*

All oligonucleotides purified by HPLC were purchased from Sangon Biotech Co., Ltd (Shanghai, China). The nucleotide sequences of oligonucleotides were listed in table 1. Type II endonucleases *Bam*HI (15 units/μl) and *Bgl*II (10 units/μL) were purchased from Takara Biotechnology Co., Ltd (Dalian, China). *Bam*HI was in a storage buffer containing 10 mM Tris-HCl (pH 7.5), 400 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.01% BSA, 0.15% Triton-X, and 50% Glycerol. *Bgl*II was in a storage buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.01% BSA, and 50% Glycerol. All other chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China), and prepared with double-distilled water at the concentration of 100 μM. All the substrates are prepared at the concentration of 10 μM for the experiment.

2.2 *FRET-based assay for endonuclease cleavage*

Three components of endonuclease, the template, F-ON and Q-ON, were prepared at the final concentration of 50 nM in 100 μL working buffer (20 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 1 mM Dithiothreitol and 100 mM KCl for *Bam*HI; 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM Dithiothreitol and 100 mM NaCl for *Bgl*II). Enzymes solutions were diluted in the reaction buffer and added immediately before measurement. The fluorescence signal measurements were carried out on Fluorometer (F4500, Hitachi, Japan) and Microplate Reader (Infinite 200, Tecan, Sweden) with the excitation at 520 nm and emission at 585 nm for 6-FAM. The fluorescence intensity of A-ON and FA-ON was detected for about 10 min to a steady status before the addition of Q-ON. Until the fluorescence intensity reached equilibrium again, restriction endonuclease was added into the solution. Immediately, the fluorescence intensity of sample was recorded. The fluorescence signals were collected every 15 s with an integration time of 20 μs, and measurements were performed by monitoring the fluorescence emission with time over ~20 min. The samples were diluted to 10 μM. All experiments were performed in a final volume of 100 μL at 37°C in triplicate.

2.3 *Design of experimental models*

Here, we employed FRET to assay the enzymatic cleavage efficiency and to study the relevant kinetics. This FRET-based method is rapid, sensitive and high-throughput. In principle, the detection process comprises four steps: (i) hybridization of F-ON with the template, (ii) hybridization of Q-ON with the template, (iii) the fluorescence of F-ON quenched when the donor is closed to the acceptor, and (iv) cleavage of the substrate by the restriction endonuclease. *Bam*HI was taken as an example. Figure 1 illustrates the principle of this FRET-based assay briefly. The substrates of the enzyme are composed of three oliogonucleotides, namely, F-ON (labelled 6-FAM at 5′-termini), Q-ON (labelled Dabcyl at 3′-termini), and A-ON (the complementary strand containing *Bam*HI recognition sequence). When they are hybridized to form a stable duplex, the fluorophore was so close to the quencher that no fluorescence signals could be detected because the fluorescence energy transfers from the fluorophore to the quencher. Once the recognition sequences of the duplex are cleaved by the endonuclease, the hydrolysed fragments could no longer be hybridized with the template and released, and the

Table 1. Sequences of DNA templates and nucleotide derivatives templates

Index	Sequence(5′-3′)*
A-ON	5′-ATACGCATACCTGTGGATCCTGGCTAAAAGCACACGCACGGAGAC
A-G1	5′-ATACGCATACCTGT**g**GATCCTGGCTAAAAGCACACGCACGGAGAC
A-G2	5′-ATACGCATACCTGTG**g**ATCCTGGCTAAAAGCACACGCACGGAGAC
A-A3	5′-ATACGCATACCTGTGG**a**TCCTGGCTAAAAGCACACGCACGGAGAC
A-T4	5′-ATACGCATACCTGTGGA**t**CCTGGCTAAAAGCACACGCACGGAGAC
A-C5	5′-ATACGCATACCTGTGGAT**c**CTGGCTAAAAGCACACGCACGGAGAC
A-C6	5′-ATACGCATACCTGTGGATC**c**TGGCTAAAAGCACACGCACGGAGAC
G-ON	5′-ATACGCATACCTGTAGATCTTGGCTAAAAGCACACGCACGGAGAC
G-A1	5′-ATACGCATACCTGT**a**GATCTTGGCTAAAAGCACACGCACGGAGAC
G-G2	5′-ATACGCATACCTGTA**g**ATCTTGGCTAAAAGCACACGCACGGAGAC
G-A3	5’-ATACGCATACCTGTAG**a**TCTTGGCTAAAAGCACACGCACGGAGAC
G-T4	5′-ATACGCATACCTGTAGA**t**CTTGGCTAAAAGCACACGCACGGAGAC
G-C5	5′-ATACGCATACCTGTAGAT**c**TTGGCTAAAAGCACACGCACGGAGAC
G-T6	5′-ATACGCATACCTGTAGATC**t**TGGCTAAAAGCACACGCACGGAGAC
FA-ON	3′-TATGCGTATGGACACCTAGGACCGAT-6-FAM
FG-ON	3′-TATGCGTATGGACATCTAGAACCGAT-6-FAM
Q-ON	3′-DABCYL-TCGTGTGCGTGCCTCTG

*The restriction endonuclease recognition sequences are underlined and the different nucleotide derivatives modified at the recognition site are highlighted in boldface and uppercase

separation of the fluorophore and the quencher leads to a regeneration of the fluorescence signals.

2.4 *Determination of kinetic parameters*

The substrates were prepared at the concentration of 25–2000 nM. Initial velocities (V_0) of each reaction were determined from the slope of the intensity curves of FI–Time as transverse and longitudinal axis. Then, the initial velocities were plotted by using linear curve fitting (Lineweaver-Burk), and V_{max} and K_m were calculated from the Michaelis-Menten equation. Data here were calculated with averaged values for independent measurements.

3. Results

3.1 *Fluorescence emission of the nuclease cleavage process*

Figure 2 shows fluorescence spectra of the substrate at three conditions. First, the *Bam*HI template A-ON was mixed with 6-FAM-labelled oligonucleotide FA-ON. The fluorescence spectrum showed a strong peak at 520 nm, the characteristic of fluorophore 6-FAM emission. Then, the Dabcyl-labelled oligonucleotide QA-ON was added into the solution, and the fluorescence signal decreased remarkably, indicating that the fluorescence emission of 6-FAM was quenched by the fluorescence acceptor

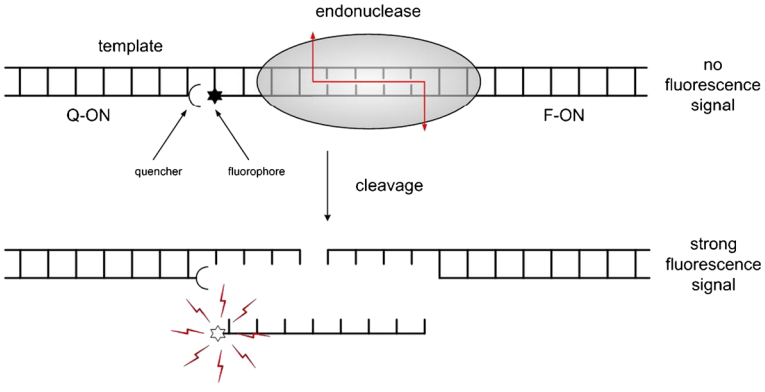

Figure 1. Schematic diagram for monitoring the cleavage of restriction endonuclease.

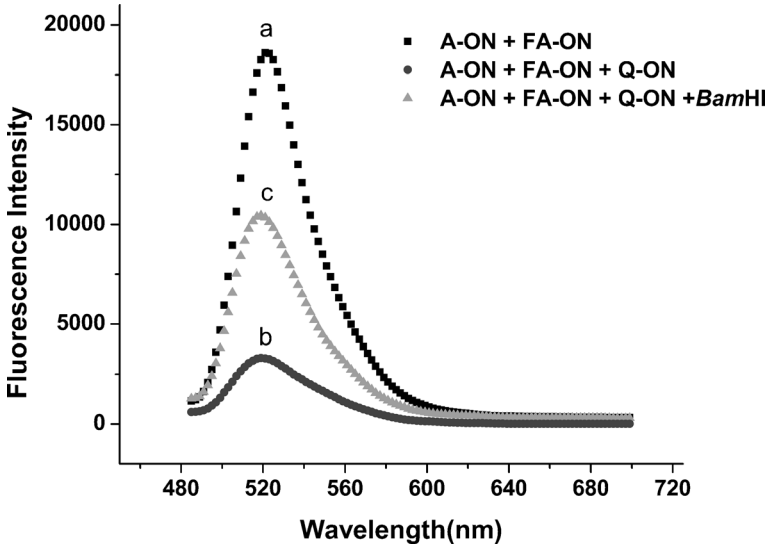

Figure 2. Emission spectra of *Bam*HI endonuclease cleavage process. All the spectra are monitored with an excitation wavelength of 485 nm at 37°C: (**a**) The square curve for a scan of fluorescence intensity of the A-ON with FA-ON. (**b**) The circle curve for the fluorescence intensity of the duplex which is composed of A-ON, FA-ON and QA-ON. (**c**) The triangle curve for emission spectra of *Bam*HI added to the duplex.

Dabcyl. A tiny fluorescence peak was probably due to a small portion of FA-ON not hybridized with QA-ON. Finally, once the restriction endonuclease *Bam*HI was mixed with the substrate, the fluorescence intensity was restored almost to the original level. The incomplete recover of fluorescence intensity was to be expected because the energy wasted in the process of energy transfer. Clearly, it showed that the fluorescence energy was released because the fluorescence donor and fluorescence acceptor were separated after the nuclease cleavage.

3.2 *Concentration-dependent of enzymatic cleavage*

We characterized the endonuclease cleavage behavior using different concentrations of *Bam*HI and *Bgl*II, respectively. Curves in figure 3a represent the *Bam*HI cleavage processes at the concentrations in the range of 9.4 U/mL to 600 U/mL, and the curves in figure 3b represent the *Bgl*II cleavage processes at the concentrations in the range of 18.8 U/mL to 600 U/mL, respectively. As illustrated, at lower enzyme concentrations, the fluorescence intensity increased linearly with the cleavage reaction progress, and at higher enzyme concentrations, the fluorescence intensity increased rapidly at the beginning and became saturated later. These curves clearly exhibited the time courses of substrate cleavage catalysed by different amounts of *Bam*HI or *Bgl*II (figures 3c and 3d).

3.3 *Position-dependent of enzymatic cleavage*

To probe the interactions between the DNA substrate and the endonucleases, we used 2′-OMeN to substitute the six nucleotides of the recognition sequences one by one, and determined their cleavage efficiencies. Figure 4a shows the relative cleavage rates of *Bam*HI when the relevant nucleotides were substituted by 2′-OMeN. In comparison with the DNA substrate, 2′-OMeN substituted DNA duplexes generated different cleavage efficiencies: some substitutions enhanced the cleavage, while others retarded. For example, substitutions at the fifth and sixth position of the GGATCC sequence (known as A-C5 and A-C6) enhanced the initial cleavage velocity by 3- to 4-folds. In contrast, when the fourth nucleotide of the GGATCC sequence (known as A-T4) was replaced by 2′-OMeN, the cleavage was almost completely repressed.

We have also observed a similar phenomenon for *Bgl*II cleavage. As shown in figure 4b, substitution at the first and third positions of the sequence (aGATCT known as G-A1 and AGaTCT known as G-A3) of *Bgl*II enhanced the initial cleavage velocity by ~1.5-fold, whereas substitution at the fourth position of the sequence (AGAtCT known as G-T4) inhibited the cleavage significantly. These results indicated

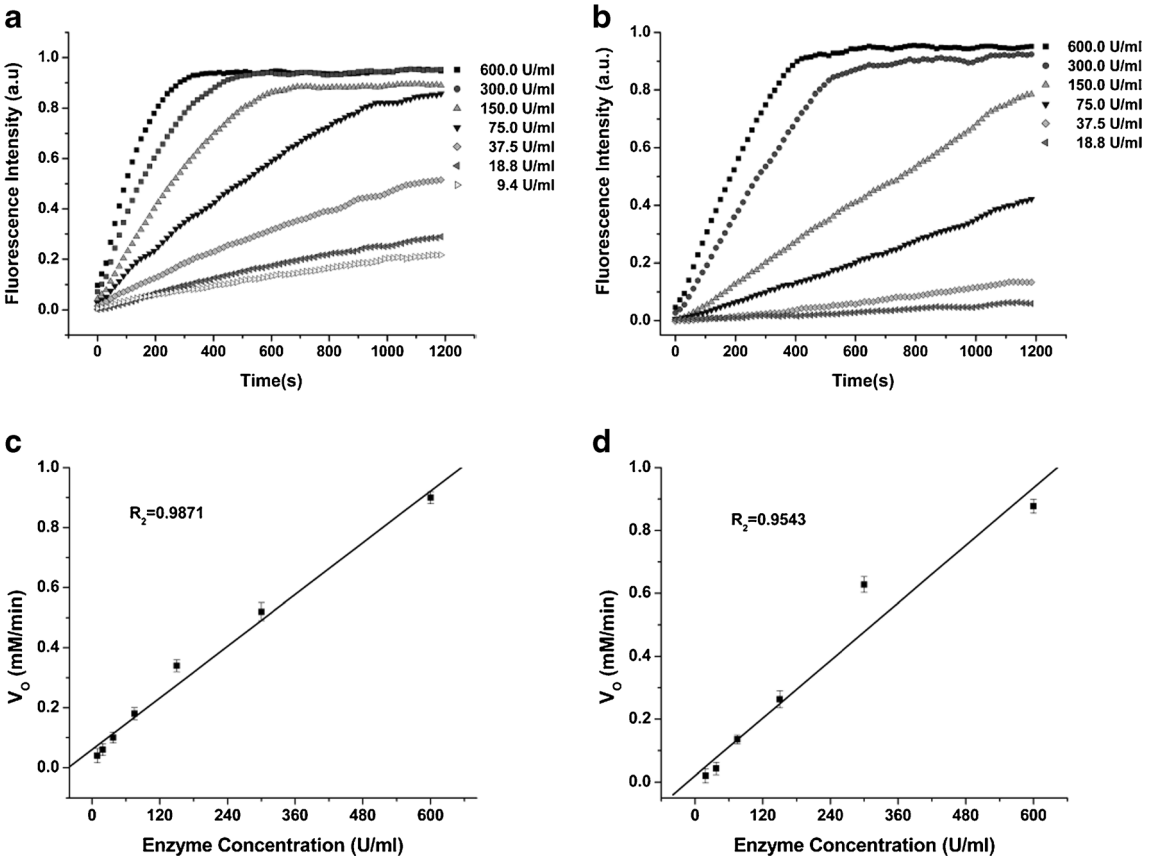

Figure 3. Time curves of various amounts of *Bam*HI or *Bgl*II endonuclease. (**a**) Time course of monitoring of DNA cleavage catalyzed by different amounts of *Bam*HI. (**b**) Time course of monitoring of DNA cleavage catalyzed by different amounts of *Bgl*II. (**c**) Linear plots between initial velocities and different amounts of *Bam*HI. (**d**) Linear plots between initial velocities and different amounts of *Bgl*II.

clearly that the cleavage efficiencies had a strong position-dependent behaviour.

3.4 *Determination of kinetic parameters*

Furthermore, we have determined the kinetic parameters for each different OMeN-substituted substrate. Keeping the enzyme concentration at a constant and varying the substrate concentrations from 25 nM to 2000 nM, we measured the initial cleavage velocity in each situation (figures 4c and d). Fitting these data to the Michaelis-Menten equation, two kinetic parameters, V_{max} and K_m, were determined (tables 2 and 3). As expected, these 2′-OMeN-substituted duplex substrates showed significant changes in kinetic parameters. The substrate GGAtCC had no measurable numbers due to the complete inhibition, whereas the substrate GGATCc had a significantly increased V_{max} value. All these results of *Bam*HI and *Bgl*II were consistent with their cleavage efficiencies shown in figures 4a and 4b.

4. Discussion

Type II REs play an important role in recombinant DNA technology (Engler *et al.* 2001). They are also good models for studying the DNA enzymology and the protein–DNA interactions since a large number of endonucleases with a great diversity of recognition sequences have been characterized well in terms of biological functions and spatial structures (Pingoud and Jeltsch 2001). The present study intended to use nucleotide derivative substitutions for the specific nucleotide of the recognition sequences and to obtain an understanding about the interactive patterns between the DNA duplexes and endonucleases.

4.1 *Effect of nucleotide derivatives on the cleavage*

Previous studies showed that removal or substitution of functional groups of a recognition site can influence recognizing the endonuclease significantly (Nardone *et al.* 1990). Even a single base pair change within the recognition

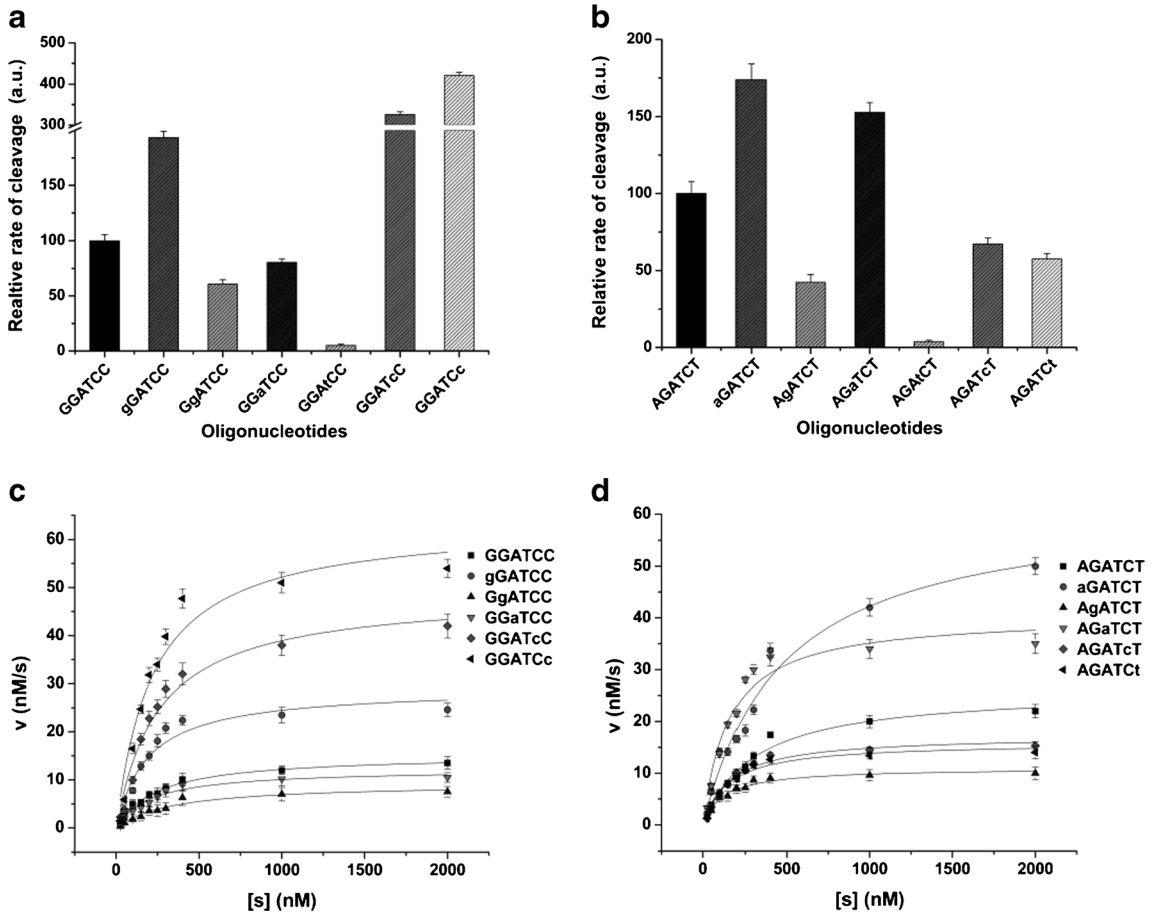

Figure 4. Cleavage assay of *Bam*HI or *Bgl*II with different substrates and the kinetics of different substrates. (**a**) *Bam*HI cleavage efficiencies of with different substrates (A-G1, A-G2, A-A3, A-T4, A-C5 and A-C6) (**b**) *Bgl*II cleavage efficiencies of with different substrates (G-A1, G-G2, G-A3, G-T4, G-C5 and G-T6). (**c**) Plots of cleavage of different substrates by *Bam*HI endonuclease. (**d**) Plots of cleavage of different substrates by *Bgl*II endonuclease.

sequence could lead to over a million fold loss in activity (Lesser *et al.* 1990). Restriction endonucleases have to adapt changes when the substrate recognition sequence is altered (Wenz *et al.* 1998; Schottler *et al.* 1998).

X-ray crystallographic data show that 2′-modification of nucleotides constrain the ribose to the C3′-endo pucker

Table 2. The kinetics parameters of *Bam*HI cleavage

Modified Sites	V_{max}(nM/s)	K_m(nM)	V_{max}/K_m(1/s)
GGATCC	15.21±0.49	249.38±21.56	0.061
gGATCC	29.11±2.49	189.20±26.85	0.154
G**g**ATCC	9.24±0.74	342.45±37.11	0.027
GG**a**TCC	12.25±0.73	216.62±25.84	0.057
GGA**t**CC	N/A	N/A	N/A
GGAT**c**C	48.88±3.01	255.21±21.50	0.192
GGATC**c**	63.73±4.62	221.74±34.23	0.287

Table 3. The kinetics parameters of *Bgl*II cleavage

Modified Sites	V_{max}(nM/s)	K_m(nM)	V_{max}/K_m(1/s)
AGATCT	26.07±1.34	300.61±28.93	0.087
aGATCT	45.34±4.35	283.11±50.21	0.157
A**g**ATCT	11.01±0.42	120.73±15.37	0.091
AG**a**TCT	40.48±2.49	153.31±29.21	0.264
AGA**t**CT	N/A	N/A	N/A
AGAT**c**T	17.25±0.72	161.82±20.56	0.107
AGATC**t**	15.89±0.78	147.96±22.86	0.104

*The different nucleotide derivatives modified at the recognition site are in boldface and uppercase

conformation which is characteristic of A-form DNA or RNA (Guschlbauer and Jankowski 1980; Teplova *et al.* 1999). Crystal structure shows that the 2′-*O*-methyl group resides in the minor groove of duplexes and reduces the amount of water molecules, increasing the hydrophobicity (Adamiak *et al.* 2001). The 2′-*O*-methyl group also generates a strong steric hindrance. All these effects influence the binding affinity of 2′-OMeN modified DNA substrates toward the endonucleases (Yan *et al.* 2012). Interestingly, when incorporated in oligonucleotide probes, 2′-OMeN probes demonstrated a higher binding affinity towards RNA targets rather than DNA targets. Furthermore, 2′-OMeN can provide a faster hybridization dynamics than DNA nucleotides (Majlessi *et al.* 1998). Previous investigations have concluded that 2′-OMeN can reduce the non-specific binding *in vitro* (Kierzek *et al.* 2005). 2′-*O*-methyl nucleotides have been used as a key element in antisense-based therapeutic applications (Burmeister *et al.* 2005; Chen *et al.* 2009).

In comparison with the modified bases and modified phosphate group, the effect of modified-ribose nucleotides within the recognition sites has largely been unexplored. The previous researches demonstrated that *Rsa*I was an efficient enzyme capable of recognizing and cleaving LNA-modified DNA oligonucleotides when the cleavage sites of duplexes contain LNA nucleotides in both top and bottom strands (5′-GTA^{L}C-3′). On the contrary, the LNA-modified cleavage site efficiently inhibited cleavage by *Pvu*II, *Pst*I, *Sac*I, *Kpn*I and *Eco*RI restriction endonucleases (Crouzier *et al.* 2012). Moreover, the rate of cleavage was decreased when a DNA contained a phosphorothioate modification at the recognition sites (Lesser *et al.* 1992; Kurpiewski *et al.* 1996; Rosati *et al.* 2002).

According to the results, G2, A3, T4 sites with 2′-methoxyl nucleotide restrict the cleavage of *Bam*HI. On the basis of *Bam*HI crystal structure, the major groove binds amino terminus primarily by the interactions of α4 and α6, and GC base outside is connected by Arg155 and Asp154, which is located in a spiral ring made of 152-157 bases before α6. GC base pairs in the middle are combined with Asp154, Arg122 and Asn116. AT base pairs inside are mainly based on hydrogen bonding by water-mediated and Asn116 pairing. After the substrates was modified with 2′-OMeN, the group will affect the relevant ions between the base pairs, which leads to the three-dimensional comformational changes of the main and side chains and hydrogen bonds formed by amino acid and recognition sequence. The interpretation is evidence-based. When G:C becomes A:T, the active site residues (Asp94, Glu111, Glu113) from the scissile phosphodiester bond is about 0.7 nm. Due to extremely strict specificity of cleavage, the restriction enzyme will not cut DNA (Jen-Jacobson *et al.* 2000). G2, A3, T4 sites with 2′-OMeN nucleotide increased the cleavage efficiency of *Bam*HI, the cleavage are more effective. That is because DNA and the enzymes were combined more stable due to a stronger electrostatic interaction.

4.2 *Effect of 2′-OMeN substitutions on enzymatic cleavage*

Surprisingly, 2′-OMeN substitutions at different recognition sites showed significantly different effects on the cleavage rate. As shown in figure 4a, the GGATCc sequence showed the highest cleavage rate, and the GGAtCC sequence showed the almost completely repressed cleavage rate. 2′-OMeN substitutions for the *Bgl*II substrate demonstrated a similar position-dependent behaviour. However, *Bam*HI and *Bgl*II have different cleavage patterns. The endonuclease cleavage event has been known to hydrolyse the phosphodiester linkages of DNA duplexes.

The specific structure of a DNA duplex is usually characterized by six torsion angles along its backbone, each of which has been confined in a specific region for a particular form of DNAs and RNAs. When the recognition sequence is substituted by 2′-OMeN, the C3'-endo sugar pucker conformation of 2′-OMeN influence the torsion angles directly since the C3′ atom is involved in the torsion angles of δ and ε. Consequently, the substrate–enzyme interactions must be altered inevitably.

The methoxyl group attached to the C2′ atom is relatively bulky in comparison with the H atom of the DNA nucleotides. Thus, a strong steric hindrance must be introduced. Furthermore, the methoxyl group also exerted a strong hydrophobicity. The combination of these three types of interactions influences the interaction pattern of the DNA substrate and enzymes, creating a complex cleavage mechanism.

It has been widely known that a water molecule was activated to generate a hydroxide ion which affects the nucleophilic attack of specific phosphorus atom of the DNA substrate (Pingoud and Jeltsch 2001). In the last decade, a phenomenon known as substrate-assisted catalysis has been recognized, where the substrate contains a functional group that can participate in catalysis in a way that is plausible from the mechanistic, structural, and sterochemical standpoints. Representative examples include H64A subtilisin BPN', Ras p21 GTPase and *Eco*RV. Considering these facts, it is not surprising to see the enhanced enzymatic cleavage rates (Jeltsch *et al.* 1993; Dall'Acqua and Carter 2000).

4.3 *Enzymatic kinetics of isocaudamers*

In the study, the nuclease cleavage reactions were monitored over a wide range of substrate concentrations from 25 to 2000 nM. Plotting V_0 against substrate concentration [s]

yielded a hyperbolic curve (figure 4), and the data fitting procedure confirmed that these two enzymes obeyed the Michaelis-Menten equation. From the Michaelis-Menten curves, we have determined V_{max} and K_m of 15.21 nM/s and 249.38 nM for *Bam*HI and its unsubstituted sequence, which are in a good agreement with previous studies (Kang *et al.* 1995). However, in the case of 2′-OMeN substitution, the corresponding V_{max} and K_m values of *Bam*HI exhibited great variations. Taking gGATCC as an example, its K_m has reduced by 20% and its V_{max} has been almost doubled. In contrast, the GgATCC substrate showed the increased K_m and reduced V_{max}. The increased V_{max} values suggest a fast conversion of the reactants to the products, and the increased K_m values imply the reduced binding affinities in general. Many earlier reports have proposed the mechanism for *Bam*HI cleavage. The endonuclease binds the substrate duplex randomly, then slides along the duplex to the recognition sequence and pauses, and cleaves the duplex substrate finally. Thus, the measured cleavage rate should be the overall rate of these three steps. Unfortunately, it could not be resolved the reaction rate of each step at the moment.

Cleavage kinetics of *Bam*HI has been investigated extensively in many previous studies, and the K_m value has been found to be dependent strongly on the cognate sequence as well as the size of the substrate (Kang *et al.* 1995; Mukhopadhyay and Roy 1998). In comparison with the reported values, the K_m data obtained in this study are comparable to the results for synthetic oligomers (Kang *et al.* 1995).

In the case of *Bgl*II, V_{max} and K_m of 26.07 nM/s and 300.61 nM were determined for the normal substrate, respectively. When substituted by 2′-OMeN, *Bgl*II showed different values of V_{max} and K_m for different substituted substrates. But no comparison could be made, as *Bgl*II has not been studied well previously.

4.4 *Differences between BamHI and BglII*

Although *Bam*HI and *Bgl*II are mutually isocaudamers, they use different mechanisms to recognize the common core GATC sequence according to the crystal structures reported (Newman *et al.* 1995). Previous investigations have studied the *Bam*HI binding in the presence of divalent metal, the optimal oligonucleotide substrate length, and the number of flanking base-pairs on each side (Engler *et al.* 2001). A comparison of the free *Bam*HI and *Bam*HI-DNA complex structures had suggested that DNA duplex is located in the concave binding site of the enzyme dimer (Viadiu *et al.* 2000). *Bam*HI makes most of its base pair interactions in the major groove by side chains and backbone atoms near the N-terminal of the parallel four helix bundle. The subunits move outward the complex to allow the DNA to enter in the case of free *Bam*HI dimer and clamp onto the DNA by a ~10° rotation around the DNA axis.

*Bgl*II is a 223-amino-acid endonuclease that creates a GATC sticky end identical to that of *Bam*HI (Anton *et al.* 1997). *Bgl*II approaches the DNA duplex substrate also from the major groove side, and contacts are made to the edges of the bases in the minor groove (Pingoud and Jeltsch 2001). *Bgl*II has shown some unique structural features. Its β-sandwich subdomain encircles fully the DNA substrate, and the *Bgl*II dimer opens and closes like a 'scissor' parallel to the DNA helical axis, which is accompanied by a complete rearrangement of the α-helices at the dimer interface. The enzyme is a loose dimer with a wide cleft enough to accommodate DNA in the free state (Lukacs *et al.* 2001).

Structural analysis showed that residues Asp94, Glu111 and Glu113 of *Bam*HI are located in the putative catalytic site with an arrangement almost identical with that of the active-site residues, Asp84, Glu93 and Gln95 in *Bgl*II structure. In *Bam*HI, Glu113 is in the position to promote the activation of the attacking H_2O (Newman *et al.* 1994).

Based on the similarities and the differences between *Bam*HI and *Bgl*II as mentioned, we speculated that the modified substrates affected the structures of enzyme substrates and then led to different performances on the cleavage efficiencies. Correspondingly, values of V_{max} and K_m were different. It is worth mentioning that both the digestions of isocaudamers were limited when the fourth position was substituted. The mechanism cannot be detailed but it is mainly related to the structures of enzyme–substrates compounds.

4.5 *Biological significance*

Type II restriction endonucleases have served as models for understanding the site-specific DNA recognition and cleavage by nucleases. These enzymes have also been used extensively as the effective tools in DNA cloning. Furthermore, recent studies have revealed the significance of the RE protection in therapeutic treatments of certain diseases caused by DNA mutations (Srivastava and Moraes 2001). Substitution of recognition sequences with nucleotide derivatives could affect the DNA structures, interactions with ligands and dynamic processes, providing an opportunity for us to regulate the enzymatic reaction behaviour without changing the native endonucleases. Previously, we have examined the effects of different 2′-modified nucleotides on endonuclease cleavage, exonuclease digestion and ligation efficiency. In addition, we have found that the 2′-modified nucleotides could make structure conversion of G-quadruplex. These results lay a solid foundation to use site-specific substitutions as an effective strategy to regulate the enzymatic activity. It is our belief that appropriate usage of nucleotide derivatives could achieve a great success in

personalized medicine, targeted genome engineering, and bioengineering utilization.

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