Binding Kinetics and Footprinting of *Taq*I Endonuclease: Effects of Metal Cofactors on Sequence-Specific Interactions†

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ABSTRACT: Restriction endonucleases achieve sequence-specific recognition and strand cleavage through the interplay of base, phosphate backbone, and metal cofactor interactions. In this study, we investigate the binding kinetics of *Taq*I endonuclease using the wild-type enzyme and a binding proficient, catalysis deficient mutant *Taq*I-D137A both in the absence of a metal cofactor and in the presence of Mg^{2+} or Ca^{2+}. As demonstrated by gel mobility shift analyses, *Taq*I endonuclease requires a metal cofactor for achieving high-affinity specific binding to its cognate sequence, TCGA. In the absence of a metal cofactor, the enzyme binds all DNA sequences (*Taq*I cognate site, star site, and nonspecific site) with essentially equal affinity, thereby exhibiting little discrimination. The dissociation constant of the cognate sequence in the presence of Mg^{2+} at 60 °C is 0.26 nM, a value comparable to our previously reported K_m of 0.5 nM measured under steady-state conditions. The *Taq*I−TCGA−Mg^{2+} complex is stable, with a half-life of 21 min at 60 °C. The boundary of the protein−DNA interface is approximated to be about 18 bp as determined by DNase I footprinting. Data from this study support the notion that a metal cofactor plays a critical role for achieving sequence-specific discrimination in a subset of nucleases, including *Taq*I, *Eco*RV, and others.

*Taq*I endonuclease recognizes a short palindromic double-stranded DNA sequence, T↓CGA, and cleaves the double-stranded DNA with exquisite specificity. Using modified substrates, base contacts within the major groove and phosphate backbone contacts have been identified (*1*, *2*). Preliminary results from filter binding assays and gel retardation assays demonstrate that *Taq*I, like *Eco*RV, requires Mg^{2+} as a cofactor for specific binding to its recognition sequence (*3*). In a kinetic study using cognate, star (sites which differ from the cognate sequence by a single base pair), and nonspecific DNA sequences along with Mg^{2+} or Mn^{2+} as the metal cofactor, we have observed differential modes of binding toward different DNA sequences (*4*). With Mg^{2+} as the metal cofactor, *Taq*I exerts a specific binding mode toward its cognate sequence, forming a tight, activated tertiary complex, while binding to star sequences is weak and similar to nonspecific sequences. With Mn^{2+} as the metal cofactor, *Taq*I forms a highly activated specific complex with its cognate sequence, partially activated complexes with star sequences, and an inactive complex with a nonspecific DNA sequence. As a result, *Taq*I achieves high specificity toward its cognate site in the presence of Mg^{2+}, while exhibiting star activity with Mn^{2+} (*4*). However, the requirement of Mg^{2+} for specific binding prevents us from using the wild-type enzyme for further binding studies, as well as defining the protein−DNA interface.

Recently, through Fe^{2+}-mediated cleavage and site-directed mutagenesis, we have identified a single mutation at D137 which uncouples specific DNA binding from DNA cleavage (*5*). The initial characterization in which partially purified mutant *Taq*I-D137A was used suggests that D137 is involved in coordinating a catalytic Mg^{2+} metal ion. Elimination of the carboxyl group (D137A) at this position renders the enzyme inactive in catalysis with the enzyme still retaining full binding affinity for the *Taq*I cognate sequence. Thus, this mutant shares some features with mutants at D91 in *Eco*RI (*6*, *7*), D74 in *Eco*RV (*6*), D94 in *Bam*HI (*8*), D124 and D246 in *Bso*BI (*9*), and D450 and D467 in *Fok*I (*10*).

It has been proposed that restriction endonucleases can be classified on the basis of two modes of achieving specific binding. *Eco*RI and *Bam*HI represent a group of restriction enzymes which do not require a metal cofactor for specific and high-affinity binding to the cognate sequence, while *Eco*RV and *Taq*I represent another group of restriction enzymes which require a metal cofactor for specific and high-affinity binding (*3*, *11*−*13*). Recently, some have debated whether *Eco*RV type enzymes indeed constitute a "new paradigm" for sequence-specific interactions, questioning whether a metal cofactor plays any significant role in sequence-specific interactions (*14*, *15*). In this work, we take advantage of the catalytic deficiency of *Taq*I-D137A to study the binding behavior of *Taq*I endonuclease toward different DNA substrates using gel mobility shift assays. We compare the binding of *Taq*I and *Taq*I-D137A in the absence or presence of Ca^{2+} or Mg^{2+}, determine the dissociation constant (K_d) and the dissociation rate constant (k_{off}) for dissociation of *Taq*I from its cognate site, and define the

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protein−DNA interface in the specific *Taq*I−TCGA−Mg^{2+} complex by DNase I footprinting. Finally, we discuss the differential effects a metal cofactor has on sequence-specific interactions of *Taq*I endonuclease.

EXPERIMENTAL PROCEDURES

Materials. T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). Sequenase 2.0 was from U.S. Biochemicals (Cleveland, OH). $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ were from Fisher Scientific (Fair Lawn, NJ). $MnCl_2 \cdot 4H_2O$ was from Alfa Products (Danvers, MA). Acetic acid and methanol were from J. T. Baker (Philipsburg, NJ). [γ-^{32}P]ATP was from NEN (Boston, MA). *Taq*I endonuclease and its mutant, *Taq*I-D137A, were purified as previously described (*16*). Oligonucleotides were synthesized using an Applied Biosystems 381 DNA synthesizer and purified on a denaturing sequencing gel (7 M urea/10% polyacrylamide) (*17*). Kinase-ligase buffer (kin-lig buffer) consisted of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA,[1] and 6 mM β-mercaptoethanol. Gel shift electrophoresis running buffer consisted of 90 mM Tris, 90 mM boric acid, and 10 mM metal cofactor ($CaCl_2$ or $MgCl_2$) or 2 mM EDTA. MS buffer (1×) consisted of 10 mM Tris-HCl (pH 8.0) at 60 °C, 100 μg/mL BSA, 50 mM NaCl, and 10 mM DTT. The gel fixer solution consisted of 10% (v/v) glacial acetic acid and 10% (V/V) methanol. The DNase I stop buffer consisted of 7 μL of 3 M sodium acetate (pH 5.0), 1.5 μL of 1 mg/mL tRNA, and 231 μL of 100% ethanol. The sequencing stop buffer consisted of 95% formamide, 20 mM EDTA (pH 8.0), 0.05% bromophenol blue, and 0.05% xylene cyanol.

Gel Shift Binding Assay. Oligodeoxynucleotide 201 with the single *Taq*I recognition site underlined was 5′ end labeled as previously described (*4*) and annealed with a 5-fold excess of 202 to form a duplex DNA substrate for the binding experiment:

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201   5'-ACTCACCAATCGATTTACCGGGTGGCAGCCTGGCATGCCT-3'
202   3'-TGAGTGGTTAGCTAAATGGCCCACCGTCGGACCGTA-5'
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Wild-type *Taq*I and the *Taq*I-D137A mutant were incubated with above duplex DNA substrate (250 pM) in 20 μL of binding buffer containing 10 mM Tris-HCl (pH 9.2 at 23 °C or room temperature and pH 8.0 at 60 °C), 10 mM DTT, 50 mM NaCl, 0.1 mg/mL BSA, 10 mM metal cofactor ($CaCl_2$ or $MgCl_2$) or 2 mM EDTA, 10% glycerol, and 0.005% bromophenol blue at 60 °C for 15 min. The free and bound duplex oligonucleotides were separated by electrophoresis in a Bio-Rad mini-Protean II gel apparatus carried out at 80 V for 50 min on a 6% polyacrylamide gel (30 acrylamide:0.8 *N*′,*N*′-methylene bis-acrylamide) prepared with the buffer containing 89 mM Tris, 89 mM boric acid, and 10 mM $CaCl_2$ or $MgCl_2$ or 2 mM EDTA (*18*). The gel was wrapped with SaranWrap and exposed in a PhosphorImager screen for 2 h. The image of the gel was visualized on a PhosphorImager (Molecular Dynamics). The star site oligonucleotides (101 and 102) are identical to 201 and 202 except that the *Taq*I recognition sites were changed to 5′-TCAA-3′ and 3′-AGTT-5′, respectively. The nonspecific oligonucleotides (201-NS and 202-NS) are identical to 201 and 202 except that the *Taq*I recognition sites were changed to 5′-TGCA-3′ and 3′-ACGT-5′, respectively.

K_d Measurement. The following duplex oligodeoxynucleotides were synthesized for K_d measurement because multiple radioactive NTPs were needed for this assay:

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201p  5'-ACTCACCAATCGATTTACCGG-3'
202   3'-TGAGTGGTTAGCTAAATGGCCCACCGTCGGACCGTA-5'
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The oligonucleotides were purified by denaturing (7 M urea) polyacrylamide gel electrophoresis according to a technical manual from The Applied Biosystems Division of Perkin-Elmer. 201p was mixed with a 5-fold excess of 202, and the oligonucleotides were denatured at 85 °C for 10 min and then transferred to a room-temperature environment so they could cool to room temperature for 30 min. The primer extension reaction mixture (20 μL) contained 0.75 μM duplexed 201p/202, dATP, dCTP, and dTTP (each at 25 μM), 5 mM DTT, 5× sequanase buffer (U.S. Biochemicals), 8.25 μM [α-^{32}P]dGTP (6000 Ci/mmol, Du Pont-NEN, Wilmington, DE), and 5 units of Sequenase version 2.0 (U.S. Biochemicals). The reaction mixture was incubated at 37 °C for 1 h, and then chased with 2 μL of 500 μM dGTP and 1 μL each of dATP, dCTP, and dTTP at 37 °C for 30 min. The labeled duplex oligonucleotides (201p-FI/202) were separated from dNTPs by G-25 Sephadex size exclusion chromatography (*4*). Various amounts of *Taq*I-D137A ranging from a 10- to a 100-fold excess over the labeled substrate (201p-FI/202) were incubated in 20 μL of binding buffer containing 10 mM Tris-HCl (pH 9.2 at room temperature), 10 mM DTT, 50 mM NaCl, 0.1 mg/mL BSA, 10 mM $MgCl_2$, 10% glycerol, and 0.005% bromophenol blue at 60 °C for 15 min. Samples were loaded with a Hamilton syringe onto a 6% polyacrylamide gel prepared as described above and prerun at 80 V for 1 h in a 60 °C water bath. After electrophoresis at 80 V for 50 min at the same temperature, the gel was soaked in gel fixer solution for 30 min, and then wrapped with SaranWrap and exposed in a PhosphorImager screen for overnight. The intensity of ^{32}P-labeled DNA bands was quantified using a PhosphorImager (Molecular Dynamics). The K_d values were calculated with the following equation (*19*)

$$\frac{r}{E_0} = \frac{1}{K_D} - \frac{r}{K_D}$$

where E_0 is the total amount of enzyme and r is the ratio of bound 201p-FI/202 to total 201p-FI/202. Results were reported as the average of three independent experiments.

k_{off} Measurement. *Taq*I-D137A (1.25 nM) was mixed with 0.25 nM labeled duplex 201/202 oligonucleotide substrate (see Gel Shift Binding Assay) in 200 μL of binding buffer as described in K_d Measurement. After incubation at 60 °C for 15 min, 20 μL of binding mixture was removed at time zero, and 18 μL of 275 nM unlabeled 201/202 duplexed oligonucleotides (100-fold excess of labeled 201/202) was quickly incorporated into the remaining binding mixture. Samples of 22 μL each were withdrawn at the indicated time points, and loaded directly onto a 6% polyacrylamide gel without interrupting the electrophoresis. The k_{off} values were

[1] Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; dNTP, deoxyribonucleoside triphosphate; DTT, dithiothreitol; wt, wild-type.

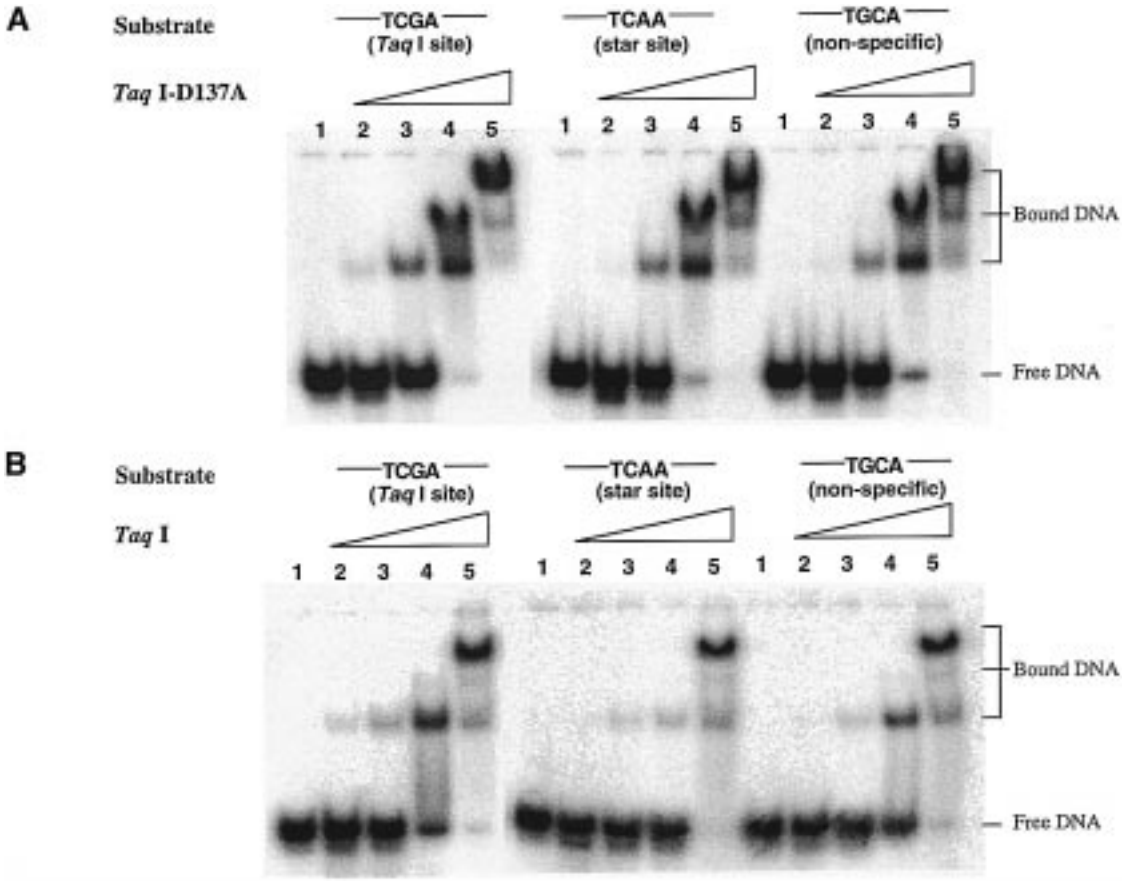

FIGURE 1: Gel mobility shift of *Taq*I endonuclease at 60 °C without a metal cofactor. The binding mixture and conditions are described in Experimental Procedures. EDTA (2 mM) was used in place of a metal cofactor. The star site oligonucleotides (101/102) are identical to 201 and 202 except that the *Taq*I recognition site was changed to 5′-TCAA-3′ and 3′-AGTT-5′, respectively. The nonspecific oligonucleotides (201-NS and 202-NS) are identical to 201 and 202 except that the *Taq*I recognition site was changed to 5′-TGCA-3′ and 3′-ACGT-5′, respectively. One of the oligonucleotide (0.25 nM) was 5′ end labeled: lane 1, no *Taq*I enzyme or *Taq*I-D137A mutant; lane 2, 0.25 nM *Taq*I enzyme or *Taq*I-D137A mutant; lane 3, 2.5 nM *Taq*I enzyme or *Taq*I-D137A mutant; lane 4, 25 nM *Taq*I enzyme or *Taq*I-D137A mutant; and lane 5, 250 nM *Taq*I enzyme or *Taq*I-D137A mutant. (A) Binding of the *Taq*I-D137A mutant to DNA sequences. (B) Binding of the *Taq*I enzyme to DNA sequences.

Chart 1

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201L   5'-TTGTAGGGACGGACTAACCGCACTCACCAATCGATTTACCGGGTGGCAGCCTGGCATGCCTAAC-3'
202L   3'-AACATCCCTGCCTGATTGGCGTGAGTGGTTAGCTAAATGGCCCACCGTCGGACCGTACGGATTG-5'
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calculated with the following equation (*19*)

$$\ln\left(\frac{DNA_B}{DNA_T}\right) = k_{off}t + C$$

where DNA_B is the amount of bound DNA substrate and DNA_T is the total amount of DNA substrate. Results were reported as the average of three independent experiments.

DNase I Footprinting. Two long oligodeoxynucleotides containing a single *Taq*I recognition site as shown in Chart 1 were synthesized and purified. The two oligonucleotides were 5′ end labeled and purified by G-25 Sephadex size exclusion chromatography (*4*). Duplexed 201L/202L was formed by mixing labeled oligonucleotide with a 5-fold excess of unlabeled complementing strand at 85 °C for 10 min and annealing at room temperature for 30 min.

The binding mixture (20 μL) contained 100 nM *Taq*I-D137A, 3 nM labeled 201L/202L, 10 mM Tris-HCl (pH 9.2 at room temperature), 10 mM DTT, 50 mM NaCl, 0.1 mg/mL BSA, 10% glycerol, 5 mM $MgCl_2$, and 2.5 mM $CaCl_2$. After incubation at 60 °C for 15 min, 1.5 μL of 2 μg/mL DNase I was added and the nuclease digestion was performed at room temperature for a predetermined time. The reaction was terminated by the addition of 240 μL of DNase I stop buffer and 65 μL of deionized water. The digested oligonucleotides were precipitated at −70 °C for 15 min, and then collected by centrifugation. The pellet was washed twice with 70% ice-cold ethanol and dried in a Speedvac evaporator (*20*). The sample was suspended in 10 μL of sequencing gel stop buffer, and 5 μL was loaded onto a 10% polyacrylamide (20:1) gel containing 7 M urea. The electrophoresis was conducted at 70 W for 80 min in a BRL model S2 sequencing gel apparatus. The gel retained on a glass plate was wrapped with SaranWrap and exposed under a PhosphorImager screen for 2 h. The image was visualized by scanning on a PhosphorImager (Molecular Dynamics).

RESULTS

Binding without a Metal Cofactor. We initially studied binding of the wt *Taq*I enzyme and the *Taq*I-D137A mutant in the absence of any metal cofactor by gel mobility shift assays. The amount of proteins used in the assay ranged from

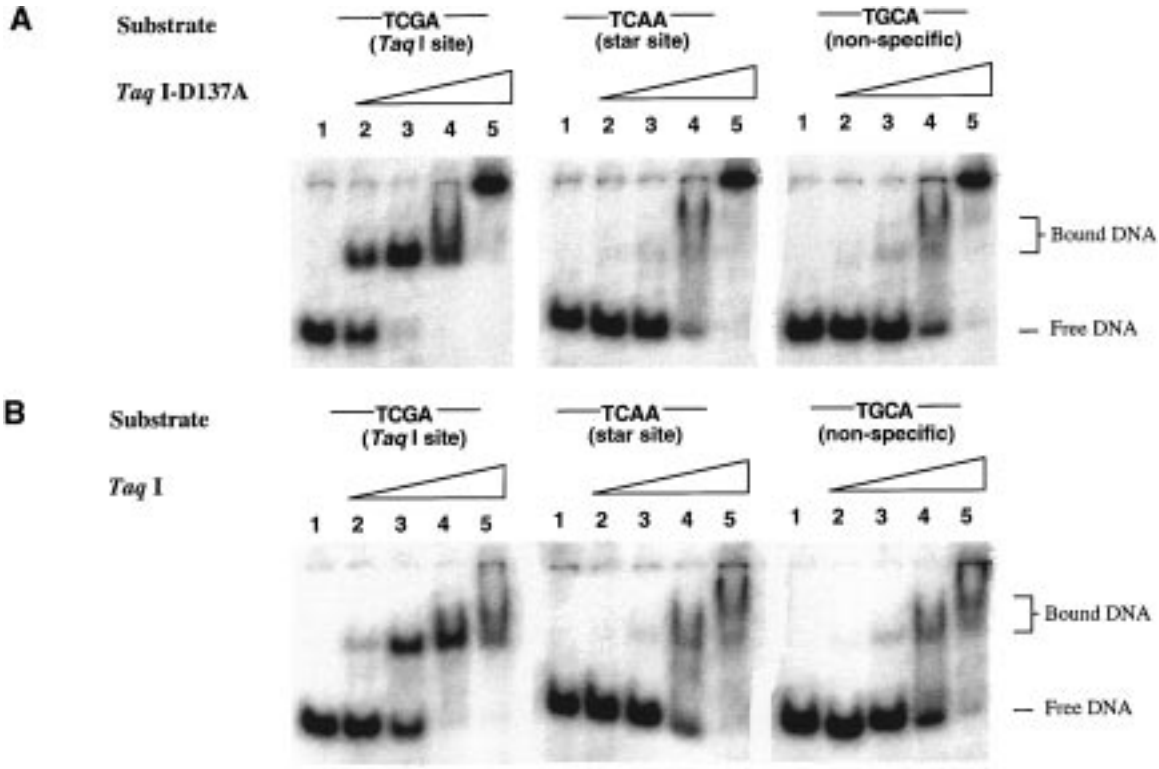

FIGURE 2: Gel mobility shift of *Taq*I endonuclease at 60 °C with Ca^{2+} as the metal cofactor. The binding mixture and conditions are described in Experimental Procedures: lane 1, no *Taq*I enzyme or *Taq*I-D137A mutant; lane 2, 0.25 nM *Taq*I enzyme or *Taq*I-D137A mutant; lane 3, 2.5 nM *Taq*I enzyme or *Taq*I-D137A mutant; lane 4, 25 nM *Taq*I enzyme or *Taq*I-D137A mutant; and lane 5, 250 nM *Taq*I enzyme or *Taq*I-D137A mutant. (A) Binding of the *Taq*I-D137A mutant to DNA sequences. (B) Binding of the *Taq*I enzyme to DNA sequences.

a 1-fold to a 1000-fold excess over the substrate concentration so all possible species bound to this 36/40mer oligonucleotide substrate could be observed. As shown in Figure 1, both the wt *Taq*I enzyme and the *Taq*I-D137A mutant bound to all DNA species, the cognate site (TCGA), the star site (TCAA), and the nonspecific sequence (TGCA), essentially equally in the absence of a metal cofactor. At a relatively low protein concentration, a single bound enzyme−DNA complex was observed with all DNA species, indicating that the wt *Taq*I and the mutant enzyme interacted nondiscriminatively with a DNA sequence at a 1:1 ratio (Figure 1, lane 3). When the protein concentration was further increased, the enzyme not only formed more 1:1 ratio complex but also formed a discreet higher-molecular weight complex, indicating more than one enzyme molecule was bound to the DNA sequences (Figure 1, lane 4). Since the length of this duplexed DNA was 36 bp, and footprinting results shown later demonstrated a region of an 18 bp DNA−protein interface, it is likely that this secondary complex is an enzyme−DNA complex formed by two enzyme molecules over one duplex DNA sequence. This secondary complex was predominantly formed with the mutant enzyme, suggesting a tighter nonspecific binding to DNA by *Taq*I-D137A. Elimination of the negatively charged carboxyl side chain may ameliorate the electrostatic clash of D137 with the negatively charged phosphate backbone in the absence of a metal cofactor. When this short DNA sequence was oversaturated with the enzyme, multiple enzyme molecules were complexed with a DNA sequence [possibly a ≥3:1 E−S (enzyme−substrate) complex] so that its mobility was further retarded. This multiple-enzyme complex may result from nonspecific DNA−protein interaction as well as from weak protein−protein interaction between dimeric *Taq*I enzymes.

Binding with Ca^{2+} at 60 °C. An alternative approach to studying the binding in the presence of a metal cofactor is to use a metal cofactor which supports specific binding but not DNA cleavage. Ca^{2+} has been found to be the choice of the catalytically inactive metal cofactor for *Eco*RV (*21*), *Nae*I (*22*), and *Taq*I studies (*5*). Both the *Taq*I-D137A mutant and the wt enzyme bound differentially to different DNA sequences with Ca^{2+} as the metal cofactor (Figure 2). The binding can be characterized by formation of a stable specific complex with the cognate sequence (Figure 2, *Taq*I site) and weakening of the nonspecific complex as seen with the star site and nonspecific complexes (Figure 2, star site and nonspecific site). In contrast to results observed in the absence of a metal cofactor (Figure 1), the enzymes appeared to form only a 1:1 complex at an enzyme excess up to 100-fold; more enzyme would lead to a ≥2:1 E−S complex. Discreet 2:1 or 3:1 complexes were not observed, suggesting that the specific binding of the enzymes to an 18 bp region of the 36mer duplex DNA may effectively occlude addition of another enzyme molecule to the complex. The *Taq*I-D137A mutant appeared to form a tighter and more stable complex. The nonspecific complexes described in the absence of a metal cofactor (Figure 1) were weakened and destabilized in the presence of Ca^{2+} (Figure 2). Hence, only a weak smear was observed with star site and nonspecific complexes. The lack of a difference in binding between the star site and the nonspecific sequence suggests that the enzymes interacted with the star site in a manner similar to that of a nonspecific sequence in the presence of Ca^{2+},

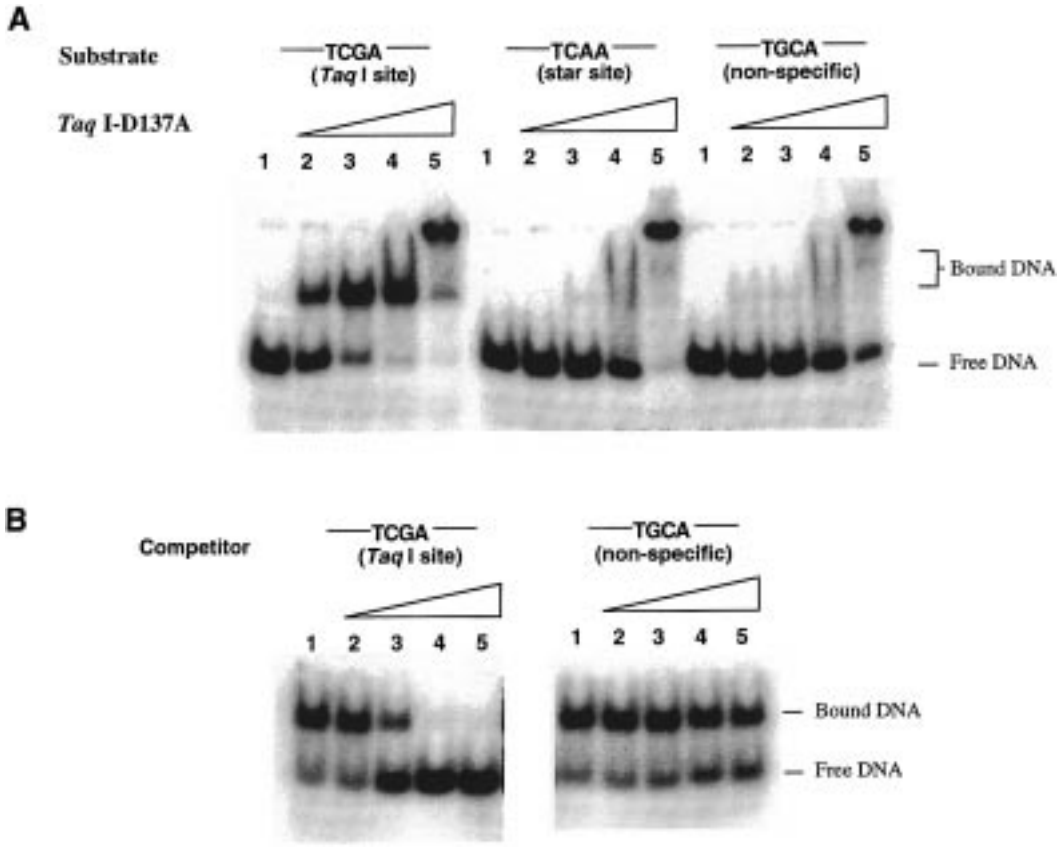

FIGURE 3: (A) Gel mobility shift assay of *Taq*I-D137A to its cognate sequence at 60 °C with Mg^{2+} as the metal cofactor. The binding mixture and conditions are described in Experimental Procedures: lane 1, no *Taq*I-D137A mutant; lane 2, 0.25 nM *Taq*I-D137A mutant; lane 3, 2.5 nM *Taq*I-D137A mutant; lane 4, 25 nM *Taq*I-D137A mutant; and lane 5, 250 nM *Taq*I-D137A mutant. (B) Competition of specific binding with unlabeled specific or nonspecific DNA substrates. Specific binding was performed with 2.5 nM *Taq*I-D137A, 0.25 nM 201/202 oligonucleotide substrate, 10 mM $MgCl_2$, and the indicated amount of either unlabeled specific (201/202) or nonspecific DNA substrate (201-NS/202-NS) as described in Experimental Procedures: lane 1, no competitor; lane 2, 0.25 nM unlabeled 201/202 or 201-NS/202-NS; lane 3, 2.5 nM unlabeled 201/202 or 201-NS/202-NS; lane 4, 25 nM unlabeled 201/202 or 201-NS/202-NS; and lane 5, 250 nM unlabeled 201/202 or 201-NS/202-NS.

confirming our previous conclusion drawn from kinetic studies with Mg^{2+} (*4*). Different from the binding to the cognate site, the enzymes formed a very weak ≥2:1 E−S complex with the star site and the nonspecific sequence when a 1000-fold excess of protein was used (Figure 2, lane 5).

Binding with Mg^{2+} at 60 °C. We further compared the binding of *Taq*I-D137A to the three DNA sequences. The enzyme was able to form a tight, specific complex with its cognate site with Mg^{2+} as the metal cofactor (Figure 3A). This complex was absent with the star site and the nonspecific sequence. Similar to what we had observed using Ca^{2+} as the metal cofactor, the nonspecific interactions between the enzyme and the noncognate sequences were weakened and the complexes destabilized.

To prove the 1:1 complex formed in the presence of Mg^{2+} was indeed a complex specific to the cognate sequence, a competition experiment was performed. As shown in Figure 3B, the cold competitor DNA containing the *Taq*I cognate site effectively competed away the specific binding complex, while the cold competitor without the cognate site did not. Thus, the discreet 1:1 complex we observed in the presence Mg^{2+} represented specific interactions between the *Taq*I enzyme and its cognate site.

Kinetics of Specific TaqI Binding. To understand the kinetics associated with this specific binding, the dissociation constant and dissociation rate constant were determined. The K_d was measured by first forming the specific complex in the presence of Mg^{2+} with different concentrations of the *Taq*I-D137A mutant (Figure 4A). The K_d calculated from the plot shown in Figure 4B was 0.26 nM. This value is half of the K_m value obtained under a similar experimental condition, reaffirming the tight binding of the *Taq*I enzyme to its cognate sequence.

The dissociation rate constant (k_{off}) was determined by first allowing formation of the specific complex under the same condition that was used in the K_d measurement, and then excess unlabeled substrate was added to capture the dissociated protein to prevent reannealing of *Taq*I with the labeled substrate (*23*−*25*). The k_{off} of 5.5×10^{-4} s^{-1} translates into a half-life of 21 min, suggesting that the specific *Taq*I−DNA−Mg^{2+} complex is very stable and the dissociation proceeds at a very slow rate (Figure 5).

DNase I Footprinting. To define the region of the substrate with which the enzyme interacts, we performed DNase I footprinting using a 64mer duplexed DNA with a *Taq*I recognition site located in the middle of the sequence. The substrate concentration (201L/202L) was raised to 3 nM per reaction so that the intensity of each individual band was readily visible, and the enzyme:substrate ratio was adjusted to 33:1 so that the vast majority of the labeled substrate was maintained in the bound state. An 18 bp region was strongly protected from DNase I digestion by *Taq*I-D137A (Figure

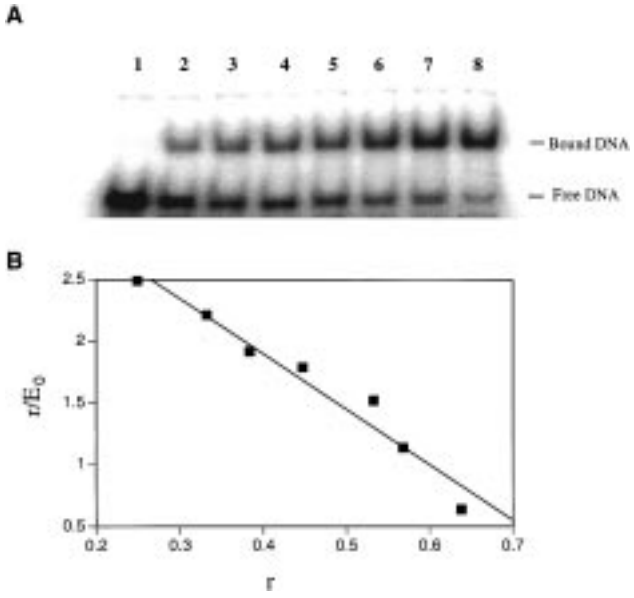

FIGURE 4: Dissociation constant of the *Taq*I-D137A mutant with Mg^{2+} as the metal cofactor. The binding mixture and conditions are described in Experimental Procedures: lane 1, no *Taq*I-D137A mutant; lane 2, 0.1 nM *Taq*I-D137A mutant; lane 3, 0.15 nM *Taq*I-D137A mutant; lane 4, 0.2 nM *Taq*I-D137A mutant; lane 5, 0.25 nM *Taq*I-D137A mutant; lane 6, 0.35 nM *Taq*I-D137A mutant; lane 7, 0.5 nM *Taq*I-D137A mutant; and lane 8, 1 nM *Taq*I-D137A mutant. (A) Representative experimental data. At least three independent experiments were performed. (B) Plot of the experimental data.

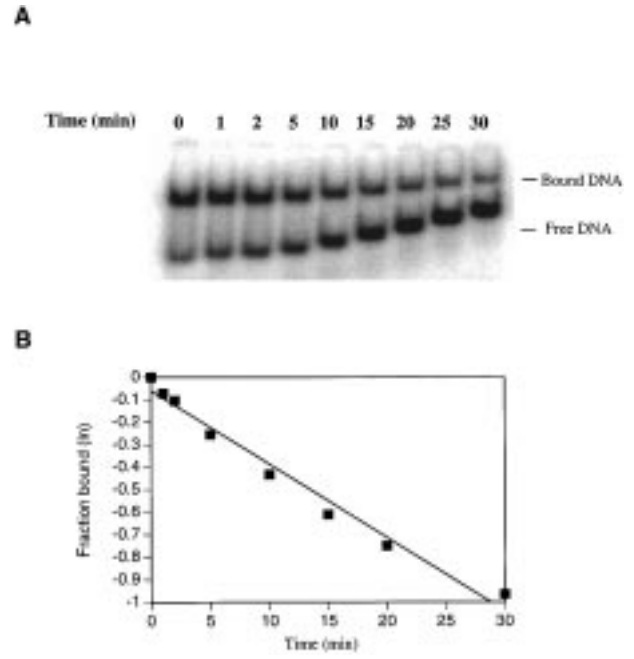

FIGURE 5: Dissociation rate constant of the *Taq*I-D137A mutant with Mg^{2+} as the metal cofactor. The binding mixture and conditions are described in Experimental Procedures. Samples were withdrawn from the binding mixture at the indicated time points and directly loaded onto a running polyacrylamide gel without interrupting electrophoresis. (A) Representative experimental data. At least three independent experiments were performed. (B) Plot of the experimental data.

6). In both strands, this protected region spanned from five nucleotides 5′ to the TCGA (*Taq*I cognate site) to nine nucleotides 3′ to the TCGA. In addition, five nucleotides further upstream of the extensively protected region exhibited

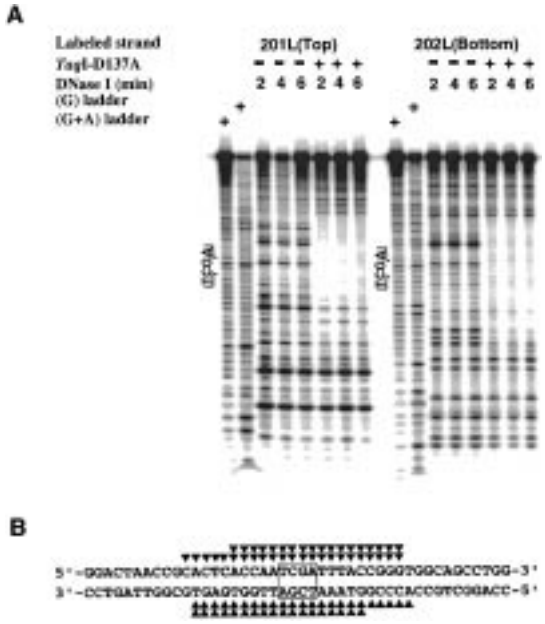

FIGURE 6: DNase I footprinting of *Taq*I-D137A with the *Taq*I recognition sequence. See Experimental Procedures for a detailed description of binding and DNase I cleavage conditions. (A) Separation of DNase I-digested oligonucleotides on a 10% sequencing gel. (B) Protection region on the DNA substrate containing a *Taq*I cognate site (201L/202L). The cognate site is boxed. Double triangles denote nucleotides fully protected by *Taq*I-D137A. Single triangles denote nucleotides weakly protected by *Taq*I-D137A. Oligonucleotides 201L and 202L are shortened by 10 nucleotides each from both ends.

a lower degree of DNase I digestion, suggesting that the five-nucleotide region (10 nucleotides from the TCGA site) was less accessible to DNase I (Figure 6B). The symmetry of protection reflects the symmetric interaction of the dimeric *Taq*I protein with its cognate sequence. The 3′ four-base staggering of the protection pattern suggests that the enzyme binds to the minor groove (*26*).

DISCUSSION

It has been proposed that restriction enzymes may fall into two categories depending upon their requirements of metal cofactor for sequence-specific binding (*11*, and references therein). *Eco*RI and *Bam*HI bind their respective cognate sequences with high affinity without a metal cofactor, while *Eco*RV and *Taq*I may bind their respective cognate sequences with equal affinity in the absence of a metal cofactor. This view is challenged recently by a binding study of the *Eco*RV enzyme in which filter binding and gel retardation assays were used (*14*). The main conclusion from this study is that specific binding of *Eco*RV with Mg^{2+} could be observed with altered assay conditions, such as pH and salt, and the previous observation that *Eco*RV binds its cognate and noncognate sequences equally as determined by gel retardation assay is due to a pH shift from 7.5 in the binding equilibrium to pH 8.3 in the electrophoresis buffer. In reexamining the binding behavior of *Eco*RV in the absence of Mg^{2+}, Erskine and Halford (*15*) found the binding affinity of *Eco*RV is essentially no different between cognate and noncognate sequences by measuring the equilibrium constants in solution with fluorescence resonance energy transfer (FRET) and fluorescence depolarization (FD) techniques.

TaqI Binds Its Cognate and Noncognate Sequences with Essentially Equal Affinity without a Metal Cofactor. In this study, we performed a gel mobility shift experiment with both the D137A mutant and the wild-type enzyme, both of which exhibited essentially no preference for binding to the cognate site in the absence of a metal cofactor (Figure 1). Our DNase I footprinting data suggest that the DNA−protein interface between *Taq*I and its recognition site TCGA is about 18 bp (Figure 6). Thus, the 36 bp duplex we used for the gel shift assay (201/202) should at least allow for binding of two enzyme molecules to one molecule of the duplexed oligonucleotide. The enzyme indeed exhibited two discrete shifted bands with increasing protein concentrations. Similar banding pattern and band intensities indicate that the enzyme interacts with the three DNA species (the cognate sequence, star site sequences, and nonspecific sequences) with essentially equal affinity. We have performed the binding assay at both room temperature and 60 °C using Tris-HCl buffer. The binding behaviors of *Taq*I toward different DNA species are similar under both conditions, suggesting that the lack of high-affinity binding to the cognate site without a metal cofactor is unlikely due to a pH shift between the equilibrium and electrophoresis buffers. Although we did not intend to compare *Taq*I and *Mun*I systematically, it appears that *Taq*I-D137A is different from *Mun*I-D83A and *Mun*I-E89A in that the latter binds its cognate sequence specifically in the absence of Mg^{2+} while the former does not (Figure 1 and ref *27*). These results suggest that elimination of the negative charge(s) at the catalytic center does not confer binding specificity to *Taq*I.

TaqI Binds Its Cognate Site Preferentially in the Presence of a Metal Cofactor. We have studied the binding behavior of *Taq*I toward different DNA species under various conditions (Figures 1−3). Consistent with our previous studies (*4*), results from these experiments suggest that the enzyme forms a catalytically active tertiary complex, *Taq*I−TCGA−Mg^{2+}. For the wild-type enzyme, the complex will proceed to DNA cleavage. For the mutant *Taq*I-D137A, the catalysis is disabled but the specific *Taq*I−TCGA−Mg^{2+} complex remained stable. The gel mobility shift data from the wild-type *Taq*I with Ca^{2+}, *Taq*I-D137A with Ca^{2+}, and *Taq*I-D137A with Mg^{2+} corroborate each other with respect to *Taq*I binding (Figures 1−3). First, under all conditions, *Taq*I shows a strong bias toward binding to its cognate sequence TCGA in the presence of a metal cofactor. Its binding specificity is high as the binding intensity only decreases with increasing concentrations of the cognate site competitor (Figure 3B), and its binding affinity is high as demonstrated by a K_d value of 0.26 nM at 60 °C (Figure 4). Second, the metal cofactor not only locks the enzyme into its cognate site with high affinity but also destabilizes the noncognate complexes substantially, generating a smearing effect on a mobility shift gel (Figures 2 and 3). The two opposing effects observed on different DNA sequences by the addition of a metal cofactor strongly suggest that $metal^{2+}$ plays an important role in forming the specific *Taq*I−TCGA−$metal^{2+}$ complex. In the case of *Taq*I, a metal cofactor thus facilitates sequence-specific recognition by two means: enhancing the specific complex as well as weakening the nonspecific complexes (*28*).

It is still puzzling how Mg^{2+} mediates sequence-specific binding, and the structural basis of these interactions. In *Eco*RV enzyme, it appears that amino acid residue(s) coordinating the binding metal may be distal from the ones positioning the catalytic metal ion(s), as the triple mutant E45A/D74A/D90A which removes all active site acidic residues still maintains wild-type binding to its cognate sequence (*29*). A tyrosine residue Y219 is proposed for *Eco*RV, yet this seems to need further study. For *Taq*I, the lack of a three-dimensional structure precludes a global view of protein−DNA interactions and the catalytic center. However, D142 has been shown to be a residue critical for binding and catalysis (*5*). It remains to be determined how D142 or other residue(s) mediated the formation of the specific *Taq*I−TCGA−$metal^{2+}$ complex.

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