Identification of TaqI Endonuclease Active Site Residues by Fe$^{2+}$-mediated Oxidative Cleavage*

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Metal cofactors (Mg$^{2+}$ and Mn$^{2+}$) modulate both specific DNA binding and strand cleavage in the TaqI endonuclease (Cao, W., Mayer, A. N., and Barany, F. (1995) Biochemistry 34, 2276–2283). This work attempts to establish the structural basis of TaqI-DNA-metal$^{2+}$ interactions using an affinity cleavage technique. The protein was cleaved by localized hydroxyl radicals generated by oxidizing Fe$^{2+}$ within the metal binding sites. Cleavage fragments were separated by SDS-polyacrylamide gel electrophoresis, and cleavage sites were determined using micropeptide sequencing. Eleven amino acid residues in the vicinity of cleavage sites were selected for site-directed mutagenesis. The negative charge at Asp$^{197}$ is essential for DNA cleavage but not required for sequence specific binding. Mutations at Asp$^{197}$ abolish both specific binding and catalysis, except for D142E, which converts TaqI into a completely Mn$^{2+}$-dependent endonuclease. The positive charge at Lys$^{158}$ appears to be important for both specific binding and catalysis. Mutations at other sites affect binding and/or catalysis to different degrees, except Trp$^{113}$ and Glu$^{135}$, which appear to be nonessential for the TaqI enzyme activity. The critical residues for TaqI function are distinct from the PDX$_{14–20}$(E/D)KX catalytic motif elucidated from other endonucleases.

TaqI endonuclease recognizes and cleaves a short palindromic double-stranded DNA sequence, T$ar{G}$A, with exquisite specificity. Its recognition mechanism has been probed with 2′-deoxyribonucleic acid; wt, wild-type; PVDF, polyvinylidene difluoride.

The active site organization of a number of restriction enzymes has been elucidated by high resolution crystallographic analysis or genetic means (9–19). One of the common features among these enzymes is that two or more carboxylates of Asp or Glu are involved in coordinating the metal cofactor in the active site. TaqI enzyme requires Mg$^{2+}$ or Mn$^{2+}$ as the metal cofactor for its activity (20). Therefore, replacement of the metal cofactor with a redox-active metal ion could lead to specific cleavage at the proximity of the active site. This study reports Fe$^{2+}$-mediated cleavage of TaqI protein and site-directed mutagenesis analysis of 11 amino acid residues. The resulting TaqI mutants were grouped into four categories based on their binding and catalysis properties. The active site of TaqI was compared with that of other known type II endonucleases.

EXPERIMENTAL PROCEDURES

Materials—PCR$^1$ amplification and dideoxy terminator sequencing kits were obtained from Perkin-Elmer. T4 polynucleotide kinase was

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The abbreviations used are: PCR, polymerase chain reaction; CAPS, 3′-(cyclohexylamino)-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; wt, wild-type; PVDF, polyvinylidene difluoride.

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RESULTS

Cleavage of TaqI Protein—Successful application of Fe$^{2+}$-mediated cleavage depends on specific interactions between the metal ion and TaqI protein. Previously, we have established by kinetic analysis that Mn$^{2+}$ metal ion may interact with TaqI protein specifically in a DNA-independent fashion (3). To determine whether a redox-active metal such as Fe$^{2+}$ can serve as a metal cofactor, we tested TaqI activity with Fe$^{2+}$ along with other divalent metal ions. As shown in Fig. 1A, the only alkaline metal that supported TaqI cleavage was Mg$^{2+}$. Mar-

FIG. 1. Cleavage activity of TaqI endonuclease with various Group 2A and period 4 transition metal ions. A, assay of TaqI enzyme activity with some Group 2A divalent ions. B, assay of TaqI enzyme activity with period 4 transition divalent ions. Reactions were performed under single turnover conditions as described previously (3), with modifications. The final concentration of purified TaqI enzyme was 0.25 μM; $^{32}$P-labeled oligonucleotide substrate, 5 nM; MnCl$_2$, 10 mM. The reaction mixtures were incubated at 60 °C for 60 min. Cleavage activity was quantified on a PhosphorImager. Arrows indicate that the cleavage reactions had completed long before the incubation at 60 °C (1 h) was stopped.

mm Tris-HCl (pH 8.0 at 60 °C), 10 mM dithiothreitol, 50 mM NaCl, 0.1 mg/ml bovine serum albumin, 10 mM CaCl$_2$, 0.02% bromphenol blue at 60 °C for 15 min. The reaction mixtures were incubated at 60 °C for 60 min. Cleavage activity was quantified on a PhosphorImager. Results were reported as the averages of three independent experiments.

Cleavage Activity Analysis by Cleavage Assay—A plasmid substrate containing a single TaqI recognition site (pFB76, 20 μM) was digested with 2 nM of partially purified wild-type or mutant enzymes in the 1× SDS-PAGE sample buffer supplemented with 20 mM EDTA.

SDS-PAGE—A 0.1% SDS-15% polyacrylamide gel was prerun at 25 V for 1 h in a Bio-Rad Protein II apparatus filled with 1× SDS-PAGE running buffer. The caps of the 0.5-ml reaction tubes were pierced by a 25 1⁄2 gauge needle to allow oxygen flow. The reaction tubes were placed in a low speed vortex to ensure proper mixing. In the case in which a DNA substrate was included in the cleavage reaction, 5 μM of annealed duplex oligonucleotide substrate (AMC201/202, see below for sequence) containing 32P-labeled DNA oligonucleotide fragment (30:0.8) containing 89 mM Tris-borate and 10 mM CaCl$_2$ by electrophoresis in a Bio-Rad Protean II apparatus at 100 V for 1.5 h (27). The intensity of 32P-labeled DNA bands was quantified using a PhosphorImager (Molecular Dynamics). Results were reported as the averages of three independent experiments.

Cleavage of TaqI Protein—TaqI activity with period 4 transition divalent ions. Reactions were performed under single turnover conditions as described previously (3), with modifications. The final concentration of purified TaqI enzyme was 0.25 μM; $^{32}$P-labeled oligonucleotide substrate, 5 nM; MnCl$_2$, 10 mM. The reaction mixtures were incubated at 60 °C for 60 min. Cleavage activity was quantified on a PhosphorImager. Results were reported as the averages of three independent experiments.

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Figure 2. Time course of Fe$^{2+}$-mediated cleavage of TaqI protein. A, the cleavage mixture consisted of 18 μg of purified TaqI protein, 20 mM HEPES (pH 7.6), 2 mM FeCl$_2$, 25 mM sodium ascorbate, 10% glycerol in a total volume of 50 μl. After incubating at room temperature for a period of time as indicated, cleavage reactions were stopped by addition of 40 μg of 2% SDS-PAGE sample loading buffer supplemented with 20 mM EDTA. See under “Experimental Procedures” for details. B, comparison of cleavage of native and denatured TaqI protein. Lane 1, cleavage of TaqI protein in cleavage buffer. Lane 2, TaqI protein was treated with 0.1% SDS at room temperature for 20 min before cleavage reaction was performed. Lane 3, TaqI was heat-treated at 95 °C for 15 min before cleavage reaction was performed.

Original activity was observed with Ca$^{2+}$ after extended incubation at 60 °C for 1 h. For the fourth period transition metals, Fe$^{2+}$ showed significant activity, suggesting that it could interact with TaqI protein specifically to form both ground state and transition state complexes (Fig. 1B). From Mn$^{2+}$ to Zn$^{2+}$, the cleavage activity generally followed a descending order.

We first determined the optimal time required for the cleavage of TaqI protein. A time course of TaqI protein cleavage was conducted with 2 mM Fe$^{2+}$ and 25 mM sodium ascorbate (Fig. 2A). Primary cleavage fragments appeared at the earliest time point (30 min) and accumulated to its maximum after about 2 h of incubation. At later time points, the reaction kinetics is dominated by concurrent cleavage of both intact protein and secondary degradation of primary cleavage products.

To demonstrate the specificity of Fe$^{2+}$-mediated cleavage of TaqI protein, we denatured the protein by incubating with 0.1% SDS for 20 min or heating at 95 °C for 15 min (Fig. 2B). Upon denaturation with SDS, discrete cleavage bands were no longer formed, although cleavage of intact TaqI protein appeared to be significant. This result suggests that when TaqI protein is denatured and linearized by SDS treatment, the cleavage reaction becomes essentially nonspecific. In the case of thermal denaturation, TaqI protein remained largely uncleaved. This result indicates that the protein may have undergone unfolding and refolded into a random coil-like state that disorganizes the metal binding site(s) and becomes more resistant to cleavage. By and large, the data demonstrate that the cleavage pattern shown in Fig. 2A is dependent on the proper tertiary or even quaternary structure of TaqI native protein.

Peptide Sequencing of Cleavage Fragments—To obtain enough fragmented peptides for N-terminal sequencing, the cleavage reaction was scaled up by using 75 μg of purified TaqI protein, 20 mM HEPES (pH 7.6), 2 mM FeCl$_2$, 25 mM sodium ascorbate, 10% glycerol in a total volume of 50 μl. After incubating at room temperature for a period of time as indicated, cleavage reactions were stopped by addition of 40 μg of 2% SDS-PAGE sample loading buffer supplemented with 20 mM EDTA. See under “Experimental Procedures” for details of reaction conditions.

These fragments were electroblotted onto PVDF membrane, and each individual band was excised out for N-terminal sequencing. For some faint bands, two identical fragments were loaded to a protein sequencer. Sequence data were obtained from at least five cycles, and in some cases eight cycles. Three fragments (F2, F3, and F8) corresponded to the original N terminus of TaqI protein, which had a seven-amino acid PhoA signal sequence (MKQSGGN) fused onto the gene (Table I). F4a and F5a uniquely generated from substrate-induced cleavage also corresponded to the original N-terminal sequences, from which little information about cleavage sites can be extracted. Internal cleavage sequences were obtained from minor amino acid residues in fragments F1, F4, F5, and F7 and from major amino acid residues in fragment F6 (Table I). Some fragments with single internal sequence data available (F1, F4, and F6) were readily mapped to the correct TaqI sequence. For fragments with several internal amino acid residues identified in each cycle, all the possible combinations with a match of sequence more than four amino acids were selected (Fig. 4).

Figure 3. Scale-up of Fe$^{2+}$ cleavage with or without DNA substrate. Left lane, protein molecular weight standards. +, Fe$^{2+}$ cleavage without DNA substrate. +, Fe$^{2+}$ cleavage with DNA substrate. See under “Experimental Procedures” for details of reaction conditions.

Seven cleavage sites were mapped to TaqI sequence: Asp$^{47}$ | Leu$^{48}$ | Ile$^{112}$ | Trp$^{113}$ | Glu$^{135}$ | Leu$^{136}$ | Ala$^{138}$ | Gln$^{139}$ | Gly$^{140}$ |
Table 1
Amino acid residues of cleavage fragments identified by micropeptide sequencing

| Cleavage | Cycle 1           | Cycle 2           | Cycle 3           | Cycle 4           | Cycle 5           | Cycle 6           | Cycle 7           | Cycle 8           |
|----------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| fragment | Maj\(^a\) Min\(^b\) | Maj \(^a\) Min\(^b\) | Maj \(^a\) Min\(^b\) | Maj \(^a\) Min\(^b\) | Maj \(^a\) Min\(^b\) | Maj \(^a\) Min\(^b\) | Maj \(^a\) Min\(^b\) | Maj \(^a\) Min\(^b\) |
| F1       | Met Leu           | Lys Tyr           | Gln Glu           | Ser His           | Gly Tyr           | Gly Trp           | Asn Lys           | Minor product(s)  |
| F2       | Met Lys           | Lys Gln Val       | Gln Glu           | Ser His           | Gln Glu           | Gly Trp           | Asn Lys           | Minor product(s)  |
| F3       | Met Trp           | Lys Ala           | Lys Glu           | Ser His           | Lys Glu           | Gly Trp           | Asn Lys           | Minor product(s)  |
| F4       | Met Lys           | Lys Tyr           | Gln Ala           | Ser His           | Lys Glu           | Gly Trp           | Asn Lys           | Minor product(s)  |
| F4a      | Met Trp Lys Ala   | Lys Tyr           | Gln Ala           | Ser His           | Lys Glu           | Gly Trp           | Asn Lys           | Minor product(s)  |
| F5       | Ser Met Lys Ala   | Lys, Glu Val Arg  | Asp, Gly Lys Ala  | Ser His           | Asp, Gly Lys Ala  | Gly Trp           | Asn Lys           | Minor product(s)  |
| F5a      | Ser Met Lys Ala   | Lys, Glu Val Arg  | Asp, Gly Lys Ala  | Ser His           | Asp, Gly Lys Ala  | Gly Trp           | Asn Lys           | Minor product(s)  |
| F6       | Met Lys           | Lys Trp           | Arg Glu           | Gly Ser Lys Ala   | Gly Trp           | Asn Lys           | Minor product(s)  |
| F7       | Met, Ser Ala      | Lys, Val Gly Lys  | Asp, Gly Lys Ala  | Ser His           | Asp, Gly Lys Ala  | Gly Trp           | Asn Lys           | Minor product(s)  |
| F8       | Met               | Lys               | Lys, Glu Val Arg  | Asp, Gly Lys Ala  | Asp, Gly Lys Ala  | Gly Trp           | Asn Lys           | Minor product(s)  |

\(^a\) Major product(s).
\(^b\) Minor product(s).

Identification of TaqI Active Site

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Our results shown in Fig. 1A demonstrated that Ca$_2^+$ is also unable to support significant catalytic activity (>10%) with TaqI. This technique is particularly useful in characterizing wild-type and mutant binding because both EcoRV and TaqI require Mg$_2^+$ for binding and catalysis. To evaluate the binding property of these mutants, we used a binding condition in which wt enzyme achieved less than 100% binding to the oligonucleotide. Relative binding affinity was quantified by taking the intensity ratio of each bound mutant enzyme over bound wt enzyme. The mutants can be classified into three categories (Table II). Category 1: wild-type binding (Fig. 5A), including all mutants at Trp113 and Glu135, one at Gln139 (Q139N), three at Lys157 (K157R, K157S, and K157C), two at Asp137 (D137A and D137G), one at Asp48 (D48V), and one at Lys174 (K174R). Category 2, reduced binding (20–90% wild-type binding), including three at Asp48 (D48E, D48A, and D48G), two at Asp137 (D137A and D137G), one at Asp48 (D48V), and one at Lys174 (K174R). Category 2, reduced binding (20–90% wild-type binding), including three at Asp48 (D48E, D48A, and D48G), two at Asp137 (D137A and D137G), one at Asp48 (D48V), and one at Lys174 (K174R). Category 3, severely reduced binding (<20% wild-type binding), including four mutants at Lys158.

Cleavage assay conditions were designed such that limited cleavage occurs on a plasmid substrate (pFB76) containing a single TaqI recognition site. The ratio of linearized product formed by a particular mutant over wt TaqI was used to determine relative cleavage activity. The majority of mutations (Asp48, Trp113, Glu135, Gln139, Gly140, Lys157, Arg173, and Lys174) retained wt or reduced activity (Table II). Most mutations at Asp137, Glu142, and Lys158 completely lost DNA cleavage activity even after incubation with 10-fold more enzymes (data not shown). The effects of individual mutations on binding and cleavage are summarized in Table II and described as follows.

Asp48—DNA binding was lowered by 20% by changing Asp to Glu, albeit D48E retains a negative charge at this position. Elimination of the negative charge (D48A and D48G) further lowered the binding to 50%, whereas catalysis was comparable with the wt enzyme, suggesting that the influence of mutations at Asp48 mainly takes effect on the formation of ground state ES complex.

Trp113 and Glu135—Mutations at these two positions did not seem to affect binding or catalysis, although they are located at or near the Fe$_2^+$-mediated cleavage sites and are conserved among four homology groups of TaqI isoschizomers (29). These results suggest that Trp113 and Glu135 may not be involved in direct DNA binding and catalysis. However, some of the mutants (W113C and E135V) may disturb catalysis when Mn$_2^+$ is used as the metal cofactor.

Asp137—Conservative change from Asp to Glu led to about 25% loss of binding affinity but 80% loss of catalytic activity (Fig. 5). The other three mutants (D137A, D137V, and D137G), which eliminated the negative charge, led to complete loss of catalysis. Thus, mutations at Asp137 in general do not affect substrate binding, suggesting that Asp137 is a critical amino acid residue for catalysis, possibly by binding to a catalytic metal ion in the formation of transition state complex.

Gln139—A conservative change (Q139N) seemed to affect neither binding nor catalysis. Failure to retain the hydrogen bond donor and acceptor property (Q139D and Q139Y) reduced binding or both binding and catalysis. Some Asn or Gln residues in restriction endonucleases and other proteins are involved in DNA recognition. Mutations at Gln139 caused only limited reduction in substrate binding, suggesting that Gln139 is not a direct role in DNA recognition. However, Gln139 may be part of a hydrogen bond network in the vicinity of the active site.

Gly140—A conservative change (Q139N) seemed to affect neither binding nor catalysis. Failure to retain the hydrogen bond donor and acceptor property (Q139D and Q139Y) reduced binding or both binding and catalysis. Some Asn or Gln residues in restriction endonucleases and other proteins are involved in DNA recognition. Mutations at Gly140 led to loss of binding affinity, which roughly corresponded with the bulkiness of the amino acid side chain. As to DNA cleavage, whereas a methyl side chain exhibited little effect, an isopropyl or a hydroxylethyl side chain reduced cleavage by 60%. These results indicate that Gly140 may influence both binding and catalysis by providing...
flexibility to the protein confirmation in the vicinity of the active site.

Asp142—Unlike Asp137, which retained binding affinity and some catalytic activity with a conservative change (D137E), both binding with Ca\(^{2+}\) and catalysis with Mg\(^{2+}\) are completely abolished by any replacement at this position. We considered two scenarios to explain the role of Asp142. First, the carboxylate of Asp142 may be involved in forming a metal binding site to coordinate Mg\(^{2+}\), which is required for both ground state ES and transition state interactions. The inability to bind a Mg\(^{2+}\) metal cofactor results in loss of binding and catalysis. An alternative explanation is that Asp142 is a critical determinant for the TaqI three-dimensional structure, such that mutations at the Asp142 position would drastically distort the protein conformation, resulting in a total loss of enzyme activity. However, the high cleavage rate of D142E mutant with Mn\(^{2+}\) suggests that the protein conformational alteration is unlikely to occur globally, at least not by the Asp to Glu substitution, for a global structural change would likely impede cleavage with Mn\(^{2+}\) as metal cofactor as well. On the other hand, small conformational changes could have occurred with D142E, which altered the metal binding site in such a way that Ca\(^{2+}\) or Mg\(^{2+}\) may no longer be able to coordinate, but still could accommodate Mn\(^{2+}\) binding.

Lys157—Mutations at Lys157 in general led to better than wt binding with the exception of K157G, which may cause some conformational disturbance due to the complete elimination of the side chain. Catalysis in general was diminished, particularly when the positive charge was not maintained (K157S, K157C, and K157G). In light of its vicinity to the catalytic center residue Lys158 described below, the positively charged Lys157 may play a supportive role in catalysis.

Lys158—Mutations at Lys158 reduced both substrate binding and DNA cleavage drastically. Even the conservative change from Lys to Arg resulted in complete loss of catalysis using either Mg\(^{2+}\) or Mn\(^{2+}\) as a metal cofactor, suggesting that the precise configuration of Lys could not be achieved by Arg at Lys158. The weak binding affinity observed in K158R indicates that Arg may slightly compensate the role of Lys in ES complex formation, but it failed to interact with the DNA sequence to form an active transition state complex. Therefore, the positive charge at Lys158 is critical for the formation of ES complex as well as transition state interactions. The weak binding and...

| Mutant | Binding (Ca\(^{2+}\)) | Cleavage (Mg\(^{2+}\)) | Cleavage (Mn\(^{2+}\)) | Category |
|--------|----------------------|----------------------|----------------------|---------|
| wt     | 100                  | 100                  | 100                  | 1       |
| D48E   | 78 ± 2               | 122 ± 5              | 219 ± 67             | 2       |
| D48A   | 53 ± 6               | 117 ± 8              | 218 ± 48             | 2       |
| D48V   | 113 ± 3              | 120 ± 5              | 146 ± 47             | 1       |
| D48G   | 47 ± 10              | 132 ± 7              | 184 ± 51             | 2       |
| W113Y  | 113 ± 5              | 122 ± 3              | 124 ± 40             | 1       |
| W113S  | 91 ± 3               | 128 ± 9              | 100 ± 46             | 1       |
| W113C  | 115 ± 5              | 109 ± 8              | 62 ± 27              | 1       |
| E135D  | 100 ± 11             | 112 ± 5              | 104 ± 34             | 1       |
| E135A  | 118 ± 8              | 110 ± 4              | 123 ± 80             | 1       |
| E135V  | 116 ± 9              | 102 ± 5              | 61 ± 31              | 1       |
| E135G  | 115 ± 7              | 101 ± 7              | 89 ± 23              | 1       |
| D137E  | 76 ± 1               | 21 ± 4               | 107 ± 53             | 4       |
| D137A  | 100 ± 3              | 0                    | 0                    | 3       |
| D137Y  | 85 ± 8               | 0                    | 0                    | 3       |
| D137G  | 119 ± 10             | 0                    | 0                    | 3       |
| Q139N  | 106 ± 7              | 110 ± 7              | 144 ± 65             | 3       |
| Q139D  | 47 ± 9               | 83 ± 6               | 113 ± 43             | 4       |
| Q139Y  | 59 ± 13              | 120 ± 10             | 179 ± 39             | 2       |
| G140A  | 51 ± 9               | 98 ± 14              | 148 ± 32             | 2       |
| G140V  | 27 ± 4               | 38 ± 10              | 196 ± 22             | 4       |
| G140E  | 26 ± 8               | 40 ± 10              | 153 ± 22             | 4       |
| D142E  | 0                    | 0                    | 130 ± 34             | 4       |
| D142A  | 0                    | 0                    | 0                    | 4       |
| D142V  | 0                    | 0                    | 0                    | 4       |
| D142G  | 0                    | 0                    | 0                    | 4       |
| K157R  | 129 ± 12             | 72 ± 6               | 233 ± 45             | 3       |
| K157S  | 130 ± 8              | 40 ± 4               | 89 ± 17              | 3       |
| K157C  | 145 ± 17             | 44 ± 4               | 70 ± 9               | 3       |
| K157G  | 70 ± 5               | 35 ± 6               | 48 ± 14              | 3       |
| K158R  | 14 ± 0               | 0                    | 0                    | 4       |
| K158S  | 0                    | 0                    | 0                    | 4       |
| K158C  | 0                    | 0                    | 0                    | 4       |
| K158G  | 11 ± 2               | 19 ± 8               | 39 ± 12              | 4       |
| K173K  | 58 ± 1               | 116 ± 3              | 143 ± 13             | 2       |
| K173I  | 79 ± 9               | 53 ± 4               | 57 ± 22              | 2       |
| K173M  | 68 ± 8               | 36 ± 4               | 70 ± 24              | 2       |
| K173T  | 63 ± 6               | 44 ± 4               | 56 ± 13              | 3       |
| K173N  | 66 ± 9               | 38 ± 2               | 66 ± 14              | 3       |
| K174R  | 110 ± 9              | 102 ± 9              | 209 ± 69             | 1       |
| K174I  | 82 ± 8               | 35 ± 7               | 73 ± 29              | 4       |
| K174M  | 64 ± 8               | 44 ± 4               | 63 ± 11              | 4       |
| K174T  | 63 ± 3               | 56 ± 3               | 79 ± 8               | 4       |
| K174S  | 51 ± 4               | 59 ± 4               | 73 ± 23              | 4       |

* 1, affect neither binding nor catalysis; 2, affect binding but not catalysis; 3, affect catalysis but not binding; 4, affect both binding and catalysis.
Identification of TaqI Active Site

| Enzyme | Sequence |
|--------|----------|
| TaqI   | D- 86 aa  |
|        | D-G-D-----|
|        | 137 142 |
|        | 157 158 |
| EcoRV  | E---------|
|        | 74 90 92 |
| PsmI   | P--EG-D----|
|        | 55 58 68 70 |
| EcoRI  | D---------|
|        | 59 77 91 |
|        | 111 113 |
| BamHI  | E---------|
|        | 450 467 |
|        | 469 476 |
|        | P----------|
|        | 95 97 |
|        | DCK  |
| FokI   | E---------|
|        | 83 98 |
|        | 100 106 |
|        | P----------|
|        | EIK  |

**Fig. 6. Comparison of restriction endonuclease active sites.** A short dash represents one amino acid residue. The proposed active site amino acid residues are numbered.

cleavage observed in K158G are seemingly contradictory at first glance; however, the replacement of the Lys at 158 by a Gly may confer enough flexibility such that the adjacent Lys at 157 could fulfill the role of Lys158 for both binding and catalysis to a certain extent. As a result, limited DNA cleavage was observed.

**Arg**173 and Lys174—The positive charges at positions 173 and 174 are important for binding and catalysis. Although the role of Arg173 in substrate binding could not be entirely replaced by Lys, Lys and Arg are interchangeable at 174 without affecting either binding and catalysis. Both Arg and Lys residues at 174 have been observed in several TaqI isoschizomers (29). Whether the positive charges interact with DNA substrate directly or are required for structural integrity remains to be investigated.

**DISCUSSION**

**Fe2+-mediated Cleavage**—This study attempts to elucidate the structural basis of TaqI endonuclease catalytic center by Fe2+-mediated oxidative cleavage. The observation that Fe2+ could replace Mg2+ as the metal cofactor validates the use of this technique for TaqI. Comparison of Fe2+-mediated cleavage with native and denatured protein further ensures that the cleavage is specific to the three-dimensional structure of the protein. Although the cleavage may progress in many buffer conditions, such as TES, Tris-HCl, and HEPES, phosphate buffer was found to be inhibitory to the cleavage reaction (data not shown). Hydrogen peroxide, which is used for the generation of hydroxyl radicals in some studies, was excluded from the final protocol because it diminished the accumulation of cleavage products. All of the cleavage fragments analyzed were products of a single break in the TaqI protein by hydroxyl radicals, as indicated by their amino acid sequences and molecular weights. In some cases, the amino acid sequences of both fragments from a single cleavage were identified, where the molecular weights of the two fragments add up to that of intact TaqI protein. In other cases, only one sequence was found, suggesting that some fragments may not be able to maintain their native conformation. The unfolded peptide fragments became vulnerable to nonspecific cleavage. There was only one minor fragment identified as a multiple cleavage product (data not shown).

Two additional cleavage fragments (F4a and F5a) were identified when the oligonucleotide substrate containing a single TaqI recognition site was incorporated into the cleavage reaction. These two fragments, when sequenced, corresponded to the original N terminus of TaqI. We have not found the C-terminal fragments from these substrate-dependent cleavage events. More experimentation is needed to purify these two fragments and identify their C-terminal cleavage sites by other means.

**Active Site of Other Endonucleases**—The active site amino acid residues of several restriction endonucleases have been identified by high resolution crystallographic analysis, as well as by genetic and biochemical approaches. On the basis of the structural and biochemical studies of EcoRI and EcoRV, a PDX14-20(E/D)K motif (where X means any amino acid residue) was proposed as the Mg2+-binding site and catalytic center (Fig. 6). This motif is also found in PvuII (16, 17), FokI (33), NaeI (32), and MunI (18). Although some restriction enzymes indeed contain such a sequence element, many restriction endonucleases do not possess this motif (34). The catalytic center of BamHI endonuclease was initially identified by genetic selection (13), later confirmed by structural studies (14, 15). The organization of the BamHI catalytic center can be considered as a degenerate form of the PDX14-20(E/D)K motif, where an amino acid is inserted into the PD doublet and the positively charged Lys is replaced by a negatively charged Glu in the triplet (E/D/KX) (Fig. 6). In PvuII, the ELK triplet (amino acids 68–70) was proposed as part of the catalytic center (Fig. 6). However, a PD doublet was not found within the upstream sequence (16, 17). Glu95 and Asp98 are implicated as playing a role similar to the PD doublet in metal cofactor binding. Glu95 is followed by a Gly, which bears some resemblance to the PDG triplet (amino acids 90–92) in EcoRI. It was suggested that Pro before and Gly after the Asp may properly position the Asp for metal binding (17).

These examples strongly suggest that the catalytic centers of restriction endonucleases are more degenerate than originally thought. In TaqI, a complete PDX14-20(E/D)KX motif does not exist. A PD doublet (amino acids 47–48) is conserved among TaqI isoschizomers (29). Fe2+-mediated cleavage also occurred right after the doublet, PD↓L. Nevertheless, mutations at Asp48 appeared to affect mainly DNA binding. These results suggest that although Asp48 may play some roles in DNA binding or regulating ES complex formation, it does not seem to play a critical role in coordinating the metal cofactor required
for catalysis.

**Role of Asp**<sup>137</sup> **and Asp**<sup>142</sup>—Based on the binding and catalysis studies of the mutants, we propose that Asp<sup>137</sup>, Asp<sup>142</sup>, and Lys<sup>158</sup> are the essential active site amino acid residues in TaqI endonuclease (Fig. 6). Mutations at Asp<sup>137</sup> generate a binding-proficient, catalysis-deficient mutant, which is equivalent to Asp<sup>74</sup> in EcoRV (35), Asp<sup>90</sup> in EcoRI (35), and Asp<sup>74</sup> in BamHI (13). These negatively charged amino acid residues in EcoRV, EcoRI, and BamHI are involved in coordinating a catalytic metal cofactor at the catalytic center; a similar role is expected for Asp<sup>137</sup> in TaqI. The failure to cleave DNA substrate by these mutants could be attributed to the loss of binding affinity to the catalytic metal cofactor, which may serve as a Lewis acid to polarize the nonbridging P<sup>γ</sup>-phosphate of the GMP. Asp<sup>137</sup> in DAQG of TaqI, as discussed above, could play a similar role as Asp<sup>90</sup> of p21<sup>ras</sup>, albeit it may or may not be mediated through a localized water.

In homing endonucleases, the LAGLI-DADG motif is implicated in DNA cleavage, but the roles of each individual residue have not been established (42). It remains to be seen whether the acidic residue(s) in LAGLI-DADG motif also coordinates catalytic Mg<sup>2+</sup> directly or indirectly. Recently, some catalytic residues of BsoBI have been identified by random mutagenesis (43). Interestingly, two catalytically deficient acidic residues, Asp<sup>124</sup> and Asp<sup>246</sup>, are located in a DXXG sequence context. It appears that the DXXG motif may have evolved as a Mg<sup>2+</sup> ion coordinator for DNA hydrolysis.

In summary, this study has identified a few active site residues and revealed some unique features of the TaqI catalytic center. Although the thermophilic TaqI endonuclease shares many important catalytic features with other well studied enzymes, it may also have evolved some unique means to achieve efficient substrate turnover. The hypothetical model will evolve as more experimental data accumulate. Further structural and biochemical characterization of TaqI endonuclease will shed more light on the mechanistic understanding of endonucleases in particular and nucleases in general.

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