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ARTICLE *in* JOURNAL OF MOLECULAR BIOLOGY · APRIL 2001

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Catalytic Efficiency and Sequence Selectivity of a Restriction Endonuclease Modulated by a Distal Manganese Ion Binding Site

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Crystal structures of *Eco*RV endonuclease bound in a ternary complex with cognate duplex DNA and manganese ions have previously revealed an Mn^{2+}-binding site located between the enzyme and the DNA outside of the dyad-symmetric GATATC recognition sequence. In each of the two enzyme subunits, this metal ion bridges between a distal phosphate group of the DNA and the imidazole ring of His71. The new metal-binding site is specific to Mn^{2+} and is not occupied in ternary cocrystal structures with either Mg^{2+} or Ca^{2+}. Characterization of the H71A and H71Q mutants of *Eco*RV now demonstrates that these distal Mn^{2+} sites significantly modulate activity toward both cognate and non-cognate DNA substrates. Single-turnover and steady-state kinetic analyses show that removal of the distal site in the mutant enzymes increases Mn^{2+}-dependent cleavage rates of specific substrates by tenfold. Conversely, the enhancement of non-cognate cleavage at GTTATC sequences by Mn^{2+} is significantly attenuated in the mutants. As a consequence, under Mn^{2+} conditions *Eco*RV-H71A and *Eco*RV-H71Q are 100 to 700-fold more specific than the wild-type enzyme for cognate DNA relative to the GTTATC non-cognate site. These data reveal a strong dependence of DNA cleavage efficiency upon metal ion-mediated interactions located some 20 Å distant from the scissile phosphodiester linkages. They also show that discrimination of cognate *versus* non-cognate DNA sequences by *Eco*RV depends in part on contacts with the sugar-phosphate backbone outside of the target site.

Keywords: metal ion catalysis; protein-nucleic acid recognition; indirect readout; protein engineering

Introduction

Restriction endonucleases are superb models for the study of enzyme-DNA interactions owing to their exceptionally high degree of sequence specificity, coupled to approximately 10^{15}-fold rate enhancements for phosphodiester bond cleavage (Kovall & Matthews, 1999). The reaction mechanism of the homodimeric type II enzymes, which cleave 4-8 base-pair dyad-symmetric DNA target sites, exhibits an absolute requirement for divalent metal ions (Roberts & Halford, 1993). High-resolution structures of divalent metal-ion binding sites in these enzymes are available for *Eco*RV (Kostrewa & Winkler, 1995; Perona & Martin, 1997; Horton *et al*., 1998, 2000), *Bam*HI (Viadiu & Aggarwal, 1998), *Bgl*I (Newman *et al*., 1998), *Pvu*II (Horton & Cheng, 2000), and *Ngo*MIV (Diebert *et al*., 2000). Although there are some differences in detail, these structures suggest that phosphoryl transfer proceeds by variations of the two-metal ion mechanism first proposed for the 3′-5′ exonuclease activity of *Escherichia coli* DNA polymerase I, and for alkaline phosphatase (Beese & Steitz, 1991; Kim & Wyckoff, 1991). In this mechanism one metal ion (perhaps together with a general base) likely generates the attacking hydroxide ion, while the other metal(s) function to: (i) compensate the incipient negative charge in the pentacovalent transition state; (ii) stabilize the leaving 3′O anion; and (iii) stabilize the active site structure in part by properly orienting the required enzyme carboxylate groups. In addition to their direct role in catalysis, divalent metal ions also enhance both the DNA binding affinity and/or sequence specificity

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0022-2836/01/040851–11 $35.00/0

of *Eco*RV, *Pvu*II, *Mun*I and other enzymes (Vipond & Halford, 1995; Martin *et al*., 1999a; Nastri *et al*., 1997; Lagunavicius *et al*., 1997). This is likely accomplished by shielding of the negatively charged DNA phosphates and conserved enzyme active-site carboxylate groups from each other.

*Eco*RV has emerged as the best-studied type II enzyme, and is the only example for which detailed kinetic analyses coupled to the structural studies have been performed. This enzyme cleaves the target site GATATC at the center TA-step, generating blunt ends with 5′-phosphate groups (Schildkraut *et al*., 1984). Strong functional evidence implicating at least two divalent metals in catalysis has been obtained from stopped-flow fluorescence studies and from metal-reconstitution experiments (Vipond *et al*., 1995; Baldwin *et al*., 1995). In agreement with these findings, crystal structures of the enzyme bound to specific DNA reveal three distinct divalent metal-binding sites near to the scissile and 3′-adjacent phosphates. The pH-rate profile for the Mg^{2+}-dependent bond-making and bond-breaking steps in phosphoryl transfer shows a sharp bell-shaped curve with an optimum at pH 8.5, indicating general base catalysis for the nucleophilic attack of hydroxide ion on the scissile phosphate, and general acid catalysis for protonation of the leaving 3′O anion (Sam & Perona, 1999a). Together, these kinetic and structural data are consistent with a mechanism in which two distinct metal-ligated water molecules are ionized to generate the attacking hydroxide ion nucleophile and the proton for donation to the leaving 3′-oxygen anion, respectively.

In *Eco*RV each of the three cations Mg^{2+}, Mn^{2+} and Co^{2+} is capable of supporting high levels of activity (Vermote & Halford, 1992; Vipond *et al*., 1995), with Mg^{2+} preferred. However, the substitution of magnesium ions with manganese gives rise to several phenomena of interest. First, the DNA sequence selectivity of *Eco*RV is markedly decreased in the presence of Mn^{2+}, mirroring earlier observations made with *Eco*RI (Hsu & Berg, 1978). In *Eco*RV the preference for GATATC compared with the non-cognate GTTATC site on plasmid substrates is decreased 10^4-fold by Mn^{2+} (Vermote & Halford, 1992). Second, deleterious effects on Mg^{2+}-dependent rates caused both by enzyme mutations in the DNA binding cleft and by DNA backbone analogs can be reconstituted to varying degrees by Mn^{2+} (Jeltsch *et al*., 1995; Vipond *et al*., 1996; C. Otey & J.J.P., unpublished data). Third, while steady-state rates of the wild-type enzyme at cognate sites are slower with Mn^{2+} (Vermote & Halford, 1992), the rate of the chemical step in catalysis is faster with this metal ion (Sam & Perona, 1999b; Baldwin *et al*., 1999). Pre-steady-state measurements on short oligodeoxynucleotide substrates reveals a steep burst for Mn^{2+}-dependent reactions, indicating that the physical step of product release is rate limiting and accounting for the slower overall rate with this cofactor. This has been attributed to a higher intrinsic affinity of Mn^{2+} for phosphates (Hogfeldt, 1979), consistent with a crystal structure of the *Eco*RV-product DNA complex revealing two metals bound to the scissile phosphate (Kostrewa & Winkler, 1995; Sam & Perona, 1999b).

Crystal structures of ternary complexes of *Eco*RV bound to DNA and divalent metal ions show that Mg^{2+}, Mn^{2+} and Ca^{2+} bind in the active site, adjacent to important carboxylate groups at Asp90, Asp74 and Glu45 (Selent *et al*., 1992; Kostrewa & Winkler, 1995; Perona & Martin, 1997; Horton *et al*., 1998, 2000). However, we have recently observed that Mn^{2+} also bind at several distal sites (Horton *et al*., 2000; N.C.H. & J.J.P., unpublished results). In each subunit of *Eco*RV bound to a deoxyribo-3′-*S*-phosphorothiolate DNA analog, a manganese ion bridges a DNA phosphate outside the GATATC target site with the imidazole side-chain of His71 (Horton *et al*., 2000; Figure 1). To explore the possible functional roles of this distal site, we mutated the His71 residue to glutamine and to alanine, purified the recombinant mutant enzymes, and measured single-turnover and steady-state reaction rates toward cognate and non-cognate duplexes in the presence of either Mg^{2+} or Mn^{2+}. We find that removal of the distal Mn^{2+} site generates enzymes with significantly improved activities for steady-state cleavage of cognate DNA duplexes. Further, the mutants also exhibit enhanced discrimination against the non-cognate GTTATC site under Mn^{2+}-conditions. Models for the structural origins of rate enhancement and the generation of sequence selectivity by *Eco*RV are considered in light of these data. Significant differences in the conformations of catalytically active complexes of *Eco*RV with cognate *versus* non-cognate DNA are suggested.

Results and Discussion

*Eco*RV endonuclease has been previously cocrystallized with a deoxyribo-3′-S-phosphorothiolate DNA analog and manganese ions, and the structure of this ternary complex determined to 1.9 Å resolution (Horton *et al*., 2000). The undecamer duplex DNA contains sulfur in place of the 3′-bridging oxygen atom at each of the two scissile phosphates (3′S DNA), and is fully refractory to cleavage regardless of the identity of the divalent metal cofactor present in the reaction. Unlike structures solved in the presence of Mg^{2+} or Ca^{2+} (also determined at 2.0 Å resolution or better), in the *Eco*RV/3′S DNA/Mn^{2+} complex divalent metal ions were identified at the protein-DNA interface outside of the GATATC sequence, as well as in the active sites (Figure 1(a)). These distal sites are consistently observed in complexes of wild-type and mutant *Eco*RV enzymes cocrystallized with specific DNA and Mn^{2+} ions (N.C.H. & J.J.P., unpublished results).

The distal Mn^{2+} are bound adjacent to the minor groove, between the imidazole ring of His71 in the enzyme Q-loop (Winkler *et al*., 1993) and a DNA

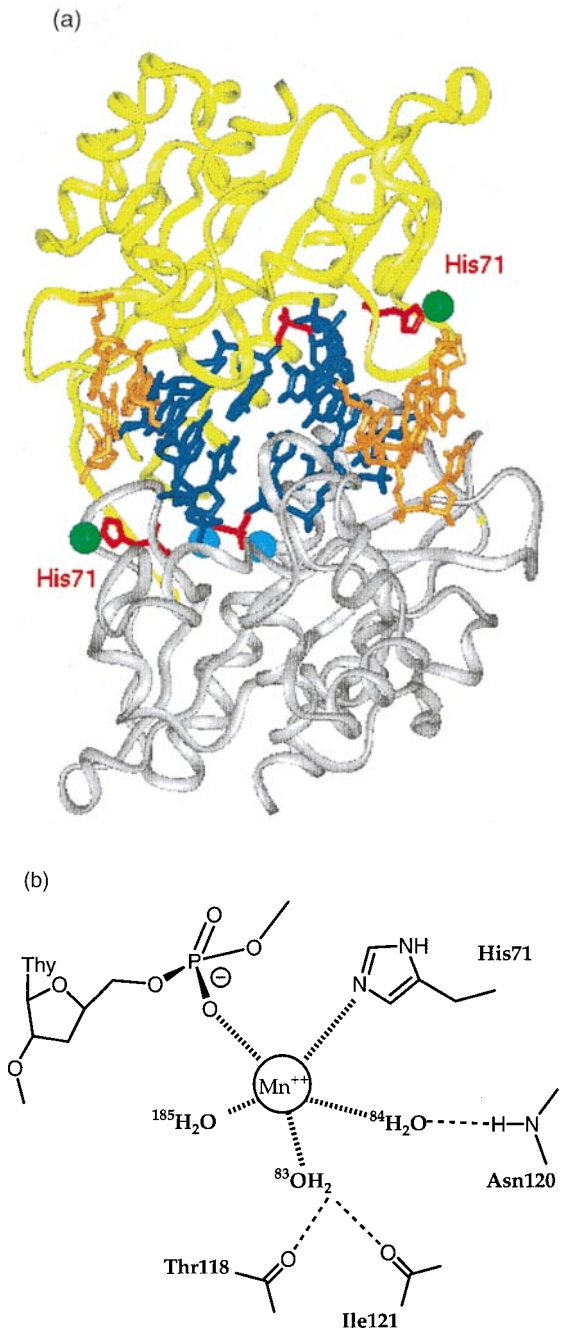

Figure 1. (a) Crystal structure of *Eco*RV bound to a duplex deoxyribo-3′S-phosphorothiolate DNA analog and Mn^{2+} (Horton *et al.*, 2000). The protein backbone is shown in a ribbon representation with the two monomers colored in yellow and gray. The specific GATATC site is colored dark blue with the flanking base-pairs of the DNA in orange. Mn^{2+} binding sites at the DNA flanks (the subject of this study) are shown as green spheres. Light blue spheres show two Mn^{2+} bound in one of the active sites adjacent to the scissile phosphate group (red). The side-chains of His71 in each subunit are also shown in red. (b) Ligand sphere of the manganese ion as visualized in subunit B of *Eco*RV bound to phosphorothiolate-modified DNA. The Mn^{2+} is bound to the pro-S oxygen atom of the phosphate at GATATCT*p*T. Hatched lines represent inner-sphere metal-ligand contacts ranging from 1.9 Å to 2.4 Å. Broken lines indicate hydrogen bonds. In subunit A of this structure, the DNA phosphate shown is not visible in electron density maps and is presumed disordered, but Mn^{2+} is nevertheless bound to His71 and water molecules.

phosphate group located 3′ to the cleavage site at 5′-AAAGATATCT*p*T-3′ (Figure 1(a)). They are easily distinguished from water molecules owing to their much higher electron density and short ligand distances between 1.9 Å and 2.4 Å, and refine to *B*-factors of 42 $Å^2$ and 32 $Å^2$ in subunits A and B, respectively (Horton *et al.*, 2000). In this and other structures, the asymmetric unit is the enzyme dimer bound to duplex DNA, allowing for differences in the manganese ion ligand sphere between the two subunits. In subunit B, Mn^{2+} is ligated directly to both the ε-nitrogen atom of His71 and a nonesterified phosphate oxygen atom (Figure 1(b)). In subunit A this flanking DNA phosphate is disordered and not visible in electron density maps; but Mn^{2+} is still bound to His71 and water molecules (Horton *et al.*, 2000). Other crystal structures of *Eco*RV active-site mutants bound to DNA and Mn^{2+} show ligation to both His71 and the DNA phosphate in both subunits. There is also one example (*Eco*RV mutant K38A) where in one subunit the Mn^{2+} cation interacts with the equivalent phosphate oxygen through a water molecule instead of directly (N.C.H. & J.J.P., unpublished results).

Effects of the distal Mn^{2+} site on cognate site cleavage

Functional roles of the distal Mn^{2+}-binding sites were examined by site-directed mutagenesis. His71 is the only protein or DNA group consistently observed to directly ligate the manganese ion, and several considerations suggest that its substitution with other amino acids is very likely to eliminate the binding site. No occupancy by either Mg^{2+} or Ca^{2+} cations is observed at the distal site, suggesting that the polarizable interaction of the histidine imidazole with a transition metal d-orbital shell is necessary to promote binding. Further, it is important to note that manganese binds at this site even when the flanking DNA phosphate is disordered, as observed in subunit A of *Eco*RV bound to 3′S DNA and Mn^{2+} (Horton *et al.*, 2000; see the legend to Figure 1(b)). The *Eco*RV K38A/DNA/Mn^{2+} structure provides another example where the only direct contact that the metal makes to either the protein or DNA is with His71 (N.C.H. & J.J.P., unpublished results). In both of these cases, all of the other inner-sphere positions are occupied by water molecules. Together, these data indicate that His71 alone is sufficient to promote binding. In the absence of the imidazole, there can then be no energetic advantage to rearranging the optimal

octahedral geometry of the manganese water sphere that is present in solution; thus, Mn^{2+} cannot bind to the mutant complexes at the same site observed in the wild-type.

The H71A mutant tests the consequences of removing all functional groups which might ligate Mn^{2+}, while H71Q was chosen to test the specific importance of the imidazole ring. The flexible glutamine side-chain can adopt multiple conformations in place of a histidine residue. If the NH_2 group of the amide is positioned equivalently to the imidazole N^{ε} atom, then a similar Mn^{2+} ligation is unlikely because this moiety is disfavored to occupy an inner-sphere position (Glusker, 1991). However, the amide oxygen atom is, in principle, capable of ligating to Mn^{2+} in place of the imidazole nitrogen atom. Nonetheless, we find that cleavage rates of *Eco*RV H71A and *Eco*RV H71Q are very similar to each other and distinct from the wild-type enzyme (see Tables 1 and 2). This demonstrates that it is the specific histidine-metal ion interactions bridging to flanking DNA which are crucial to conferring Mn^{2+}-dependent cleavage properties.

Cleavage of the cognate 16-mer duplex DNA substrate 5′-GGGAAAGATATCTTCC-3′ was monitored under both multiple-turnover and single-turnover conditions, in the presence of either Mg^{2+} or Mn^{2+} cofactors (Table 1 and Figure 2). Steady-state reactions carried out under conditions of substrate excess were analyzed by the Michaelis-Menten equation using Eadie-Hofstee plots, to derive values of k_{cat} and K_m. These reactions were carried out under conditions of saturating Mg^{2+} (10 mM) and saturating Mn^{2+} (2.5 mM; Sam & Perona, 1999a,b), at constant ionic strength. Reaction velocities of the mutants at selected DNA concentrations were monitored at higher concentrations of divalent metal ions to verify saturation. To maintain multiple turnover conditions ($[E] \ll [S]$) and practicable rates for these cognate-site reactions possessing low K_m values, most substrate concentrations were chosen above the K_m. However, correlation coefficients of the Eadie-Hofstee plots were maintained in the range 0.97 to 0.99 with good reproducibility (Table 1). There were no significant differences in the derived k_{cat} and K_m values for cleavage of the two different DNA strands, as monitored by the appearance of distinct 9-mer and 7-mer products (Figure 2). Identical rates for cleavage of the two strands were also found under single-turnover conditions with enzyme in molar excess over DNA, at saturating DNA and metal ion concentrations.

Analysis of H71A and H71Q cognate-site reactions in the presence of Mg^{2+} shows that this amino acid side-chain is not crucial to mediating the formation of the specific complex. The H71A mutant is reduced by only twofold in k_{chem} (the rate of the chemical step in the catalytic pathway) and by fivefold in k_{cat}/K_m, with the latter effect arising entirely at the level of K_m (Table 1). H71Q is reduced slightly more, implying some structural interference at the protein-DNA interface by the differently shaped glutamine side-chain. These data are consistent with a random mutagenesis study suggesting that single mutations at His71 produce enzymes with sufficient activity to maintain cell lethality, when expressed *in vivo* in the absence of *Eco*RV methylase (Vipond & Halford, 1996). The high levels of activity strongly suggest that neither mutation perturbs the structure of the protein-DNA interface sufficiently to disrupt formation of the required metal-binding sites directly in the vicinity of the scissile phosphates.

Under these steady-state experimental conditions, comparison of k_{cat}/K_m values shows that wild-type *Eco*RV cleaves cognate duplexes 15-fold faster when utilizing Mg^{2+} as the divalent metal ion cofactor, as compared with Mn^{2+} (Table 1). This is consistent with results reported for steady-state reactions toward shorter 12-mer substrates (Baldwin *et al*., 1999), and shows a smaller discrepancy than found with plasmids (where Mg^{2+} is preferred by 75-fold over Mn^{2+}; Vermote & Halford, 1992). However, the H71A and H71Q mutants instead prefer Mn^{2+} over Mg^{2+} by threefold and sixfold, respectively (Table 1). Strikingly, under Mn^{2+} conditions both mutant enzymes are improved by approximately tenfold in k_{cat}/K_m compared to the wild-type, with the effects expressed primarily at the level of K_m. Thus, removal of the distal manganese ion binding site shifts the metal cofactor preference of *Eco*RV by 45 to 90-fold in favor of Mn^{2+} relative to Mg^{2+}, such that Mn^{2+}-dependent catalysis by His71 mutants proceeds as efficiently as Mg^{2+}-dependent reactions of the wild-type enzyme (Table 1). These data show that occupancy of the distal Mn^{2+} site inhibits cleavage by *Eco*RV.

Table 1. Kinetic analysis of wild-type and mutant *Eco*RV enzymes toward cognate DNA sites

Enzyme	Metal ion	k_{chem} (sec^{-1})	k_{cat} (sec^{-1})	K_m (M)	k_{cat}/K_m ($sec^{-1}\ M^{-1}$)
WT	Mg^{2+}	0.6 ± 0.06[a]	0.14 ± 0.01	$9.8(\pm1.8) \times 10^{-9}$	$1.4(\pm0.2) \times 10^{7}$
	Mn^{2+}	4.1 ± 0.2[a]	0.033 ± 0.002	$3.5(\pm0.2) \times 10^{-8}$	$9.5(\pm1.3) \times 10^{5}$
H71A	Mg^{2+}	0.36 ± 0.08	0.14 ± 0.01	$4.6(\pm1.0) \times 10^{-8}$	$3.0(\pm0.8) \times 10^{6}$
	Mn^{2+}	3.4 ± 0.07	0.023 ± 0.001	$2.6(\pm0.1) \times 10^{-9}$	$8.9(\pm0.1) \times 10^{6}$
H71Q	Mg^{2+}	0.07 ± 0.002	0.063 ± 0.17	$3.6(\pm0.3) \times 10^{-8}$	$1.8(\pm0.4) \times 10^{6}$
	Mn^{2+}	2.4 ± 0.49	0.021 ± 0.001	$2.0(\pm0.4) \times 10^{-9}$	$1.1(\pm0.3) \times 10^{7}$

[a] These data were taken from Sam & Perona (1999b).

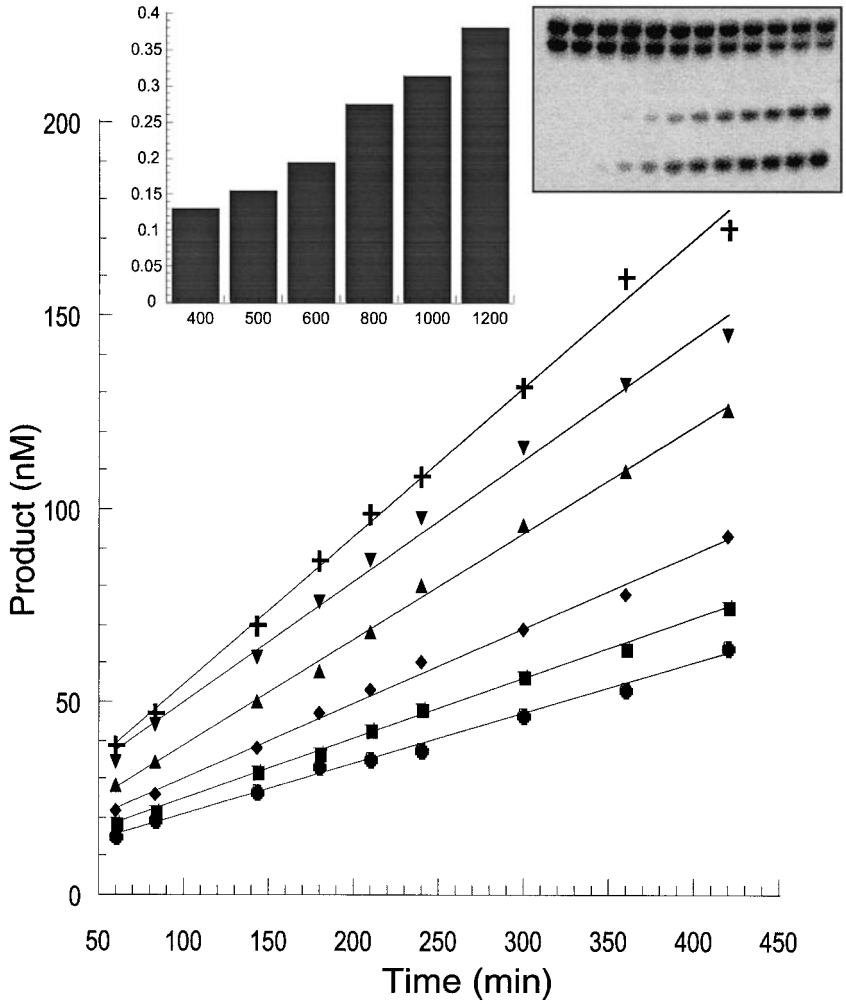

Figure 2. Steady-state kinetic analysis showing cleavage of the non-cognate GTTATC site by *Eco*RV H71A (20 nM) in the presence of Mn^{2+}. The GTTATC sequence is positioned off-center on the 16-mer substrate, so that 9-mer and 7-mer labelled products are formed (upper right). The two labelled substrate strands at the top of the denaturing gel are separated because one is purine-rich and the other pyrimidine-rich. The plots at bottom give time courses for product formation, with DNA concentrations corresponding to: ✷, 400 nM; ■, 500 nM; ◆, 600 nM; ▲, 800 nM; ▼, 1000 nM; †, 1200 nM. The gel shows the time-course for the reaction carried out with 500 nM DNA. Only k_{cat}/K_m can be determined because K_m is at least five- to tenfold above the highest DNA concentration used. This can be seen from doubling in the rate of product formation as the substrate concentration also doubles (bar graph at upper left). The bar graph plots nmol product/minute *versus* DNA concentration in nanomolar, on the ordinate and abscissa respectively.

To directly monitor the rate of the chemical step in catalysis, DNA cleavage was also measured under single-turnover conditions. Each mutant is nearly unaffected in the rate of phosphoryl transfer (k_{chem}), consistent with the notion that the active site structures are precisely maintained (Table 1). The properties of the mutants can be interpreted in the context of the following reaction scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [E \cdot S] \xrightarrow{k_{chem}} E \cdot P \xrightarrow{k_3} E + P$$

Table 2. Kinetic analysis of wild-type and mutant *Eco*RV enzymes toward the non-cognate GTTATC site

Enzyme	Metal ion	k_{chem} (sec^{-1})	k_{cat} (sec^{-1})	K_m (M)	k_{cat}/K_m (sec^{-1} M^{-1})	Specificity[d]
WT	Mg^{2+}	$<5 \times 10^{-7}$ [a]	-[b]	-	-	
	Mn^{2+}	$2.1(\pm0.36) \times 10^{-3}$	0.006 ± 0.003	$4.0(\pm1.4) \times 10^{-7}$	$1.6(\pm0.2) \times 10^{4}$	60
H71A	Mg^{2+}	$<5 \times 10^{-7}$ [a]	-[b]	-	-	
(9-mer)	Mn^{2+}	$6.5(\pm1.3) \times 10^{-4}$	-[c]	-	$8.1(\pm0.5) \times 10^{2}$	11,000
(7-mer)	Mn^{2+}	$1.3(\pm0.3) \times 10^{-3}$	-[c]	-	$1.3(\pm0.1) \times 10^{3}$	6800
H71Q	Mg^{2+}	$<5 \times 10^{-7}$ [a]	-[b]	-	-	
(9-mer)	Mn^{2+}	$3.0(\pm0.3) \times 10^{-5}$	-[c]	-	$2.7(\pm0.1) \times 10^{2}$	41,000
(7-mer)	Mn^{2+}	$7.0(\pm0.3) \times 10^{-5}$	-[c]	-	$9.1(\pm0.3) \times 10^{2}$	12,000

[a] Estimated from the extent of cleavage after incubation for 120 hours at 37 °C, after which the enzyme begins to lose activity under these reaction conditions (Martin *et al.*, 1999b).

[b] Reaction rates under multiple-turnover conditions are too slow to estimate Michaelis-Menten parameters.

[c] Only the second-order rate constant k_{cat}/K_m could be accurately determined for these substrates, because no saturation was observed at the highest DNA concentrations practicable.

[d] Specificity indicates the ratio $(k_{cat}/K_m)_{GATATC}/(k_{cat}/K_m)_{GTTATC}$ for Mn^{2+}-dependent reactions.

Depending upon whether chemistry or product release is rate limiting, K_m will reflect either the binding step (k_{chem} rate limiting) or a combination of the rate constants for binding and catalysis (if k_3 is at least partly rate-limiting). Although the rate limiting step is unknown for the mutants, the observation that k_{chem} values are maintained shows that the lowered K_m values must arise from improvement in the initial binding step. Thus, the manganese ion bound at the distal site exerts its inhibitory effect by destabilizing the wild-type *Eco*RV-DNA complex prior to catalysis.

Pre-steady-state measurements for Mn^{2+}-dependent cleavage by wild-type *Eco*RV show a sharp burst of product formation followed by a 100-fold slower steady-state rate, indicating that product release (k_3) is strongly rate limiting in this case (Sam & Perona, 1999b). This is in contrast to Mg^{2+}-dependent reactions, which show only a very small burst. Mn^{2+} has a higher intrinsic affinity for phosphates than does Mg^{2+} (Hogfeldt, 1979), and it was suggested previously that two Mn^{2+} ions ligated to the scissile DNA phosphate after cleavage cause the decreased DNA dissociation rate (Sam & Perona, 1999b). However, we speculate that slow product off-rates in wild-type reactions might also arise from occupancy of the additional distal sites by Mn^{2+}. If this is the case, the distal metal ion would have opposite effects on the enzyme-substrate and enzyme-product complexes, destabilizing the former (as deduced from the K_m effect on steady-state rates; Table 1) while stabilizing the latter.

Some previously characterized mutants of *Eco*RV also exhibit a shifted metal ion selectivity towards Mn^{2+}. In each of these cases the Mg^{2+}-dependent cleavage rates are severely decreased (Vermote *et al.*, 1992; Selent *et al.*, 1992; Vipond *et al.*, 1996; Groll *et al.*, 1997; C. Otey & J.J.P., unpublished results). The very low Mg^{2+}-dependent activities show that Mn^{2+} reconstitute function in these cases primarily by virtue of their ability to facilitate precise juxtaposition of required active-site moieties. That is, Mn^{2+} has a decreased requirement for precise inner-sphere ligand geometry as compared to Mg^{2+}, such that some variation in the relative positions of the scissile and 3′-adjacent DNA phosphate groups, and the active-site enzyme carboxylate groups, is allowed. By contrast, in H71A and H71Q the high Mg^{2+}-dependent cleavage rates show that the mutations do not significantly perturb this relative positioning. Thus, in the mutants the Mn^{2+} effect originates mainly with the removal of the distal site. The uncovering of the distal site now raises the possibility that its destabilization in some of the mutants may be partly responsible for the improved Mn^{2+}-dependent cleavages, although the large magnitudes of many of the reconstitutions do show that direct active-site binding must play the major role. *Eco*RV H71A and H71Q share with the mutant I91L (Vipond *et al.*, 1996) the property that their Mn^{2+} reactions proceed as efficiently as does the wild-type enzyme with Mg^{2+}. If the I91L mutant possesses an intact distal manganese site, the possibility exists that an I91L/H71A or I91L/H71Q double-mutant might be a significantly improved catalyst as compared to even the wild-type/Mg^{2+}-dependent enzyme.

Manganese ions also provide 50-fold reconstitution of *Eco*RV cleavage of DNA containing a phosphorothioate substitution at a different phosphate (p3) inside the target site, at G*p*ATATC (Jeltsch *et al.*, 1995). The manganese rescue was interpreted to indicate the existence of a Mg^{2+} binding site at this position. However, these data should be considered with caution in view of the fact that Mn^{2+} reconstitutes structural lesions caused by protein mutations located throughout the structure. The general explanations provided above, including possible destabilization of the distal Mn^{2+} site at GATATCT*p*T, could well hold in the case of the phosphorothioate-modified DNA. It should also be noted that metal ion binding at phosphate p3 has not been observed in any crystal structure, and that there are no negatively charged carboxylate groups near this phosphate to help form the proposed Mg^{2+} site. Phosphorothiolate substitution of the pro-S oxygen at the position of the distal site yields a substrate that is cleaved threefold better with a manganese cofactor as compared to magnesium (Jeltsch *et al.*, 1995).

Cleavage at a non-cognate DNA site by *Eco*RV H71A and H71Q

Manganese ions are known to relax the specificity of restriction enzymes, such that cleavages at non-cognate sites differing by one base-pair from the cognate sequence become more favorable (Roberts & Halford, 1993). This phenomenon has been investigated most closely for *Eco*RI (Hsu & Berg, 1978) and for *Eco*RV (Vermote & Halford, 1992; Vermote *et al.*, 1992). In the latter case the discrimination against the most-preferred GTTATC non-cognate site on a plasmid substrate is 3×10^5-fold for the Mg^{2+} cofactor-dependent reaction, but only sixfold for reactions carried out with the Mn^{2+} cofactor. This includes a remarkable 700-fold increase in k_{cat}/K_m for cleavage of the non-cognate site.

The role of the distal Mn^{2+} site in conferring these properties was examined by measuring cleavage rates of the H71A and H71Q mutants toward oligonucleotide substrates containing the non-cognate site GTTATC (Table 2). Cleavage rates in the presence of Mg^{2+} as cofactor are extremely slow for both wild-type and mutant enzymes, with single-turnover rate constants decreased by at least 10^6-fold compared to the cognate site. These rates are at least tenfold slower than previously reported (Engler *et al.*, 1997). The discrepancy can be accounted for in terms of the flanking sequences adjacent to the target site, as it is known that AT pairs immediately 5′ and 3′ to the GTTATC non-cognate site are disfavored for cleavage compared

with the GC pairs present in the earlier study (Taylor & Halford, 1989, 1992).

*Eco*RV cleaves GTTATC in the presence of Mn^{2+} with an efficiency 60-fold less than the Mn^{2+}-dependent hydrolysis of the cognate site, as assessed by k_{cat}/K_m (Tables 1 and 2). This is tenfold better Mn^{2+}-dependent discrimination than is observed toward plasmid substrates (Vermote & Halford, 1992). The rate of the chemical step is reduced by 2000-fold. In contrast to their effect in improving hydrolysis of the cognate site, the H71A and H71Q mutants are instead impaired in their ability to cleave GTTATC (Table 2). Unlike the wild-type enzyme, the mutants also show some differences in cleavage rates for the two strands of this asymmetric substrate: the 7-mer ''bottom'' strand is hydrolyzed two- to threefold faster than its complement. Considering this variation, at the level of k_{cat}/K_m H71Q shows 200 to 700-fold better discrimination of GATATC relative to GTTATC under Mn^{2+} conditions. For H71A the improved discrimination compared with wild-type *Eco*RV is 100 to 200-fold (Table 2). While these k_{cat}/K_m effects are similar in H71A and H71Q, the origins of the decreased Mn^{2+}-dependent catalytic efficiency toward GTTATC appear to differ in the two mutants. H71A shows only a two- to threefold decrease in k_{chem} compared to wild-type *Eco*RV, while H71Q is decreased instead by 30 to 70-fold (Table 2).

The abilities of several other *Eco*RV mutants to discriminate among DNA sequences in the presence of Mn^{2+} as cofactor have also been characterized. The mutants N185A and N188A in the major-groove binding recognition loop each have substantially decreased Mn^{2+}-dependent activities at the GTTATC non-cognate site, with the discrimination factor toward plasmid substrates improved by 40 and 400-fold, respectively (Vermote *et al*., 1992). Similarly, the active-site I91L mutant does not show the Mn^{2+}-dependent cleavages at GTTATC sites which are observed with the wild-type enzyme (Vipond *et al*., 1996). However, *Eco*RV H71A and H71Q differ qualitatively from the other mutants because their Mg^{2+}-dependent activities at cognate sites are not significantly decreased (Table 1). Further, the mechanism by which the selectivity is improved likely also differs. The properties of the H71A and H71Q mutants show directly that the distal manganese ion-binding site is crucial to conferring high rates of non-cognate site cleavage. By contrast, the decreased activities of the other mutants might be due to either destabilization of the distal site, a rearrangement of structure/metal binding in the active site, or some combination of the two. Future work combining mutations at the various positions might prove helpful in distinguishing these possibilities and perhaps in generating mutants with even better Mn^{2+}-dependent specificities.

The 20 to 60-fold decrease in k_{cat}/K_m for non-cognate site cleavage caused by the H71A mutation does not primarily arise from a decrease in the chemical step (Table 2). Thus, it appears that the distal manganese ion must either weaken binding by destabilizing the substrate complex, and/or decrease the rate of product release by stabilizing the product complex. Interestingly, these effects on the physical steps associated with cleavage of a non-cognate site are opposite to those for cleavage of the cognate site. The distinct influence of a distally bound metal ion on cognate *versus* non-cognate cleavage is consistent with previous findings which show that the conformations of these two complexes differ (Engler *et al*., 1997). In that study, it was shown that both the salt and pH-dependencies of binding vary significantly among specific, fully non-specific (uncleavable) and non-cognate (cleavable at slow rates) sites, which likely indicates that the protein-DNA conformations differ among these three types of complexes. X-ray structures also show tertiary and quaternary structure differences between the specific and non-specific DNA complexes (Winkler *et al*., 1993). These studies provide a basis for rationalizing how the distal manganese ion site can affect the conformations of the *Eco*RV non-cognate and cognate complexes in different ways. The findings reported here are also consistent with the idea of structural adaptability as a strategy for generating specificity (Jen-Jacobson, 1995, 1997). In this mechanism, the different conformation formed at the non-cognate site arises as a consequence of optimizing binding interactions, but the catalytic rate is then slowed because the resulting ground-state complex is off the pathway to the transition state. The manganese-dependent cleavage step at GTTATC is indeed substantially decreased for both wild-type and mutant enzymes (Table 2).

Additional evidence for distinct conformations of the cognate *versus* non-cognate *Eco*RV-DNA complexes is provided by the behavior of the H71Q mutant, which is substantially decreased in k_{chem} for Mn^{2+}-dependent non-cognate but not cognate reactions. In the non-cognate complex, the large decrease in k_{chem} shows that the alteration of structure in this region of the enzyme-DNA interface can influence events in the active sites some 20 Å distant. Because H71/Mn^{2+} contacts a flanking DNA phosphate rather than a base-specific functional group, this data suggests that indirect readout may play an important role in sequence discrimination against the non-cognate GTTATC site, as has been also shown for discrimination against different base-pairs placed at the center TA-step of GATATC (Martin *et al*., 1999b). A likely mechanism to explain the lowered k_{chem} is that the substitution of His71 with Gln alters the enzyme/DNA conformation inside the GTTATC site, such that manganese ions binding at the scissile phosphate groups are no longer properly juxtaposed for efficient catalysis (Table 2). Crystal structures of the *Eco*RV-DNA complex in different crystal lattices show that the minor-groove binding Q-loop (where His71 is situated) contacts an enzyme linker sequence (linker II) from the opposing subunit of

the dimer, by means of a contact between Gln69 and Thr37 (Winkler *et al.*, 1993; Kostrewa & Winkler, 1995; Perona & Martin, 1997; Horton & Perona, 2000; Figure 3). By analysis of a series of structures trapped in different lattices, it was shown that the burial of increased surface area between the *Eco*RV linker II/Q-loop segments and the DNA minor groove is correlated with increasing DNA bending angle (Horton & Perona, 2000). This correlated motion is then crucial to assembling the active sites, including a metal-binding site located between Asp74 (near the Q-loop) and Glu45 (on a crucial α-helix directly following linker II). Although a crystal structure of the enzyme bound to GTTATC is not yet available, it seems likely that the same enzyme motifs will be critical to cleavage here as well (although they may be somewhat rearranged compared with the cognate complex). We suggest then that the His71 → Gln71 substitution decreases cleavage rates at GTTATC by interfering with the required conformational adaptations.

The finding of functional importance for the distal Mn^{2+} sites in *Eco*RV also bears on two related questions. First, efforts are in progress to engineer an expansion of *Eco*RV specificity to include also one or two base-pairs flanking the target site (Wenz *et al.*, 1998; Horton & Perona, 1998; Schottler *et al.*, 1998; Lanio *et al.*, 1998, 2000). These experiments have so far acheived only modest success, although several mutants at a position near the DNA major groove do show some narrowed preference for certain bases directly adjacent to GATATC (Schottler *et al.*, 1998; Wenz *et al.*, 2000). Since the new Mn^{2+}-binding sites are in the flanking regions, it seems plausible that Mn^{2+}-dependent activities of mutants (so far not investigated) might show different specificity patterns for flanking base-pairs than found for Mg^{2+}. Inclusion of H71A or H71Q as part of a protein design strategy might also be of value. Second, some of the Mn^{2+}-dependent properties seen in *Eco*RV and other restriction enzymes also have been observed in enzymes carrying out different metal-dependent phosphoryl transfer reactions, including ribozymes, polymerases and ribonucleases (Dahm *et al.*, 1993; Pelletier *et al.*, 1996; Cirino *et al.*, 1995). Although detailed investigations of manganese ion binding sites at the structural level are largely lacking, particularly in the case of RNA enzymes it can be anticipated that many sites both proximal and distant from catalytic groups will be present. Structural similarities have also been recently found between type II restriction endonucleases and other DNA processing enzymes (Huai *et al.*, 2000). Thus, it appears that further investigation of metal-ion specific effects in these related other systems might provide substantial insight into both catalytic properties and evolutionary relationships.

Materials and Methods

Preparation of the H71A and H71Q mutants

Oligonucleotides were purchased from IDT (Coralville, Iowa) and enzymes were purchased from New England Biolabs except where noted. The H71A and H71Q mutants of *Eco*RV were constructed by site-directed mutagenesis using the QuickChange methodology (Stratagene), in which highly efficient selection for mutants derives from cleavage of methylated parental plasmid strands by *Dpn*I, leaving the newly synthesized and unmethylated mutant strands intact. The template for each mutagenesis consisted of a mixture of the expression plasmid *p*BSRV encoding the wild-type *Eco*RV gene together with an ampicillin resistance

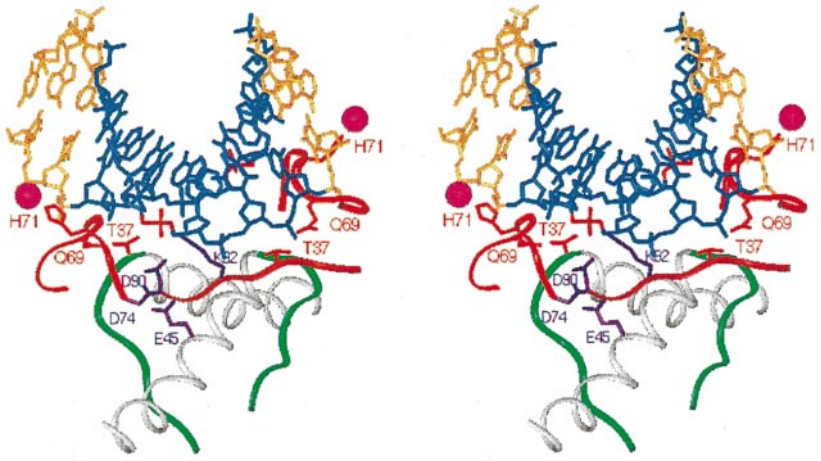

Figure 3. Divergent stereo view of a portion of the *Eco*RV structure, showing binding of protein segments in the widened minor groove of the DNA. The narrowed major groove is at the top. The distal Mn^{2+} are drawn as filled spheres in magenta. Proposed structural connectivity to the scissile phosphates (red) is shown (see the text for details). Only one active site with the important amino acid side-chains in purple is depicted. The key Gln69-Thr37 intersubunit contacts in the minor groove are shown in red. The GATATC target site is in dark blue with flanking DNA base-pairs in orange. The linker II segments from each subunit are drawn in green and the B-helices are drawn in gray (Winkler *et al.*, 1993; Horton & Perona, 2000).

marker (Vipond & Halford, 1996), and the *p*MetB plasmid encoding the *Eco*RV methyltransferase and kanamycin resistance genes (Vermote *et al*., 1992). Plasmid DNA was isolated from transformants and the sequences of both the H71A and H71Q genes verified in their entirety.

Purification of enzymes and DNA substrates

Wild-type, H71A and H71Q *Eco*RV enzymes were expressed in *E. coli* strain MM294 (*endI*$^-$, *pro*$^-$, *thi*$^-$, $r_k^- m_k^+$), purified to homogeneity by a two-column procedure, and stored as described (Perona & Martin, 1997; Martin *et al*., 1999b). Substrate DNAs comprised the wild-type non-self-complementary 16-mer: GGGAAAGATATCTTGG and the non-cognate GGGAAAGTTATCTTGG. These oligonucleotides and their complements were purified by HPLC and 5′-end labelled as described (Sam & Perona, 1999a; Martin *et al*. 1999b). The [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham.

Single-turnover cleavage assays

Single-turnover reactions in the presence of Mn^{2+} or Mg^{2+} were carried out at 37 °C in an assay buffer containing 50 mM Hepes (pH 7.5), 200 mg/ml bovine serum albumin and 1 mM DTT. Saturating concentrations of metal ion were used in all experiments (10 mM $MgCl_2$, 2.5 mM $MnCl_2$), and the ionic strength was adjusted with NaCl to a constant value of 140 mM (Sam & Perona, 1999b). For Mg^{2+} and Mn^{2+} reactions of wild-type and mutant enzymes with cognate DNA substrates, the reactions were carried out in a rapid-quench instrument (Kintek RQF-3). The enzyme and DNA were kept in separate syringes and divalent metal ions were present in each syringe, ensuring the fastest cleavage rates (Martin *et al*., 1999b). The concentrations of enzyme and DNA in these reactions is 50 nM DNA, 200 nM enzyme. Reactions carried out with the non-cognate GTTATC-containing substrate were sampled by hand, using 150 nM DNA and 450 nM enzyme (all other conditions identical). Saturation of both DNA and metal ion binding was verified for all combinations of enzyme mutant, DNA site, and metal ions. Ten time points were taken for all reactions. Reactions were quenched with a solution containing 4 M urea and 75 mM EDTA. Aliquots (10 µl) from each timepoint were mixed with an additional 8-10 µl of quench solution containing bromphenol blue dye, and separated on 8 M urea/20 % (w/v) polyacrylamide gels, followed by visualization *via* autoradiography on the Molecular Dynamics Storm840 PhosphorImager. Rate constants were determined by fitting the data to a first order exponential. Data were plotted using the program *Scientist*.

Steady-state kinetic assays

Steady-state kinetic timecourses with the cognate and non-cognate 16-mer oligonucleotide substrates were measured at DNA concentrations of 20 to 50-fold molar excess over enzyme. In different reactions oligonucleotide substrate concentrations were varied between 5 nM and 1.4 µM, and enzyme concentrations were in the range from 0.1 nM to 20 nM. Reactions were carried out at 10 mM $MgCl_2$ or 2.5 mM $MnCl_2$; increasing these concentrations for each separate enzyme/DNA combination produced no change in initial velocities, suggesting that metal-ion-binding sites are saturated at these conditions. The steady-state reactions were carried out at 37 °C in an assay buffer containing 0.2 mg/ml bovine serum albumin, 1 mM DTT, 50 mM BisTris Propane (pH 7.5). The ionic strength of all reactions was adjusted to 140 mM with NaCl. At least ten timepoints were taken for the determination of each initial velocity. Reaction aliquots were quenched in 4 M urea, 70 mM EDTA; labelled substrate and product DNAs were then separated on 20 % polyacrylamide urea gels.

Reaction velocities were quantified by phosphorimaging analysis. All kinetic parameters were determined from initial velocities at five substrate concentrations. The data were analyzed by Eadie-Hofstee plots for estimation of V_{max} and K_m. k_{cat} parameters were determined by normalizing V_{max} to the total enzyme concentration. For some reactions, the second-order parameter k_{cat}/K_m was determined directly by measurements at low substrate concentrations ($[S] \ll K_m$) using the relationship: $v = k_{cat}/K_m\,[E]_o[S]$ (Fersht, 1999).

Acknowledgments

We thank Jonathan Kohn for assistance with protein purification. This work was supported by NIH grant GM53763 and ACS-PRF grant 30427-G4 (to J.J.P.) and by American Cancer Society Postdoctoral Fellowship PF-98-015-GMC (to N.C.H.).

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Edited by K. Morikawa

(Received 17 October 2000; received in revised form 11 December 2000; accepted 26 December 2000)