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*Eco*RV Restriction Endonuclease: Communication between DNA Recognition and Catalysis†

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ABSTRACT: A genetic system was constructed for the mutagenesis of the *Eco*RV restriction endonuclease and for the overproduction of mutant proteins. The system was used to make two mutants of *Eco*RV, with Ala in place of either Asn185 or Asn188. In the crystal structure of the *Eco*RV–DNA complex, both Asn185 and Asn188 contact the DNA within the *Eco*RV recognition sequence. But neither mutation affected the ability of the protein to bind to DNA. In the absence of metal ion cofactors, the mutants bound DNA with almost the same affinity as that of the wild-type enzyme. In the presence of Mg^{2+}, both mutants retained the ability to cleave DNA specifically at the *Eco*RV recognition sequence, but their activities were severely depressed relative to that of the wild-type. In contrast, with Mn^{2+} as the cofactor, the mutant enzymes cleaved the *Eco*RV recognition site with activities that were close to that of the wild-type. When bound to DNA at the *Eco*RV recognition site, the mutant proteins bound Mn^{2+} ions readily, but they had much lower affinities for Mg^{2+} ions than the wild-type enzyme. This was the reason for their low activities with Mg^{2+} as the cofactor. The arrangement of the DNA recognition functions, at one location in the *Eco*RV restriction enzyme, are therefore responsible for organizing the catalytic functions at a separate location in the protein.

The *Eco*RV restriction endonuclease cleaves DNA at its recognition sequence, GATATC (Schildkraut et al., 1984; D'Arcy et al., 1985). The only cofactor it needs for DNA cleavage is a divalent metal ion, either Mg^{2+}, Mn^{2+}, or Co^{2+} [reviewed by Luke et al. (1987)]. Yet, in binding to DNA in the absence of metal ion cofactors, the *Eco*RV restriction enzyme shows no preference for its recognition site over other sites: all DNA sequences yield the same equilibrium constant (Taylor et al., 1991). With Mn^{2+} as the cofactor for DNA

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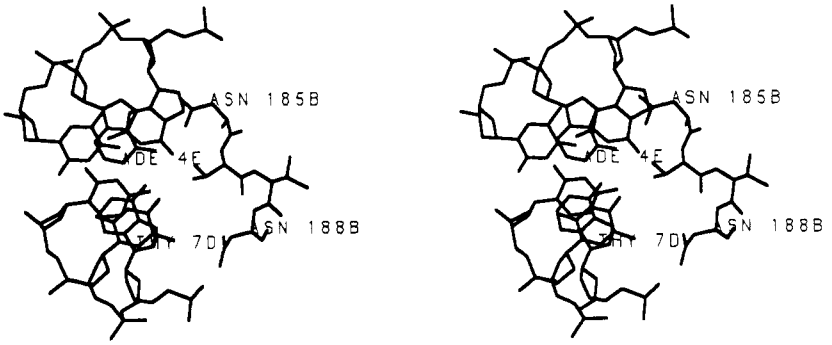

FIGURE 1: DNA recognition by *Eco*RV. The stereodiagram is from the 3-Å structure of the *Eco*RV restriction enzyme bound to duplex GGGATATCCC, using coordinates provided by F. K. Winkler. The section of the protein shown is Asn185–Thr186–Thr187–Asn188 from one subunit of the dimer. The section of duplex DNA is half of the recognition sequence: in the upper strand, 5′-pGpApT-3′ running from the back of the image to the front; in the lower, 5′-pApTpC-3′ from front to back. The 5′ phosphate in the lower strand is at the scissile bond. Both bases in the central A·T bp and both Asn residues are marked.

cleavage, the *Eco*RV endonuclease discriminates weakly between its recognition sequence and alternative DNA sequences: noncognate sites, which differ from the cognate site by 1 bp,[1] are cleaved almost as readily as the canonical site (Vermote & Halford, 1992). This is as expected for a protein that binds all DNA sequences equally well. However, with Mg^{2+}, the natural cofactor, the *Eco*RV endonuclease discriminates efficiently between its recognition sequence and other DNA sequences: noncognate sites are cleaved with at least a millionfold reduction in activity (Taylor & Halford, 1989). Hence, a protein that binds to DNA with essentially no sequence specificity can catalyze a reaction on DNA with an extremely high specificity for one particular sequence.

The gene for the *Eco*RV nuclease has been cloned, sequenced, and overexpressed (Bougueleret et al., 1984, 1985), and the protein has been crystallized (D'Arcy et al., 1985; Winkler et al., 1991). X-ray crystallography on *Eco*RV has yielded structures for not only the free protein but also the protein bound to the *Eco*RV recognition sequence and the protein bound to nonspecific DNA (Winkler, 1992; F. K. Winkler, personal communication).[2] The protein is a dimer of identical subunits. In both the specific and nonspecific complexes, 10 bp of DNA lie in a deep cleft between the two subunits. The phosphodiester backbone of the DNA is in contact with many different regions of the protein. But almost all of the contacts with the bases in the *Eco*RV recognition sequence stem from one segment of the polypeptide chain, the loop between Gly182 and Asn188.[3] In the specific complex, two of these loops, one from each subunit of the protein, are located in the major groove of the DNA (Figure 1). In the nonspecific complex, these loops are again located in the major groove but are positioned too far away from the bases for direct interactions (Winkler, 1992).

In this study, the relationship between DNA recognition and catalysis by the *Eco*RV restriction enzyme was examined by perturbing recognition functions in the protein. The recognition loop contains two asparagines (Figure 1), and each was converted by SDM to alanine. The mutant enzymes, N185A and N188A, were then characterized. Mutants of *Eco*RV in the 182–188 loop, including both N185A and N188A, have been reported previously (Thielking et al., 1991) but the examination of the mutant enzymes was incomplete: the activities of the mutants were noted as either ≈wt, <wt, ≪wt, or zero. This study provides a systematic analysis of both the catalytic and DNA-binding properties of the two mutants, with either Mg^{2+} or Mn^{2+} ions as the cofactor.

Experimental Procedures

DNA Manipulations. General manipulations of DNA were carried out as in Sambrook et al. (1989). SDM was by the procedure of Stanssens et al. (1989). DNA sequencing was by the dideoxy method (Sanger et al., 1977). Oligonucleotides were synthesized by A. Lenaerts (Plant Genetic Systems N.V., Gent) and by L. Hall (Department of Biochemistry, Centre for Molecular Recognition, University of Bristol). The Klenow fragment of DNA polymerase I (Joyce & Grindley, 1983), T4 DNA ligase (Murray et al., 1979), *Eco*RI, and *Eco*RV (Luke et al., 1987) were purified here. All other enzymes for DNA manipulation were purchased and used as advised by the supplier.

Plasmids. The plasmids, p*cI*857 (Kn^r, λ*cI*857), pLBM (Ap^r, *ecoRVM*), and pRV42p (Ap^r, *ecoRVR* from λ P_R) have been described previously (Remaut et al., 1983; Bougueleret et al., 1985; Botterman, 1986). New plasmids carrying *ecoRVM*, which were compatible with the Ap^r *ecoRVR* plasmids used here, were constructed by cloning the 1.3-kb *Eco*RI–*Hin*dIII fragment from pLBM (encompassing *ecoRVM*) at the single *Bst*EII site in p*cI*857: the termini were filled in with Klenow before blunt end ligation. Two recombinants, pMetA and pMetB, were recovered that differed only in the orientation of the 1.3-kb fragment: both were Kn^r, λ*cI*857, *ecoRVM*. A further plasmid, pCVKM (Kn^r, λ*cI*$^-$, *ecoRVM*), was constructed from pMetB by deleting a 550-bp *Hin*dII fragment from within λ*cI*857.

The SDM vectors, pMa5-8 (Ap^r Cm^s) and pMc5-8 (Ap^s Cm^r), were from Stanssens et al. (1989) as were the host strains used in conjunction with these vectors, *Escherichia coli* WK6 and WK6*mut*S and λ lysogens thereof; the lysogens were *cI*$^+$. Transformants of these strains with either pMetB or pCVKM were used for all manipulations of constructs containing *ecoRVR* expressed from λ P_R. When non-lysogens carrying pMetB were subsequently transformed with the *ecoRVR* plasmids, the strains were cultured at ≤30 °C; otherwise, overproduction of the *Eco*RV nuclease killed the cells. For procedures where the cells had to be grown at >30 °C, such as the production of single-stranded DNA (see below),

[1] Abbreviations: Ap, ampicillin (with superscripts r and s for resistance and sensitivity); bp, base pair(s); Cm, chloramphenicol; *ecoRVR* and *ecoRVM*, the genes for the *Eco*RV restriction and modification enzymes (Szybalski et al., 1988); Kn, kanamycin; kb, 1000 bp; K_D, equilibrium dissociation constant (M); K_{int}, equilibrium constant for binding to a single site on DNA (M^{-1}); M_r, relative molecular mass; SDM, site-directed mutagenesis; wt, wild-type.

[2] The coordinates for the structures of *Eco*RV and its complexes with DNA are available from Brookhaven (Ident Codes 1RVE, 2RVE, and 3RVE).

[3] The amino acid sequence of the *Eco*RV restriction enzyme is numbered from the DNA sequence of Bougueleret et al. (1984). The mature protein lacks the N-terminal methionine and starts at Ser2.

cI^+ lysogens carrying pCVKM were used. Both pMa5-8 and pMc5-8 have origins of replication from ColE1 and from phage f1. The helper phage used to recover single-stranded DNA was R408 (Russel et al., 1986). Additional vectors, pMa5-9 and pMc5-9, were constructed by substituting the 220-bp *Ava*II fragment from *amp* in both pMa5-8 and pMc5-8 with the equivalent fragment from pUC19 (Yannisch-Perron et al., 1985). The fragment in pMa5-8 contains a *Pst*I site while *amp* in pUC19 lacks this site.

Enzyme Overproduction and Purification. The derivative of pEMa3 (see below, Figure 2) coding for either the N185A or the N188A mutant of *Eco*RV was used to transform *E. coli* WK6 carrying pMetB. The transformants were grown in L-broth at 28 °C to an OD_{600} of 0.4. An equal volume of L-broth at 55 °C was added and the growth continued for 4 h at 42 °C. The cells were harvested by centrifugation and stored at −20 °C. The cells were resuspended and disrupted by sonication, and the mutant *Eco*RV enzymes were purified by chromatography, first on phosphocellulose and then on Blue-Sepharose, as described previously with the wt enzyme (Luke et al., 1987). The purifications were monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; the gels were developed either with Coomassie Brilliant Blue R-250 or by immunoblotting (Blake et al., 1984) using rabbit anti-sera against the wt *Eco*RV endonuclease (from A. Van Houtven, Plant Genetic Systems N.V., Gent). Protein concentrations were determined by the method of Bradford (1976) after calibration of the method by amino acid analysis. Concentrations of both wt and mutant *Eco*RV enzymes are given in terms of protein dimers of M_r 57 000.

DNA Binding. The binding of wt and mutant *Eco*RV enzymes to DNA was measured by gel-shift assays, as described by Taylor et al. (1991).

DNA Cleavage. The substrates were the monomeric forms of either pAT153 (Twigg & Sherratt, 1980) or pAT153b (Taylor & Halford, 1989), purified from transformants of *E. coli* HB101 that had been grown in M9 minimal salts with 1 mCi/L [*methyl*-^{3}H]thymidine (Halford & Goodall, 1988). The reactions with the mutant enzymes were carried out at 20 °C in buffer AM, with either $MgCl_2$ or $MnCl_2$ at the concentrations specified. Buffer AM is 50 mM Tris-HCl, 100 mM NaCl, 10 mM β-mercaptoethanol, 100 μg/mL bovine serum albumin, pH 7.5. More than 80% of the DNA in the preparations of the substrates was supercoiled. During each reaction, the changes in the concentrations of the supercoiled, open-circle, and linear forms of the DNA were measured as described previously (Halford & Goodall, 1988; Taylor & Halford, 1989).

Results

Mutagenesis and Expression of ecoRVR. Plasmid pRV42p carries *ecoRVR* linked to the λ P_R promoter (Figure 2). The promoter is at the optimal position for the overproduction of *Eco*RV endonuclease (Bougueleret et al., 1985; Botterman, 1986). In order to be able to mutate *ecoRVR* and to overproduce the mutant proteins, the section of pRV42p containing both λ P_R and *ecoRVR* was cloned on pMa5-8 (Stanssens et al., 1989), to create pEMa1 (Figure 2).[4] To facilitate mutagenesis of *ecoRVR*, a series of unique restriction sites were required in and around the gene on pEMa1. Mutagenic oligonucleotides were designed to create unique *Cla*I, *Sna*BI, and *Bam*HI sites within the coding region, by changing the DNA sequence without changing the amino acid sequence of the protein, and also a *Hin*dIII site 4 bp after the stop codon. However, when the oligonucleotide for the *Cla*I site was applied to pEMa1, time and again < 2% of the colonies contained the mutation, and many of these contained secondary mutations.

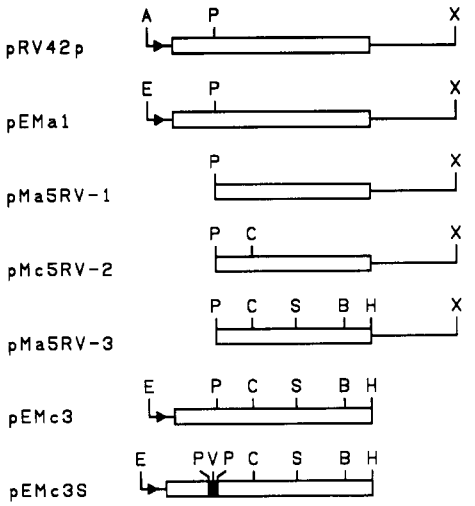

FIGURE 2: Genetic manipulations. The plasmid pRV42p (Botterman, 1986) contains a 1024-bp *Aat*II–*Xba*I segment as shown, which includes the P_R promoter from phage λ (arrowhead) and the entire coding sequence for the *Eco*RV endonuclease (open box). This section of DNA, or the *Pst*I–*Xba*I subsection, was cloned at the polylinker of pMa5–8 (Stanssens et al., 1989) to create pEMa1 and pMa5RV-1, respectively. The latter was subjected to SDM to introduce new restriction sites, giving pMc5RV-2 followed by pMa5RV-3; each round of SDM converts the vector from pMa5-8 to pMc5-8 or vice versa (Stanssens et al., 1989). Both pEMc3 and pEMc3S contain the *Eco*RI–*Pst*I fragment from pEMa1 and the *Pst*I–*Hin*dIII fragment from pMa5RV-3 at the polylinker of pMc5-9; pEMc3S also contains a 31-bp "stuffer" fragment cloned at the *Pst*I site within *ecoRVR* (filled box). Restriction sites are marked as follows: A, *Aat*II; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sna*BI; V, *Eco*RV; X, *Xba*I.

This inefficiency may be due to the potential lethality of the system: overproduction of the *Eco*RV nuclease results in cell death even in the presence of *ecoRVM* (Bougueleret et al., 1985; Taylor et al., 1990). To examine this possibility, a new construct (pMa5RV-1; Figure 2) was made by cloning on to pMa5-8 the *Pst*I-*Xba*I fragment from pRV42p (which lacks both the λ P_R promoter and the first 77 bp of the coding sequence for *ecoRVR*). When pMa5RV-1 was tested for SDM with the *Cla*I oligonucleotide, 20 out of the 24 colonies examined contained the new site. This DNA (pMc5RV-2; Figure 2) was used for further rounds of SDM to introduce the *Sna*BI, *Bam*HI, and *Hin*dIII sites (pMa5RV-3; Figure 2). An expression system for the *Eco*RV restriction enzyme, pEMc3 (Figure 2), was then regenerated with the *Eco*RI-*Pst*I fragment from pEMa1 (containing the λ promoter and the first 77 bp of *ecoRVR*) and the *Pst*I–*Hin*dIII fragment from pMa5RV-3 (with the rest of the coding sequence). The vector used for the construction of pEMc3 was pMc5-9, a derivative of pMc5-8 lacking the *Pst*I site within *amp* (Experimental Procedures), so that the only *Pst*I site on pEMc3 is that within *ecoRVR*. When tested for overexpression, transformants of *E. coli* WK6[pMetB] with pMEc3 gave the same amount of *Eco*RV activity as strains with pEMa1. Hence, before using pEMc3 for efficient SDM on *Eco*RV, it was necessary to inactivate the gene but in a way that would allow for an easy

[4] The DNA was obtained by digesting pRV42p with *Aat*II, removing the 3′ extensions with the exonuclease activity of Klenow, and then liberating the fragment with *Xba*I. The fragment was ligated to pMa5–8 that had been cut twice within its polylinker: first with *Eco*RI (the 5′ extensions were then filled in with Klenow) and subsequently with *Xba*I. The blunt end ligation of the *Eco*RI and *Aat*II termini restores the *Eco*RI site.

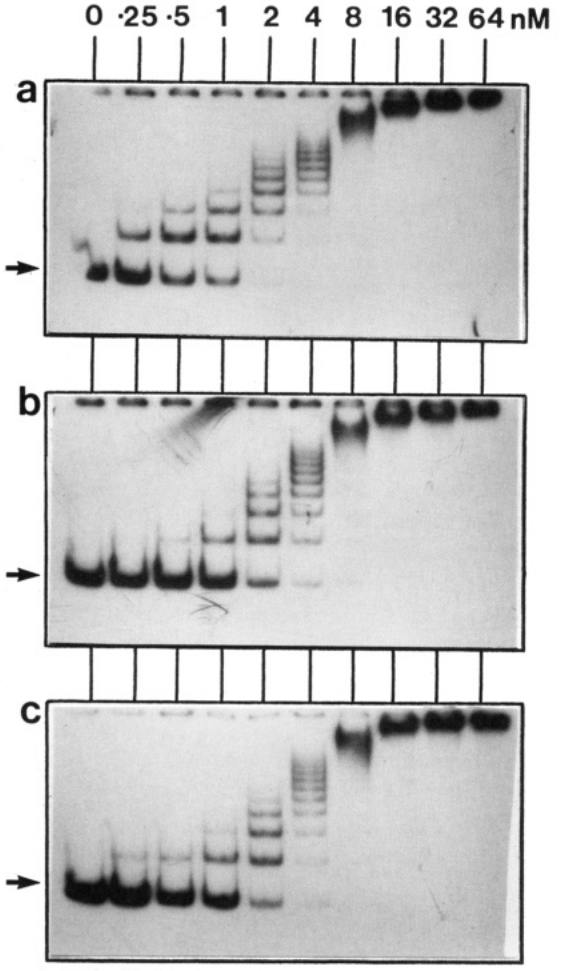

FIGURE 3: DNA binding by wild-type *Eco*RV (a), N185A (b), and N188A (c). The binding mixtures contained ≤0.1 nM DNA in buffer AM with 0.1 mM EDTA and varied concentrations of either the wt *Eco*RV restriction endonuclease (panel a), the N185A mutant (panel b), or the N188A mutant (panel c). For all three panels, the nanomolar concentration of the protein in each lane is given above panel a; lane 0 is in the absence of *Eco*RV protein. The DNA was the *Eco*RI–*Bam*HI fragment from pAT153 (381 bp with 1 *Eco*RV site), with the ends filled in by Klenow in the presence of [α-^{32}P]dATP. Samples from each mixture were subjected to electrophoresis through 6% polyacrylamide and the gels were analyzed by autoradiography. The mobility of the free DNA is marked on the left of each autoradiograph.

recovery of the active gene. This was achieved by cloning a 31-bp "stuffer" fragment at the unique *Pst*I site in pEMc3, to give pEMc3S (Figure 2).[5]

Gapped duplex mutagenesis was carried out on pEMc3S to replace either Asn185 with Ala or Asn188 with Ala. The genes were sequenced in full: no changes were found apart from the specified mutations. For both mutants, the reading frame for *Eco*RV was recovered by the removal of the "stuffer" fragment,[5] and the proteins were then generated. Levels of overproduction were the same as for wt *Eco*RV and, throughout the purifications, they behaved like the wt enzyme. The final preparations of both N185A and N188A were >95% *Eco*RV protein (data not shown).

DNA Binding. The DNA binding properties of the N185A and N188A mutants were compared with those of the wt *Eco*RV enzyme (Figure 3). The binding studies were carried out by the gel-shift method (Garner & Revzin, 1981), under conditions where the DNA cannot be cleaved due to the absence of divalent metal ions. Previous gel-shift studies had shown that wt *Eco*RV endonuclease binds to DNA with no specificity for the DNA sequence, but with some degree of cooperativity (Taylor et al., 1991). With all DNA molecules tested, with and without *Eco*RV sites, wt *Eco*RV produced multiple DNA–protein complexes containing 1, 2, 3, or n molecules of protein per molecule of DNA, with each protein covering about 15 bp of DNA. However, only one equilibrium constant (K_{int}) was needed to describe the association of the protein to all of the individual sites on the DNA, including the recognition site, though the binding of the protein to a site adjacent to one already occupied by the enzyme required an additional term for the protein–protein interaction (Taylor et al., 1991).

The DNA fragment used here was one of 381 bp and thus can accommodate about 25 molecules of *Eco*RV protein. The electrophoresis of this DNA, with increasing concentrations of either N185A or N188A, yielded the same array of DNA–protein complexes as the wt restriction enzyme: first, the DNA with one molecule of protein bound, then the resolved series with 1–10 molecules bound, and finally unresolved forms of the DNA bound to >10 molecules of protein (Figure 3). Hence, the nature of the binding equilibria with either mutant is unaltered from that of the wt. Equilibrium constants for binding to a single site on the DNA (K_{int}) were determined from the amount of complex formation as a function of the protein concentration [as in Taylor et al. (1991)]. For both mutants, the values obtained for K_{int} were smaller than the wt value, though the decreases were close to the error limit of the method (∓50%): 3-fold for N185A and 2-fold for N188A. Thus, despite the fact that Asn185 and Asn188 are located in the *Eco*RV enzyme at positions where they could directly affect the interaction with DNA (Figure 1), the DNA binding properties of the mutants are virtually unchanged from wt.

Mg^{2+}-Dependent Reactions. Thielking et al. (1991) reported that both the N185A and N188A mutants of *Eco*RV were much less active in cleaving DNA than the wt enzyme. To correlate this report with the observation (Figure 3) that neither mutation has a major effect on DNA binding, experiments were carried out to determine the mechanisms of DNA cleavage by the mutant enzymes. The plasmid pAT153, a 3.65-kb DNA with one *Eco*RV recognition site (Twigg & Sherratt, 1980), was used as the substrate and Mg^{2+} was used as the cofactor. With both mutants, DNA cleavage was detected (Figure 4), and restriction analyses of the reaction products showed that cleavage had occurred exclusively at the *Eco*RV recognition site (data not shown). However, to detect DNA cleavage by the mutant proteins, the reactions had to be carried out with higher enzyme concentrations (≥100 nM) and for longer times (≥1h) compared to the wt reaction. Under the same reaction conditions, 0.5 nM wt *Eco*RV cleaves >80% of the substrate within 20 min (Halford & Goodall, 1988). Moreover, the wt enzyme cleaves both strands of the DNA at its recognition site in a concerted fashion, converting the supercoiled substrate directly to the linear product. In contrast, both mutants cleaved the supercoiled form of pAT153 first in one strand, to produce open-circle DNA, and only later in the second strand to produce the linear form [see Figure

[5] The "stuffer" fragment was made from two synthetic oligonucleotides that annealed to each other to produce a 27-bp duplex with 4-base *Pst*I extensions at both 3′ ends. It contained stop codons in all three reading frames in both orientations and also an *Eco*RV recognition site; pEMc3S has no other *Eco*RV sites. To remove the "stuffer" fragment from pEMc3S, to regenerate pEMc3, the DNA (lacking *Eco*RV methylation) was cut with *Pst*I, then treated with DNA ligase and finally subjected to *Eco*RV. DNA retaining the "stuffer" fragment is linearized and has a low transformation efficiency.

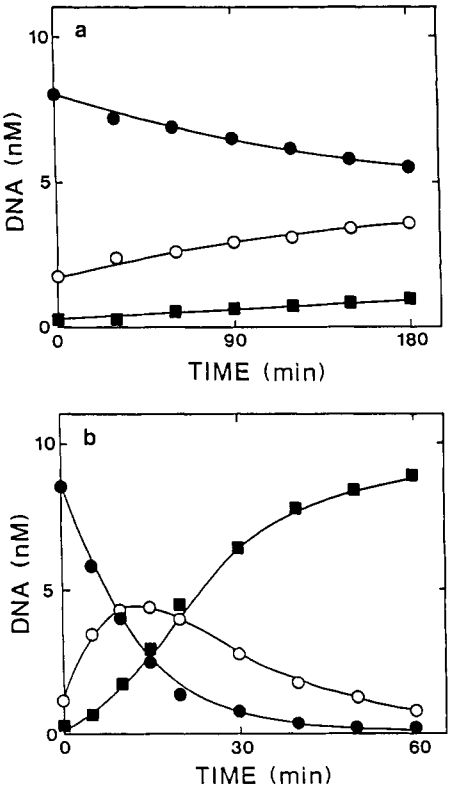

FIGURE 4: DNA cleavage by N185A (a) and N188A (b) in $MgCl_2$. The reactions contained 10 nM pAT153 (^3H-labeled) in buffer AM with 10 mM $MgCl_2$ and either 1 μM N185A protein (panel a) or 100 nM N188A protein (panel b). Aliquots were withdrawn from the solutions at timed intervals after the start of the reactions and mixed immediately with EDTA to stop DNA cleavage. [The reaction in panel a was sampled at longer time intervals than that in panel b.] The DNA in each sample was subsequently analyzed by electrophoresis through 1.2% (w/v) agarose to separate the supercoiled, open-circle, and linear forms of the DNA. The concentrations of the three forms were determined by scintillation counting on slices from the gel: for both panels, the symbols represent supercoiled DNA (●); open-circle DNA (○); and linear DNA (■).

3 for N188A; with 1 μM N185A, the conversion to linear DNA took about 24 h (data not shown)].

In the presence of Mg^{2+}, DNA cleavage by either N185A or N188A was too slow to allow the reactions to be studied steady-state conditions, with the plasmid in excess of the enzyme. However, in reactions where the mutant enzyme was at a higher concentration than the DNA substrate (as in Figure 4), the decrease in the amount of supercoiled DNA with time followed an exponential progress curve. A first-order rate constant (k_a) was determined from each reaction. For both mutants, values for k_a were measured across a range of protein concentrations and were found to increase linearly with increasing amounts of enzyme (data not shown). The values for k_a could thus be normalized against the enzyme concentration to give a parameter, $k_a/[E_0]$, that is related to k_{cat}/K_m (Vermote & Halford, 1992). The relative activities of mutant and wt enzymes against the *Eco*RV recognition sequence were assessed by comparing the values of $k_a/[E_0]$ for the mutants with k_{cat}/K_m for the wt (Table I), though the former reflects the cleavage of the first strand of the DNA and the latter the rate of double-strand breaks. In 10 mM $MgCl_2$, $k_a/[E_0]$ for the N185A mutant at the *Eco*RV recognition site was 3×10^6 times lower than k_{cat}/K_m for the wt, while the activity of the N188A mutant was (2×10^3)-fold lower than that of the wt (Table I). The lack of alteration in DNA binding by these mutants is indeed accompanied by large alterations in Mg^{2+}-dependent activity.

Table I: Cleavage of the *Eco*RV Recognition Site by Mutants of *Eco*RV

enzyme	kinetic parameter (M^{-1} s^{-1})	metal ion[a] $MgCl_2$	metal ion[a] $MnCl_2$
wild-type[b]	k_{cat}/K_m	3 (∓1) × 10^7	4 (∓1) × 10^5
N185A	$k_a/[E_0]$	10 (∓2)	5 (∓1) × 10^4
N188A	$k_a/[E_0]$	1.3 (∓0.2) × 10^4	2.5 (∓0.5) × 10^5

[a] Reactions on pAT153 were in buffer AM with either 10 mM $MgCl_2$ or 10 mM $MnCl_2$. The rates refer to the reaction at the *Eco*RV recognition site on pAT153. [b] Data from Halford and Goodall (1988) and Vermote and Halford (1992).

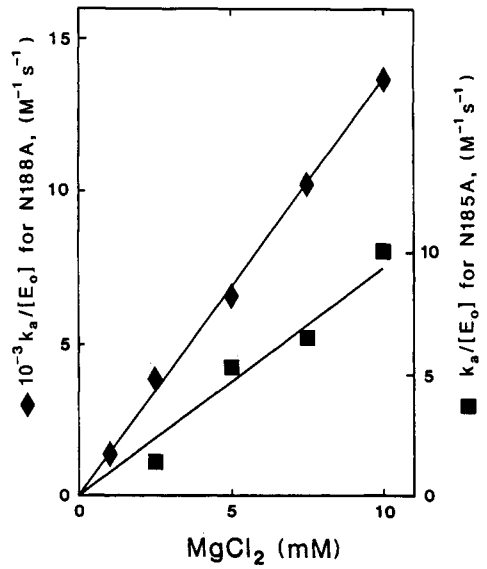

FIGURE 5: Magnesium concentration dependencies. The reactions contained 10 nM pAT153 in buffer AM with varied concentrations of $MgCl_2$ and either 3 μM N185A endonuclease (■) or 100 nM N188A endonuclease (♦). For each reaction, a first-order rate constant (k_a) was evaluated from the decline in the amount of supercoiled DNA. The rate constants were normalized against the concentration of the enzyme, to produce values for $k_a/[E_0]$. The values for the N185A enzyme are given on the righthand ordinate; those for N188A are on the lefthand ordinate (the scale for the latter is 1×10^3 times greater than that for N185A).

The rates of the reactions of the two mutants of *Eco*RV were also measured across a range of concentrations of $MgCl_2$ (Figure 5). In both cases, the rates at which the mutants cleaved the *Eco*RV recognition site on pAT153 increased *linearly* with increasing levels of Mg^{2+}. This behavior is in marked contrast to that of wt *Eco*RV: the activity of the wt enzyme at its recognition site is unaffected by varying the concentration of $MgCl_2$ from 1 to 10 mM (Halford & Goodall, 1988; Taylor & Halford, 1989). For the reactions of either N185A or N188A at the *Eco*RV recognition site, the apparent K_D for Mg^{2+} is thus $\gg$ 10 mM (Figure 5), while that for the wt is $\ll$ 1 mM.

Mn^{2+}-Dependent Reactions. With Mn^{2+} as the cofactor in place of Mg^{2+}, the wt *Eco*RV restriction enzyme has a lower activity against its recognition site, but a higher activity against noncognate sites (Vermote & Halford, 1992). However, when Mn^{2+} was used as the cofactor for the N185A and the N188A mutants of *Eco*RV, both mutants cleaved the recognition site on pAT153 at faster rates than those observed with Mg^{2+} (Figure 6). With Mn^{2+}, they also generated less of the open-circle intermediate (Figure 6). The linear form of pAT153 from the Mn^{2+} reactions of N185A and N188A was due to cleavage specifically at the *Eco*RV recognition site (data not shown). For Mn^{2+} reactions with the mutant enzymes in excess of the DNA substrate (as in Figure 6), the kinetics were

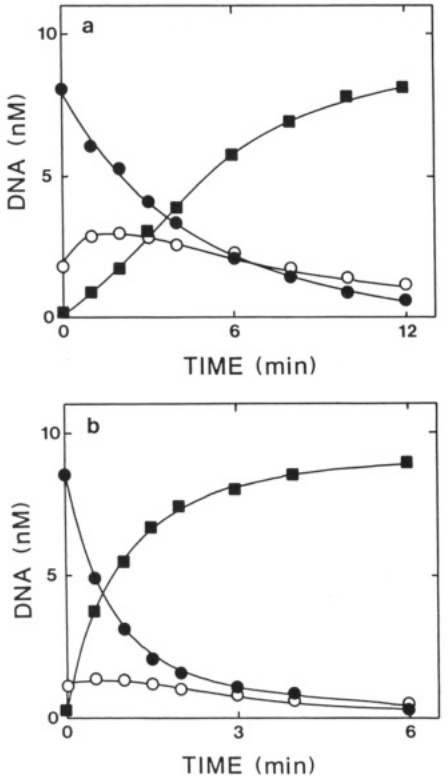

FIGURE 6: DNA cleavage by N185A (a) and N188A (b) in $MnCl_2$. The reactions contained 10 nM pAT153 (^{3}H-labeled) in buffer AM with 10 mM $MnCl_2$, and 100 nM mutant *Eco*RV protein (N185A in panel a and N188A in panel b). Aliquots were withdrawn at timed intervals after the start of the reactions and the concentrations of the supercoiled, open-circle, and linear forms of the DNA were determined at each time point as in Figure 4: for both panels, the symbols represent supercoiled DNA (●); open-circle DNA (○); and linear DNA (■).

measured as above, by evaluating $k_a/[E_0]$ from the exponential decline in the concentration of supercoiled DNA (Table I). For both N185A and N188A, the change from Mg^{2+} to Mn^{2+} caused large increases in $k_a/[E_0]$: 5000-fold for N185A and 25-fold for N188A (Table I). For the wt *Eco*RV enzyme, the change from Mg^{2+} to Mn^{2+} caused a 75-fold decrease in k_{cat}/K_m (Vermote & Halford, 1992). Consequently, Mn^{2+} ions bring the activities of the mutant enzymes to within a factor of 10 of that of the wt.

The activity of N188A in the presence of 10 mM $MnCl_2$ was high enough to allow this reaction to be studied with the substrate (10 nM pAT153) in excess of the protein (1 or 2 nM N188A). The data from these reactions (not shown) were essentially the same as the wt (see Figure 1 of the preceding paper). As with the wt, the two strands of the DNA at the recognition site were cleaved processively, though some of the supercoiled substrate was converted initially to open-circle DNA during a pre-steady-state phase. This accounts for the small amount of open-circle DNA seen in the Mn^{2+} reactions with N188A in excess of the substrate (Figure 6). Moreover, the value of k_{cat} from the steady-state phase of the N188A reaction in $MnCl_2$, 0.04 min^{-1}, was the same as wt.

The rates with which the N185A and N188A enzymes cleaved the *Eco*RV recognition site on pAT153 were examined across a range of concentrations of $MnCl_2$ (Figure 7). For N185A, the values of $k_a/[E_0]$ varied with the concentrations of $MnCl_2$ in a hyperbolic manner, and this yielded an apparent K_D of 6 mM for Mn^{2+}, much lower than that for Mg^{2+} ($\gg$10 mM). For N188A, the lowest level of $MnCl_2$ tested, 1 mM, gave the maximal value of $k_a/[E_0]$; as in the wt reaction at the *Eco*RV recognition site (see Figure 4a in the preceding paper), no further increases in rate were observed at higher

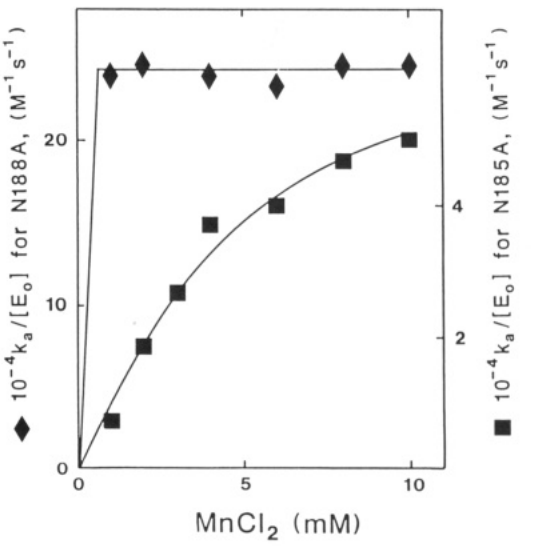

FIGURE 7: Manganese concentration dependencies. The reactions contained 10 nM pAT153 in buffer AM with varied concentrations of $MnCl_2$ and either the N185A endonuclease (■) or the N188A endonuclease (♦); the enzyme concentrations were 100 nM in both cases. For each reaction, a first-order rate constant (k_a) was evaluated from the decline in the amount of supercoiled DNA. The rate constants were normalized against the concentration of the enzyme to produce values for $k_a/[E_0]$. The values for the N185A enzyme are given on the righthand ordinate; those for N188A are on the lefthand ordinate (both ordinates have the same scalar factor). The line drawn through the data for N185A is the rectangular hyperbola for an apparent K_D of 6 mM.

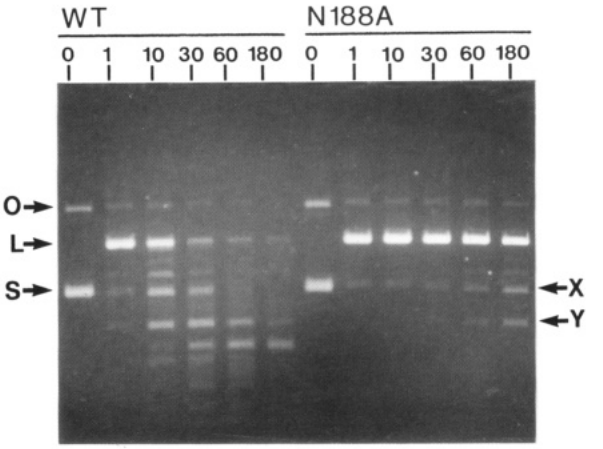

FIGURE 8: Reactions at noncognate sites on pAT153. The reactions contained 10 nM pAT153 in buffer AM with 10 mM $MnCl_2$ and either the wt *Eco*RV restriction enzyme (100 nM) or the N188A mutant (1 μM). Samples were withdrawn from the reactions at timed intervals after the addition of the enzyme, the reaction in each sample was stopped immediately, and the samples were then analyzed by electrophoresis through 1.2% agarose. Samples from the wt and the mutant reactions are marked above the gel as WT and N188A, respectively, and the times of withdrawal of the samples (in minutes) are noted above each lane; lane 0 is before the addition of enzyme. The supercoiled, open-circle, and linear forms of pAT153 are marked as S, O, and L on the left of the gel, and the two products, X and Y, are identified on the right of the gel.

levels of $MnCl_2$ (Figure 7). Hence, the apparent K_D of the N188A mutant for Mn^{2+} during its reaction at the *Eco*RV recognition site must be $\ll$ 1 mM, while that for Mg^{2+} had been $\gg$ 10 mM.

Reactions at Noncognate Sites. When pAT153 is incubated with a comparatively high concentration of wild-type *Eco*RV endonuclease in the presence of $MnCl_2$ (Vermote & Halford, 1992), the supercoiled DNA is cleaved rapidly at the *Eco*RV recognition site, to yield the full-length linear form of the

Table II: Mn^{2+}-Dependent Activities at Noncognate Sites

enzyme[a]	activity ($k_a/[E_0]$; M^{-1} s^{-1})[b]	discrimination factor[c]
wild-type	7×10^4	6
N185A	2×10^2	250
N188A	1×10^2	2500

[a] The reactions at contained 10 nM pAT153b (^{3}H-labeled) in buffer AM with 10 mM $MnCl_2$ and either wt *Eco*RV restriction enzyme (20–100 nM), N185A (1–3 μM), or N188A (1–3 μM). [b] In each reaction, a rate constant (k_a) was determined from the decline in the amount of supercoiled DNA with time, and the constants were then normalized against the enzyme concentrations. [c] The discrimination factor is the activity at the *Eco*RV recognition site (k_{cat}/K_m for the wt, $k_a/[E_0]$ for the mutants) divided by the activity at the preferred noncognate site on pAT153b ($k_a/[E_0]$ values for all enzymes).

plasmid, but this is then cleaved again: first to two fragments (labeled X and Y in Figure 8) and later to a series of smaller fragments. The reactions following that at the *Eco*RV recognition site are at sequences that differ from the canonical site by 1 bp (Halford et al., 1986). But 1 out of the 12 noncognate sites on pAT153, GTTATC at position 1734, is cleaved more rapidly than any other (Taylor & Halford, 1992).

In similar experiments with either the N188A mutant of *Eco*RV (Figure 8) or the N185A mutant (data not shown), the full-length linear form of pAT153 was again produced rapidly but, even with 10 times more of the mutant enzyme than wt, the subsequent reactions at the noncognate sites were depressed relative to that of the wt (Figure 8). Hence, with Mn^{2+} to support DNA cleavage, the activity of the N188A mutant at the *Eco*RV recognition site is similar to that of the wt (Table I), but its activity at noncognate sites is lower than that of the wt. The N185A mutant behaved similarly. However, both mutants retained the preference of the wt enzyme for one of the noncognate site on pAT153, that at position 1734, to give the same initial products from the linear plasmid (X and Y in Figure 8).

The activities of both N185A and N188A at noncognate sites were assessed quantitatively by using pAT153b as the substrate; this plasmid lacks the *Eco*RV recognition site, so the first reaction of *Eco*RV on this DNA is at the preferred noncognate site (Taylor & Halford, 1989; Vermote & Halford, 1992). The rates of the reactions at this site, GTTATC at position 1743, were measured from the decline in the concentration of the supercoiled form of the DNA (Table II). These in turn yielded discrimination factors, the ratio of the activities at cognate and noncognate DNA sequences (Vermote & Halford, 1992). With Mn^{2+} as the cofactor, the discrimination factors for the mutants were larger than that for the wt *Eco*RV endonuclease (Table II). It was impossible to evaluate discrimination factors for the mutants with Mg^{2+} as the cofactor; with Mg^{2+}, the activities of the mutants at the cognate site were low and their activities at noncognate sites were too low to measure.

Discussion

Mechanisms for Specificity. The specificity of a protein for a given DNA sequence can stem simply from the protein binding more tightly to the target sequence than to any other sequence (von Hippel & Berg, 1986). Alternatively, an enzyme can generate specificity for its substrate by utilizing the binding energy from the recognition of the cognate substrate to drive the catalytic reaction; in this case, the experimentally observed $\Delta G°$ for complex formation will be less negative than the intrinsic $\Delta G°$ from all of the interactions between enzyme and substrate, in order to reduce the energy difference between the complex and the transition state (Jencks, 1975). The *Eco*RI restriction enzyme derives much of its specificity from the first mechanism: it binds its recognition site much more tightly than any other sequence (Terry et al., 1987; Lesser et al., 1990). The *Eco*RV restriction enzyme cannot employ the same mechanism.

The crystal structure of *Eco*RV bound to its recognition sequence shows multiple interactions between protein and DNA, including 12 hydrogen bonds, that have no counterparts in the structure of *Eco*RV bound to nonspecific DNA (Winkler, 1992; F. K. Winkler, personal communication). These interactions ought to make the intrinsic $\Delta G°$ for binding specific DNA more negative than that for nonspecific DNA. Yet the *Eco*RV enzyme binds all DNA sequences, including its recognition site, with the same equilibrium constant (Taylor et al., 1991). All of the additional interactions with the recognition sequence observed by X-ray crystallography appear to contribute nothing to the binding energy. However, the complexes formed by *Eco*RV at specific or nonspecific DNA differ in both protein and DNA conformations (Winkler, 1992).[2] In particular, the DNA in the specific complex is severely distorted while that in the nonspecific complex is close to B-form. Consequently, a possible mechanism for the specificity of *Eco*RV is that the intrinsic $\Delta G°$ for binding the recognition sequence is more negative than that at nonspecific DNA, but all of the "excess" energy from the interactions with the recognition site is used up in distorting the DNA and in conformational changes in the protein.

The catalytic activity of *Eco*RV is absolutely dependent on binding a divalent metal ion to the DNA–protein complex. But the affinity of the *Eco*RV endonuclease for Mg^{2+} varies with the DNA sequence to which the protein is bound: when located at the recognition site, it has a high affinity for Mg^{2+}; when located at any other sequence, it has a low affinity for Mg^{2+} (Taylor & Halford, 1989). Therefore, an essential function of the structural reorganization, which accompanies the recognition of the cognate DNA, is to create the binding site for the Mg^{2+} ion(s). A binding site for Mg^{2+} requires the correct juxtaposition of the functional groups that coordinate the metal, from *both* the protein and the DNA (Winkler, 1992; Selent et al., 1992).

Mutagenesis. The experiments in both this and in the preceding paper were designed to probe the interrelationship between DNA recognition and catalysis by *Eco*RV: first, by changing the metal ion cofactor for catalysis and then examining whether or not this perturbed the discrimination between alternative DNA sequences; second, by changing amino acids involved in DNA recognition and then examining whether or not this perturbed catalysis, particularly the interaction with the metal ion cofactor. In this study, a genetic system was constructed for the mutagenesis of *ecoRVR* and for the overproduction of mutant restriction enzymes (Figure 2). In our hands, DNA coding for the active *Eco*RV endonuclease expressed from a high-level promoter could not be used for SDM, even when carried in strains containing both the *Eco*RV methyltransferase and a repressor for the promoter. Strains of this type are viable but, even so, it is likely that the potential lethality of *ecoRVR* caused the low efficiency. Efficient mutagenesis was only achieved by using DNA where the gene either lacked the 5′ end (pMa5RV-1) or was disrupted by the "stuffer" fragment (pEMc3S).

Asparagine 185. In the structure of *Eco*RV bound to cognate DNA (F. K. Winkler, personal communication),[2] Asn185 makes three hydrogen bonds with bases in the GATATC sequence: one with its main chain amino group to 7-N of guanine, and two with its side chain carbonyl and amino

groups to 6-NH_2 and 7-N on the adjacent adenine (Figure 1). If the adenine contacted by Asn185 is replaced by analogs that lack either of these functional groups, the rate for *Eco*RV cleavage is too slow to detect (Mazzarelli et al., 1989; Newman et al., 1990). The insertion of Ala in place of this Asn, in both subunits of the protein dimer, must abolish all 4 hydrogen bonds between the side chains and the adenines, and should this substitution alter the main chain conformation, even more hydrogen bonds will be lost. Given typical energies for hydrogen bonds in protein–ligand interactions (Fersht, 1987), the loss of at least 4 hydrogen bonds ought to produce a major reduction in the equilibrium constant for binding the recognition sequence. If each hydrogen bond contributed −1.5 kcal/mol, the loss of 4 bonds should reduce specific binding by a factor of 30000. In the *Eco*RI enzyme, mutations at residues that contact the DNA bases do indeed reduce the specific binding constant by factors of this sort (Alves et al., 1989; Hager et al., 1990).

But the N185A mutant bound DNA in the same nonspecific manner as wt, with a very similar value for K_{int} (Figure 3). The mutation did, however, cause a large reduction in catalytic activity, at least with Mg^{2+} as the cofactor (Table I). The (3×10^6)-fold reduction in Mg^{2+}-dependent activity is obviously much larger than the 3-fold reduction in K_{int}. This effect cannot be due to a direct role for Asn185 in catalysis by *Eco*RV; the side chain is not close to the scissle bond (Figure 1). However, in its reaction at the *Eco*RV recognition site, the affinity of the N185A nuclease for Mg^{2+} ions was too low to meausre ($K_D \gg 10$ mM; Figure 5), while that for the wt nuclease had been too high to measure ($K_D \ll 1$ mM; Halford & Goodall, 1988). This accounts for why the N185A enzyme has such a low Mg^{2+}-dependent activity; the activity of *Eco*RV at any DNA sequence is proportional to the fractional saturation of the enzyme–DNA complex with the metal ion cofactor (Taylor & Halford, 1989; Vermote & Halford, 1992).

The weak binding of Mg^{2+} to the complex of N185A and DNA accounts for why the initial product from its reaction at the *Eco*RV site is DNA cut in just one strand (Figure 4). A double-stand break requires Mg^{2+} to be bound to both subunits of the dimer and, while this is readily achieved for wt *Eco*RV (Halford & Goodall, 1988), it cannot be so for the mutant. Thielking et al. (1991) reported that partially purified preparations of N185A had no DNA cleavage activity in the presence of $MgCl_2$, in contrast to the very low activity observed here with the purified enzyme. The discrepancy may be due to the fact that Thielking et al. (1991) used a linear substrate (phage λ DNA), rather than supercoiled DNA, so they would have not detected products until both strands of the DNA had been cut at the same site; even with high concentrations of N185A, only a fraction of the DNA will have double-strand breaks after 24 h. A number of mutants of *Eco*RI also cleave DNA by successive single-strand breaks, under conditions where wt *Eco*RI makes double-strand breaks (Alves et al., 1989; Needels et al., 1989; Heitman & Model, 1990a; Hager et al., 1990).

Though the N185A mutant of the *Eco*RV restriction enzyme had a very low affinity for Mg^{2+} ions and a correspondingly low Mg^{2+}-dependent activity, it had a much higher affinity for Mn^{2+} ions and a much higher activity with this cofactor (Figures 5 and 6). Presumably, after the disruption of DNA recognition by the Asn185 → Ala mutation, the metal binding site in the DNA–protein complex accommodates Mn^{2+} better than Mg^{2+}. The same thing happens when the wt *Eco*RV endonuclease binds to a noncognate DNA sequence. The N185A mutant at the cognate site, with either Mg^{2+} or Mn^{2+} as the cofactor, is analogous to the wt enzyme at a noncognate site (Taylor & Halford, 1989; Vermote & Halford, 1992). Other mutants of *Eco*RV, at residues adjacent the catalytic center, also show enhanced activity with Mn^{2+} as the cofactor (Selent et al., 1992; I. B. Vipond and S. E. Halford, manuscript in preparation). A similar observation has also been made with the *Bam*HI restriction enzyme (Xu & Schildkraut, 1991).

Asparagine 188. In the crystal structure of *Eco*RV (F. K. Winkler, personal communication), the carbonyl group on the side chain of Asn188 forms a complimentary surface for the methyl group on the thymine. The methyl group is a determinant for DNA recognition by *Eco*RV (Fliess et al., 1988; Newman et al., 1990), and it is probable that the phenotype of N188A is due mainly to the loss of this van der Waal's interaction. Asn188 might also have a role in stabilizing the conformation of the 182–188 loop. In the *Eco*RI restriction enzyme, mutations at residues that buttress the amino acids contacting the DNA have similar effects to mutations at the contact points (Heitman & Model, 1990a; Rosenberg, 1991). In addition, Asn188 interacts with a water molecule adjacent to the phosphate at the scissile bond in the DNA (Winkler, 1992), so it might participate in the hydrolytic reaction. However, though the N188A mutant was less active than wt *Eco*RV in the presence of Mg^{2+} (Table I), its k_{cat} with Mn^{2+} as the cofactor was the *same* as wt. Hence, it is unlikely that this residue has any direct role in the bond-breaking step. The properties of the N188A mutant of *Eco*RV are similar to those of the N185A mutant. Like N185A, the N188A mutant had much the same DNA binding activity as wt *Eco*RV (Figure 3), but a low affinity for Mg^{2+} ions (Figure 5) and a reduced Mg^{2+}-dependent activity (though not as low as N185A; Table I). However, with Mn^{2+} as the cofactor, its binding of the metal ion became like that of the wt: i.e., too tight to measure (Figure 7). The activity of N188A with Mn^{2+} was also like that of the wt. The consequence of the N188A mutation on Mg^{2+}-dependent activity is thus likely to stem from an indirect effect, via the conformation of either DNA or protein at the metal binding site. But the catalytic center appears to be disrupted less by the N188A mutation than by N185A.

Noncognate Sites. In the presence of $MgCl_2$, the *Eco*RV endonuclease discriminates efficiently against sequences 1 bp different from its recognition sequence (Taylor & Halford, 1989). With Mn^{2+} as the cofactor, the *Eco*RV enzyme still cleaves its recognition site faster than other sites but the discrimination is much less efficient (Vermote & Halford, 1992). Both Asn185 and Asn188 contact the DNA sequence at the *Eco*RV recognition site and therefore it might have been expected that mutations at these residues would cause bigger reductions in activity at the recognition site than elsewhere on the DNA: i.e., they would lead to smaller discrimination factors. This effect has been observed with mutants of the *Eco*RI restriction enzyme (Heitman & Model, 1990b). With *Eco*RV, the opposite was observed with both N185A and N188A (Figure 8 and Table II). The replacement of amino acids that contact the first A·T bp within the GATATC recognition sequence caused a larger reduction in activity at GTTATC than that at GATATC. *Eco*RV appears to be unable to cope with both an altered substrate and an altered protein.

Molecular Communication. The Asn → Ala substitutions, at either position 185 or 188 in the *Eco*RV restriction enzyme, remove specific interactions with bases in the recognition sequence. Yet, these mutations have almost no effect on DNA binding. Instead, they perturb the binding of Mg^{2+} at a lo-

cation in the enzyme–DNA complex that is distant from the site of the mutation. Thus, there must be some communication between two spatially separate regions of the protein, one involved in DNA recognition and the other in catalysis.

For these mutants, the difference between the intrinsic and the observed free energy changes for binding the recognition site will be smaller than that for the wt enzyme. The N188A mutant has a higher activity than N185A and maybe less of the "excess" energy is lost by replacing Asn188 with Ala than by the same substitution at Asn185. This is consistent with the crystallography on *Eco*RV; Asn185 has a more central role in DNA recognition than Asn188. For the wt *Eco*RV restriction enzyme, catalysis is presumably facilitated by using some of the "excess" energy to distort the structure of the specific DNA, in order to position the scissile bond in the DNA against the Mg^{2+} ion(s) and the catalytic groups from the protein (Winkler, 1992; Selent et al., 1992). If so, the structure of the DNA itself becomes the means of communication between DNA recognition, binding Mg^{2+}, and catalysis. Perhaps the reason why N188A at the *Eco*RV recognition site fails to bind Mg^{2+} ions as readily as wt *Eco*RV is the mutant protein fails to distort the DNA to the same extent, with the result that the phosphate at the scissile bond is not in the correct position. The N185A mutant may distort the structure of the DNA by even less than N188A.

Acknowledgments

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