An Isoleucine to Leucine Mutation that Switches the Cofactor Requirement of the *Eco*RV Restriction Endonuclease from Magnesium to Manganese†

I. Barry Vipond, Byung-Jo Moon,‡ and Stephen E. Halford*

Department of Biochemistry, Centre for Molecular Recognition, University of Bristol, Bristol BS8 1TD, U.K.

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ABSTRACT: The *Eco*RV restriction endonuclease cleaves DNA at its recognition sequence more readily with Mg^{2+} as the cofactor than with Mn^{2+} but, at noncognate sequences that differ from the *Eco*RV site by one base pair, Mn^{2+} gives higher rates than Mg^{2+}. A mutant of *Eco*RV, in which an isoleucine near the active site was replaced by leucine, showed the opposite behavior. It had low activity with Mg^{2+}, but, in the presence of Mn^{2+} ions, it cleaved the recognition site faster than wild-type *Eco*RV with either Mn^{2+} or Mg^{2+}. The mutant was also more specific for the recognition sequence than the native enzyme: the noncognate DNA cleavages by wild-type *Eco*RV and Mn^{2+} were not detected with the mutant. Further mutagenesis showed that the protein required the same acidic residues at its active site as wild-type *Eco*RV. The Ile→Leu mutation seems to perturb the configuration of the metal-binding ligands at the active site so that the protein has virtually no affinity for Mg^{2+} yet it can still bind Mn^{2+} ions, though the latter only occurs when the protein is at the recognition site. This contrasts to wild-type *Eco*RV, where Mn^{2+} ions bind readily to complexes with either cognate and noncognate DNA and only Mg^{2+} shows the discrimination between the complexes. The structural perturbation is a specific consequence of leucine in place of isoleucine, since mutants with valine or alanine were similar to wild-type *Eco*RV.

Divalent metal ions, normally Mg^{2+}, are essential for both DNA cleavage by the *Eco*RV restriction endonuclease at its recognition site, GATATC (Schildkraut et al., 1984; D'Arcy et al., 1985), and also for the enzyme's discrimination between this particular sequence and all other sequences (Halford et al., 1993). In the absence of divalent metal ions, *Eco*RV has no catalytic activity though it can still bind to DNA, albeit in a nonspecific manner with no preference for its recognition site (Taylor et al., 1991). However, the complex of *Eco*RV and its cognate DNA has a high affinity for Mg^{2+}, due to the distortion of the bound DNA creating a metal-binding site between the protein and the DNA (Kostrewa & Winkler, 1995). In contrast, *Eco*RV bound to nonspecific DNA has a low affinity for Mg^{2+} (Taylor & Halford, 1989): in this case, the lack of distortion leaves the DNA too far away from the active site to allow a metal ion to be liganded by both protein and DNA (Winkler et al., 1993). The metal ion thus effectively creates the specificity of *Eco*RV for its recognition site by locking the protein onto DNA at this sequence (Thielking et al., 1992; Vipond & Halford, 1995; Cal & Connolly, 1996). Other metal ions in place of Mg^{2+} perturb both the activity and the specificity of *Eco*RV (Vipond et al., 1995). In the presence of Mn^{2+}, *Eco*RV has a lower reaction rate (k_{cat}) at its recognition site but a higher rate at noncognate sites, with the result that the ratio of DNA cleavage rates at cognate and noncognate sites alters from a value of 1×10^6 with Mg^{2+} to 6 with Mn^{2+} (Vermote & Halford, 1992). The lack of discrimination with Mn^{2+} stems from both cognate and noncognate complexes having high affinities for this ion, but why the noncognate complex should have so much higher an affinity for Mn^{2+} over Mg^{2+} has yet to be explained. The switch from Mg^{2+} to Mn^{2+} also perturbs both the mechanism and specificity of other restriction enzymes such as *Taq*I (Cao et al., 1995).

In the previous paper, Vipond and Halford (1996) randomly mutagenized the active site region of the *Eco*RV endonuclease and selected for null mutants by viability in cells lacking the *Eco*RV methyltransferase. Several independent mutants contained a leucine residue in place of isoleucine at position 91: no mutants were recovered where Ile91 had been replaced by any other amino acid. We were surprised that a single amino acid substitution as conservative as Ile→Leu should have created the null phenotype, particularly as the side chain of Ile91 points into the hydrophobic core of the protein rather than being on the DNA−protein interface (Winkler et al., 1993; see Figure 1 in the preceding paper). However, many restriction enzymes possess at their active sites a conserved sequence, P-D-...(D/E)-X-K, where X is generally a long-chain aliphatic residue (Anderson, 1993): Ile91 in *Eco*RV corresponds to "X". The amino acid immediately before Ile91, Asp90, binds a metal ion at the active site (Selent et al., 1992; Kostrewa & Winkler, 1995), and the I91L mutant seemed to interact with metal ions differently from wild-type *Eco*RV. It had very low activity with Mg^{2+} as the cofactor, but, in the presence of Mn^{2+}, it cleaved the *Eco*RV recognition site 40 times faster than the wild-type enzyme (Vipond et al., 1994). These unusual properties prompted us to carry out a more detailed examination of the I91L mutant, by analyzing the kinetics of its reactions at both cognate and noncognate DNA sequences,

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* Correspondence to this author. Department of Biochemistry, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD, U.K. Phone: +44 (0)117-928-7429. FAX: +44 (0)117-928-8274.

‡ On leave of absence from the Department of Biochemistry, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea.

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with either Mg^{2+} or Mn^{2+} as cofactors, and also its DNA-binding properties. The role of Ile91 in determining the organization of the active site of *Eco*RV was further characterized by using SDM to substitute Ile91 with Val, Ala, or Gly and to introduce secondary mutations in the I91L protein at the residues that bind the catalytic metal ions in the wild-type enzyme.

EXPERIMENTAL PROCEDURES

Plasmids. The plasmid pBSKSRV (Vipond & Halford, 1996) carries a disabled copy of the gene for the *Eco*RV endonuclease (with a 30 bp[1] "stuffer" fragment at a unique *Pst*I site close to the start of the open reading frame; Vermote et al., 1992), while pBSKSRVΔ carries an active copy (generated by using *Pst*I to remove the stuffer fragment, followed by recircularization with DNA ligase): in both cases, the reading frame for *Eco*RV is linked to the λ P_L promoter. The plasmids pRV-91L and pRV-91LΔ are identical to pBSKSRV and pBSKSRVΔ, respectively, except that the open reading frame contains one base change, specifying Leu at position 91 instead of Ile: the substitution was generated by random mutagenesis on pBSKSRV (Vipond & Halford, 1996).

Mutagenesis. The following oligodeoxynucleotides were synthesized by L. Hall (this department) on a Millipore Expedite system using reagents from Cruachem (Glasgow, U.K.).

oligo 45:

5′-TATTATTGGTCT**C**GAGAATAAC**AT**AAATATTGTGCT-3′

oligo 90:

5′-GTATATGTTGTTTTT**AGCG**CGATTGCAATTTTTTTA-3′

oligo 91:

5′-GTATATGTTGTTTT**AXC**ATC**A**ATTGCAATTTTTTT-3′

The oligonucleotides match the complementary strand of the genes for either the I91L mutant of *Eco*RV or wild-type *Eco*RV at the selected loci except for the underlined bases. For base X in oligo 91, the synthetic cycle employed equal amounts of the phosphoramidites for A, G, and C. SDM on *Eco*RV using these oligonucleotides was by the method of Taylor et al. (1985) with the mutagenesis kit from Amersham International. Oligos 45 and 90 were applied to pRV-91L in order to construct the double mutants, I91L+E45M and I91L+D90N. Oligo 91 was applied to pBSKSRV in order to replace Ile91 in wild-type *Eco*RV with either Val, Ala, or Gly in a single mutagenic reaction: the mutagenized DNA gave rise to a number of transformants, and examples of each substitution were identified among the individual colonies. The oligonucleotides also specified additional base changes that altered the restriction map of the gene for *Eco*RV without further altering the amino acid sequence of the protein: oligo 45 created a new *Xho*I site, while both oligos 90 and 91 destroyed a *Cla*I site. The DNA in the individual transformants from each preparation of mutagenized DNA were first subjected to the above restriction analysis as an initial screen for the desired mutants, and the *Eco*RV genes were then sequenced in full by the method of Sanger et al. (1977). No alterations other than the desired mutations were detected.

The mutant reading frames were subsequently activated by the removal of the stuffer fragment at the *Pst*I site and overexpressed as described previously (Vermote et al., 1992). The mutant proteins were purified from the induced cultures by chromatography on phosphocellulose (Luke et al., 1987). The peak fractions were pooled, precipitated by addition of ammonium sulfate to 75% saturation, and stored at 4 °C (Vipond & Halford, 1996). The purity of each mutant protein was assessed by polyacrylamide gel electrophoresis: with the exception of I91G that gave preparations containing about 50% *Eco*RV protein, the single chromatography column used here yielded preparations that were about 90% *Eco*RV.

DNA Cleavage and Binding Assays. Rates of DNA cleavage were measured as before (Taylor & Halford, 1989; Vermote & Halford, 1992; Vipond & Halford, 1996), as was also the binding of *Eco*RV to DNA (Taylor et al., 1991; Vipond & Halford, 1995). Unless noted otherwise, the reactions were carried out in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM BME, 100 μg of BSA/mL, supplemented with either 10 mM $MgCl_2$ (to give MG buffer), or 0.5 mM $MnCl_2$ (MN buffer), or 5.0 mM $CaCl_2$ (CA buffer), or 2.0 mM EDTA (EDTA buffer).

RESULTS

Mg^{2+}-Dependent Reactions at the Eco*RV Recognition Site.* The plasmid pAT153 has been used before as a substrate for *Eco*RV (Halford & Goodall, 1988; Taylor & Halford, 1989) and was also used here to allow for comparisons between the rates of DNA cleavage by the wild-type enzyme and the I91L mutant isolated by Vipond and Halford (1996). This plasmid is obtained from a *rec*A strain of *Escherichia coli* as a 3650 bp circle of supercoiled DNA with one copy of the *Eco*RV recognition sequence. The reaction at the recognition site converts the supercoiled circle to the full-length linear form; however, should the enzyme cleave the two strands of the duplex in separate reactions, the open-circle form of the DNA will appear as an intermediate prior to the linear form. The progress of each reaction was monitored by withdrawing samples from the reaction mixes at timed intervals, quenching these immediately with EDTA, and then separating the supercoiled, open-circle, and linear forms by electrophoresis through agarose: the amounts of each form of the DNA were measured by scintillation counting [the DNA had been labeled in vivo by the incorporation of [*methyl*-^{3}H]-thymidine]. For DNA cleavage by wild-type *Eco*RV with Mg^{2+} as the cofactor, this assay was typically used to measure steady-state rates by taking 10 samples at 2 min intervals from reactions that contained 0.5 nM enzyme and 10 nM pAT153: at these enzyme and substrate concentrations, essentially all of the DNA was cleaved within 20 min (Halford & Goodall, 1988). The steady-state rates were invariant across the range of $MgCl_2$ concentrations tested, 1−10 mM: the K_D of the complex for Mg^{2+} is therefore <1 mM (Taylor & Halford, 1989).

Similar experiments were carried out in an attempt to measure the steady-state rates of DNA cleavage by I91L, but, in buffers containing Mg^{2+}, the reactions with 0.5 nM

[1] Abbreviations: bp, base pair(s); BME, β-mercaptoethanol; BSA, bovine serum albumin; EDTA, ethylenedinitrilotetraacetic acid; K_D, equilibrium dissociation constant; SDM, site-directed mutagenesis.

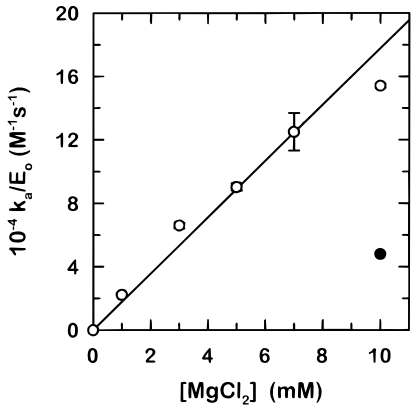

FIGURE 1: Mg^{2+}-dependence for I91L. For the data points marked ○, reactions at 25 °C contained 50 nM I91L protein and 10 nM supercoiled pAT153 (^{3}H-labeled) in 50 mM Tris-HCl, pH 7.5, 10 mM BME, 100 μg of BSA/mL, and both $MgCl_2$ and NaCl. The concentration of $MgCl_2$ is given on the *x*-axis, and the concentration of NaCl was adjusted so that the contribution of both $MgCl_2$ and NaCl to the overall ionic strength of the reaction mixtures was 100 mM. For the data point marked ●, the reaction was the same except that it contained 10 mM $MgCl_2$ and 100 mM NaCl. Samples were removed from the reactions at timed intervals and quenched immediately with EDTA, and the DNA in each sample was subsequently analyzed by electrophoresis through agarose to separate the supercoiled, open-circle, and linear forms of the DNA. The amounts of each form were determined by scintillation counting and a first-order rate constant (k_a) was fitted to the decrease in the concentration of the supercoiled DNA with time. The values for k_a, normalized against the enzyme concentration to give $k_a/[E_0]$ values, are given on the *y*-axis: the error bars on each point indicate the range of values from repeat experiments.

mutant enzyme and 10 nM DNA gave only trace amounts of DNA cleavage even when they were monitored for 4 h. DNA cleavage by I91L in the presence of Mg^{2+} could, however, be measured under single-turnover conditions with the enzyme at a higher concentration than the DNA substrate. Under these conditions, the supercoiled DNA was converted first to the open-circle form and then to the linear form (data not shown). As in previous studies with other mutants (Vermote et al., 1992), the decrease in the concentration of the supercoiled substrate with time followed an exponential progress curve defined by a first-order rate constant, k_a. The values of k_a were normalized against the enzyme concentration, $[E_0]$, to yield a parameter, $k_a/[E_0]$, that is related to the catalytic constant, k_{cat}/K_m (Taylor & Halford, 1989; Vermote et al., 1992). In contrast to wild-type *Eco*RV where the reaction rates were invariant with increasing concentrations of $MgCl_2$, the values of $k_a/[E_0]$ for I91L increased linearly as the concentration of $MgCl_2$ was increased from 1.0 to 10 mM (Figure 1).[2] The complex of the I91L protein bound to the *Eco*RV recognition site must therefore have a K_D for the binding of Mg^{2+} ions that is >10 mM. In 1.0 mM $MgCl_2$, the value of $k_a/[E_0]$ for the I91L mutant, 1×10^4 M^{-1} s^{-1}, was 3000 times smaller than the value of k_{cat}/K_m for wild-type *Eco*RV, 3×10^7 M^{-1} s^{-1}. However, at 10 mM $MgCl_2$, the difference in rates between mutant and wild-type was about 500-fold (Table 1).[2]

Mn^{2+}-Dependent Reactions at the Eco*RV Recognition Site.* When Mg^{2+} was replaced by Mn^{2+} as the cofactor for

[2] For these reactions, the concentration of NaCl was adjusted to maintain a constant ionic strength of 0.1 M from both the $MgCl_2$ and the NaCl (open circles in Figure 1). In other cases, the reactions contained 10 mM $MgCl_2$ and 100 mM NaCl (filled circle in Figure 1 and also data in Table 1).

Table 1: Activities of Mutant *Eco*RV Enzymes[a]

	relative activities	
*Eco*RV protein	MG buffer	MN buffer
wild-type	1.0	0.05
I91L	1.6×10^{-3}	1.9
I91V	0.26	0.12
I91A	0.25	0.06
I91G	2.7×10^{-4}	1.3×10^{-4}
I91L+E45M	0	3.0×10^{-4}
I91L+D90N	0	0

[a] Rates of DNA cleavage were measured from reactions at 25 °C that contained 10 nM pAT153, in either MG buffer or MN buffer, with serial dilutions of each *Eco*RV protein as indicated. The reactions were initially carried with a range of concentrations of *Eco*RV protein, from 0.1 nM to 1.0 μM, and, where possible, the rates were then evaluated from reactions containing an appropriate enzyme concentration for DNA cleavage to be measured on a 1 h time scale. All reaction rates are given relative to a value of 1.0 for wild-type *Eco*RV in MG buffer. Relative rates given as 0 indicate that no DNA cleavage was detected with 1.0 μM enzyme, the highest enzyme concentration tested. Rates that were reduced from the wild-type level by a factor of 1×10^4 would have yielded detectable amounts of DNA cleavage at 1.0 μM enzyme.

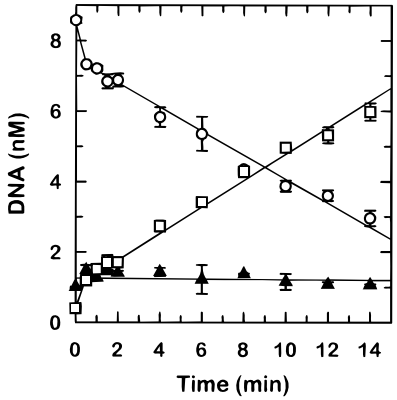

FIGURE 2: Reaction profile for I91L in $MnCl_2$. The reaction at 25 °C contained 2.0 nM I91L protein and 10 nM pAT153 (85% supercoiled) in MN buffer containing 10 mM $MnCl_2$. Samples were withdrawn from the reaction at the indicated times and quenched immediately. The samples were then analyzed by electrophoresis through agarose, and the concentrations of the supercoiled (○), open circle (▲), and linear (□) forms of pAT153 were measured. The error bars on each point indicate the range of values from duplicate experiments.

*Eco*RV, the I91L mutant again cleaved pAT153 at its *Eco*RV recognition site, but the rate of this reaction was much faster than that with Mg^{2+}. It could now be monitored under steady-state conditions with the enzyme at a lower concentration than the plasmid (Figure 2). In the presence of Mn^{2+}, I91L converted the supercoiled form of pAT153 directly to its linear form: very little of the open-circle form was produced. However, the reaction of I91L with Mn^{2+} generated an initial burst phase prior to the steady-state phase (Figure 2). Some of the supercoiled substrate was converted to linear product at a rate that was too fast to measure by the methods used here. The amount cleaved in this initial burst phase was approximately equal to the concentration of the enzyme. The burst phase, together with the lack of open-circle intermediates, shows that I91L with Mn^{2+} must rapidly cleave both strands of the DNA at the *Eco*RV recognition site to yield the enzyme-bound product and that the rate-limiting step for the complete reaction is then the final dissociation of the protein from the cleaved DNA. In this respect, the I91L mutant is similar to wild-type *Eco*RV,

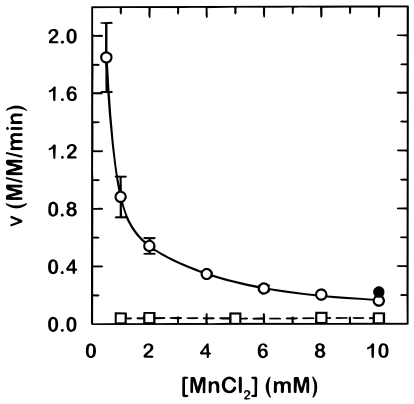

FIGURE 3: Mn^{2+}-dependence for I91L. For the data points marked ○, reactions at 25 °C contained 0.5 nM I91L protein and 10 nM supercoiled pAT153 (^{3}H-labeled) in 50 mM Tris-HCl, pH 7.5, 10 mM BME, 100 μg of BSA/mL, and both $MnCl_2$ and NaCl. The concentration of $MnCl_2$ is given on the *x*-axis, and a concentration of NaCl was adjusted so that the contribution of both $MnCl_2$ and NaCl to the overall ionic strength of the reaction mixtures was 100 mM. For the data point marked ●, the reaction was the same except that it contained 10 mM $MnCl_2$ and 100 mM NaCl. The extent of each reaction was measured at timed intervals as described in Figure 1. Steady-state velocities were measured from the linear decrease with time in the concentration of supercoiled DNA and the concomitant increase in the concentration of linearized product. The velocities were normalized against the enzyme concentration to give the k_{cat} values shown on the *y*-axis: the error bars on each point indicate the range of values from repeat experiments. The data points marked □ show the k_{cat} values for wild-type *Eco*RV at varied concentrations of $MnCl_2$ [data from Vermote and Halford (1992)].

but the kinetics of the burst phase were much slower with the wild-type enzyme (Vermote & Halford, 1992).

After the completion of the burst phase, the amount of supercoiled DNA present in the reactions of I91L with Mn^{2+} decreased linearly with time (and conversely for the linearized DNA product). The reaction velocities rates were measured from this zero-order steady-state phase. For wild-type *Eco*RV with Mn^{2+}, the K_m for pAT153 is about 1.5 nM (Vermote & Halford, 1992) but the I91L mutant gave uniform reaction velocities as the concentration of pAT153 was varied from 10 nM down to 0.5 nM, the lowest concentration that can used with the methods employed here (data not shown). The K_m of I91L for pAT153 in the presence of Mn^{2+} must therefore be <0.5 nM. The reaction velocities measured at 10 nM substrate can thus be normalized against the enzyme concentration to yield k_{cat} values, but the lack of a value for K_m precludes estimations for k_{cat}/K_m. Consequently, comparisons of the Mn^{2+}-dependent activities of wild-type and mutant *Eco*RV proteins (Table 1) refer to their k_{cat} values.

As with wild-type *Eco*RV (Luke et al., 1987), I91L showed no activity in the absence of divalent metal ions, but its change in activity with increasing concentrations of $MnCl_2$ differed markedly from wild-type (Figure 3). From an activity of zero in the absence of $MnCl_2$, it increased to its maximum at the lowest concentration of $MnCl_2$ tested, 0.5 mM, but further increases in the concentration of Mn^{2+} then caused a progressive reduction in activity. The decrease in k_{cat} as more $MnCl_2$ was added to the reactions cannot be due to an increase in ionic strength: the reactions were at a constant ionic strength, maintained by adjusting the concentration of NaCl, even though I91L gave the same rate at 10 mM $MnCl_2$ with either 70 or 100 mM NaCl (Figure 3). The insensitivity of the Mn^{2+}-dependent reactions of I91L to salt (Figure 3), compared to its Mg^{2+}-dependent reactions (Figure 1),[2] is probably due to its low K_m for pAT153 in the presence of Mn^{2+}. The wild-type enzyme also showed its maximal activity at the lowest concentration of Mn^{2+} tested, but in this case further increases produced no change in rate. The latter dependence can be related to a simple binding equilibrium: the complex of wild-type *Eco*RV at its recognition site has a high affinity for Mn^{2+} ions so that even low concentrations of this ion give the maximal rate. However, the variation in I91L activity with the concentration of $MnCl_2$ cannot be correlated to a single binding equilibrium since low levels of Mn^{2+} are needed for activity while higher levels are inhibitory.

The optimal rate for I91L at 0.5 mM $MnCl_2$, 1.9 min^{-1}, was about 40 times higher than that for wild-type *Eco*RV under the same conditions, 0.05 min^{-1}, and it was also double the k_{cat} for wild-type *Eco*RV with Mg^{2+} as the cofactor, 1.0 min^{-1} (Table 1). For wild-type *Eco*RV, Co^{2+} can support DNA cleavage at a rate in between those from Mg^{2+} and Mn^{2+} while other divalent metal ions such as Zn^{2+}, Ni^{2+}, or Ca^{2+} give very low or zero activities (Vipond et al., 1995). When each of these metal ions was tested with I91L, none apart from Mn^{2+} gave any significant amount of DNA cleavage (data not shown). The ability of Mn^{2+} to support a high rate of DNA cleavage by I91L is thus a specific effect for this particular metal ion.

Specificity of DNA Cleavage. In addition to one copy of the *Eco*RV recognition sequence, pAT153 contains 16 copies of sequences that differ from the recognition site by one bp, most of which can be cleaved, albeit slowly, by the *Eco*RV endonuclease (Halford et al., 1986). In its reaction on pAT153, the *Eco*RV endonuclease cleaves the DNA first at its recognition site, to convert the supercoiled DNA to a full-length linear product, and then at the noncognate sites, to convert the full-length linear form to a series of smaller DNA fragments (Taylor & Halford, 1989). In standard buffers containing Mg^{2+} ions, the first noncognate site to be cut, GTTATC at position 1734 on pAT153, is cleaved a million times more slowly than the recognition site and the other noncognate sites are cleaved even more slowly (Taylor & Halford, 1992). However, the wild-type enzyme shows a much weaker discrimination against noncognate sites in buffers containing Mn^{2+}: the preferred noncognate site on pAT153 is then cut only six times more slowly than the cognate site (Vermote & Halford, 1992). Consequently, in reactions with Mn^{2+} under conditions where 10 nM wild-type *Eco*RV is required to convert essentially all of the supercoiled form of pAT153 to its full-length linear form, just a 10-fold increase in the enzyme concentration to 100 nM results in the conversion of the full-length linear form into smaller fragments (Figure 4).

In the presence of Mg^{2+}, the I91L mutant produced no DNA cleavages on pAT153 other than at the recognition site (data not shown). This was as expected. With Mg^{2+}, I91L has a low activity at the cognate site (Figure 1) and, if its activity at noncognate sites is appreciably lower than its cognate activity, it would be too low to detect. In the presence of Mn^{2+}, the I91L mutant again failed to cleave pAT153 at any sites apart from the recognition site (Figure 4): a mutant of pAT153 lacking an *Eco*RV site (Taylor & Halford, 1989) was not cleaved at all under these conditions (data not shown). This was not as expected. With Mn^{2+}, I91L has a higher activity at the *Eco*RV recognition site than

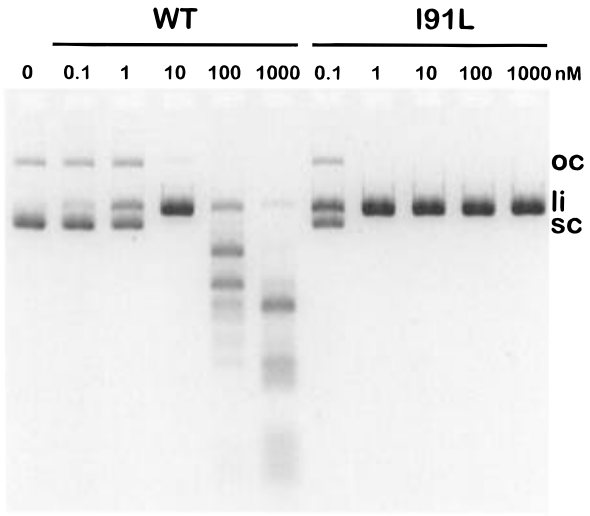

FIGURE 4: DNA cleavage at noncognate sites. The reactions contained 10 nM pAT153 in MN buffer with either the wild-type *Eco*RV endonuclease (in all of the lanes under the heading WT) or the I91L mutant (in all of the lanes marked I91L). The enzyme concentration (nM) in each reaction is noted above the lane. For the left-hand lane marked 0, no enzyme was added. After 1 h at 25 °C, the reactions were stopped and the DNA was analyzed by electrophoresis through agarose. The electrophoretic mobilities of the supercoiled, open-circle, and linear forms of pAT153 are marked on the right of the gel as **sc**, **oc**, and **li**, respectively.

wild-type *Eco*RV (Figure 3) so that, under conditions where the minimal concentration of wild-type *Eco*RV needed to linearize pAT153 was 10 nM, 1 nM I91L was sufficient (Figure 4). Yet, when the concentration of the I91L protein was increased 1000-fold, no additional cleavages of the full-length linear DNA were observed while a 10-fold increase in the concentration of wild-type *Eco*RV had been sufficient to make the additional cleavages at noncognate sites (Figure 4). In the presence of Mn^{2+}, the ability of the I91L mutant to discriminate between cognate and noncognate DNA sequences is therefore much higher than wild-type *Eco*RV. Instead, its power of discrimination with Mn^{2+} must approach the level of the wild-type enzyme with Mg^{2+}.

Other Substitutions at Position 91. In an attempt to rationalize the effect of the I91L substitution in *Eco*RV, SDM was used to make three other mutants of *Eco*RV in which Ile91 was replaced by Val, Ala, or Gly. The side chain of Ile91 is buried in the hydrophobic core of the protein structure (Winkler et al., 1993), at a position where any polar residue may well unfold the protein, so all three replacements were with nonpolar residues. Since the side chains of Ile, Val, Ala, and Gly decrease progressively in size, the perturbations to *Eco*RV activity caused by the Ile91→Leu mutation may be perturbed further, in a progressive manner, as Ile91 is substituted in turn by Val, Ala, and Gly. As with the I91L mutant (Vipond & Halford, 1996), the I91V and I91A proteins were purified to 90% homogeneity by chromatography on phosphocellulose in the same manner as wild-type *Eco*RV, but the I91G protein behaved in an aberrant fashion on phosphocellulose and gave preparations that were only 50% pure (data not shown).

All three mutants were tested for their ability to cleave the *Eco*RV recognition site on pAT153 in reaction buffers containing either $MgCl_2$ or $MnCl_2$ (Table 1). In contrast to the expectation that Val or Ala at position 91 would give proteins that progressively enhanced the deviation from wild-type behavior seen with I91L, the I91V and I91A enzymes had similar kinetic properties to wild-type *Eco*RV. With either Mg^{2+} or Mn^{2+} as cofactors, both I91V and I91A had DNA cleavage activities that were within a factor of 4 of wild-type *Eco*RV with the same cofactor, and, like the wild-type, they were both more active with Mg^{2+} than with Mn^{2+} (Table 1). In addition, the activities of both I91V and I91A were invariant across the ranges of $MgCl_2$ and $MnCl_2$ tested, 1−10 mM (data not shown), in the same manner as wild-type *Eco*RV. The I91G mutant also failed to show the enhanced activity with Mn^{2+} that had been seen with I91L, but this protein exhibited very weak DNA cleavage activities in both Mg^{2+} and Mn^{2+} buffers, several orders of magnitude less than the wild-type enzyme. However, the role of glycine at position 91 is difficult to assess: the instability of this mutant during its purification suggests that its lack of activity may be due to gross misfolding of the protein.

DNA Binding Studies. The gel shift method was used to measure DNA binding by the I91L mutant of *Eco*RV and also the I91V, I91A and I91G mutants. The DNA used in the binding studies was the same 381 bp *Eco*RI−*Hin*dIII fragment from pAT153, ^{32}P-labeled at its 3′-ends, that had been used before for binding studies on wild-type *Eco*RV (Taylor et al., 1991; Thielking et al., 1992). This fragment contains the *Eco*RV site from pAT153 and an additional 366 nonspecific sites, though, for a protein like *Eco*RV that covers about 15 bp, only 25 or so of these sites can be occupied on each DNA molecule at any given time (Taylor et al., 1991). Some binding reactions were carried out in buffers containing EDTA so that they lacked any divalent metal ions, while others contained Ca^{2+}, a metal that fails to support DNA cleavage by *Eco*RV (Vipond et al., 1995). In the absence of metal ions, gel shifts with wild-type *Eco*RV had revealed a series of DNA−protein complexes due to the binding of 1, 2, 3...25 molecules of protein per molecule of DNA, with each successive association having the same equilibrium constant as the first binding event (Taylor et al., 1991). In contrast, binding experiments in the presence of $CaCl_2$ had revealed an initial complex due to the protein binding solely to its recognition site: under these conditions, the additional binding to nonspecific sites required much higher protein concentrations than that needed for specific binding (Vipond & Halford, 1995). Calcium ions thus appear to mimic Mg^{2+} in generating a specific ternary complex containing enzyme, DNA, and metal ions, but the complex with Ca^{2+} has no DNA cleavage activity.

When the I91L protein was equilibrated with the DNA in the absence of divalent metal ions, the same series of complexes were detected (Figure 5a) that had been observed previously with wild-type *Eco*RV (Taylor et al., 1991). The I91L mutant therefore binds to DNA under these conditions in the same nonspecific manner as wild-type *Eco*RV. The strength of this binding was measured from the decrease in the amount of free DNA with increasing concentrations of I91L protein (Figure 5b). The same concentrations of I91L and of wild-type *Eco*RV reduced the level of free DNA to half of its initial value, so their general binding constants to all DNA sequences must be similar to each other. Both the I91V and I91A mutants gave the same pattern for nonspecific binding in the presence of EDTA as that shown for I91L, and the concentrations of these two mutants needed to halve the level of free DNA were again similar to the wild-type enzyme (Figure 5b). However, gel shifts with I91G in

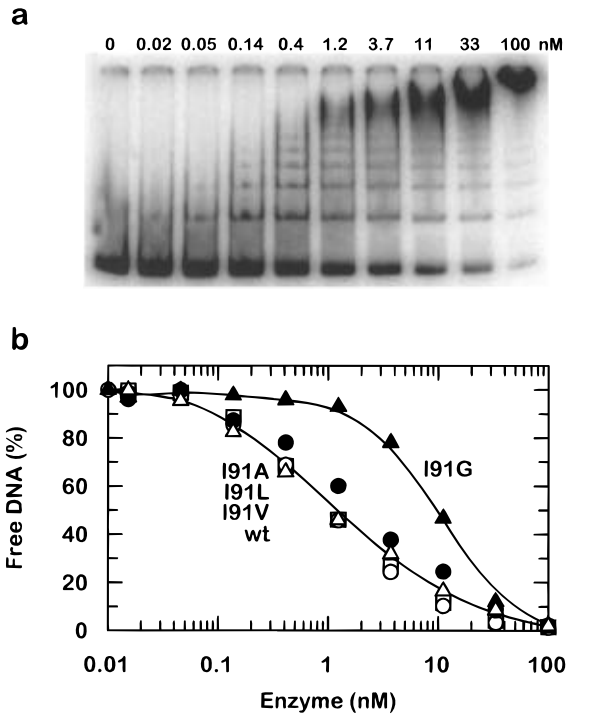

FIGURE 5: DNA binding in EDTA. Panel a: the binding reactions contained the I91L mutant of *Eco*RV at the concentration (nM) given above lane of the gel and approximately 10 pM DNA (the 381 bp *Eco*RI−*Bam*HI fragment from pAT153, ^{32}P-labeled) in EDTA buffer. The samples were subjected to electrophoresis through 6% polyacrylamide in TBE, and the gel was analyzed in a PhosphorImager. Panel b: the PhosphorImager was used to analyze gel shift experiments of the type shown in panel a in order to evaluate the fraction of the total DNA remaining as free DNA across a range of concentrations of *Eco*RV protein. The *Eco*RV proteins used were as follows: wild-type *Eco*RV, ○; the I91L mutant, △; the I91V mutant, □; the I91A mutant, ●; and the I91G mutant, ▲.

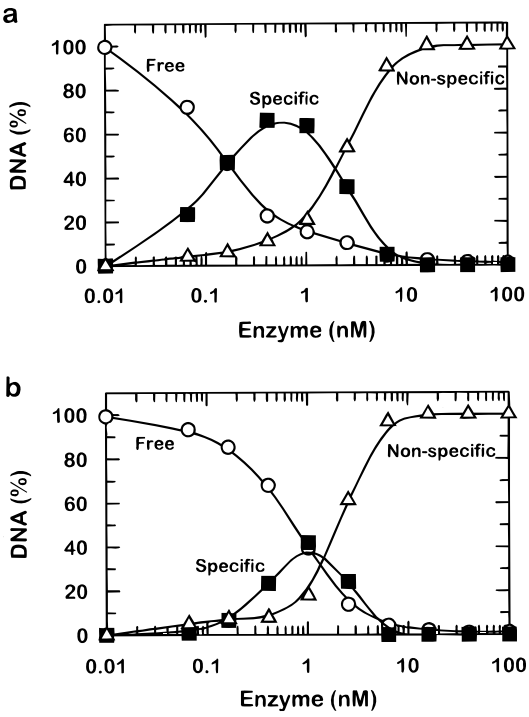

FIGURE 6: DNA binding in $CaCl_2$. The reactions contained either wild-type *Eco*RV (panel a) or the I91L mutant (panel b) at the concentrations given on the *x*-axis and approximately 10 pM DNA (the 381 bp *Eco*RI−*Bam*HI fragment from pAT153, ^{32}P-labeled) in CA buffer. The samples were subjected to electrophoresis through 6% polyacrylamide in Tris-borate buffer containing 5 mM $CaCl_2$. The gels were analyzed in a Phosphorimager in order to evaluate the fractions of the total DNA present as free DNA (○), as bound to one molecule of *Eco*RV protein (■, also marked as "specific"), or as bound to two or more molecules of *Eco*RV protein (△, also marked as "nonspecific").

EDTA failed to reveal any discrete DNA−protein complexes though high concentrations of this protein produced a reduction in the amount of free DNA and a concomitant smear of unresolved complexes running behind the free DNA (data not shown). The concentration of I91G needed to halve the level of free DNA was about 10 times higher than that for wild-type *Eco*RV (Figure 5b). The I91G protein thus binds to DNA more weakly than the other mutants, and its complexes with DNA are insufficiently stable to remain as discrete species during electrophoresis. Since the side chain at position 91 is not in contact with the DNA, the defect in nonspecific binding by I91G is consistent with the view that this mutation causes the protein to misfold.

Gel shift experiments on the binding of I91L to the 381 bp DNA fragment in the presence of Ca^{2+} ions revealed a single initial complex due to specific binding at the recognition site (Figure 6), in the same fashion as noted previously with wild-type *Eco*RV (Vipond & Halford, 1995). As with the wild-type, this Ca^{2+}-dependent complex was not formed with a DNA fragment lacking the *Eco*RV site (data not shown). However, the minimal concentration of the I91L protein needed to form a detectable amount of the specific complex was higher than that for wild-type *Eco*RV (Figure 6). In addition, at the optimal protein concentrations for generating the specific complex, the yield of this complex was lower with I91L (Figure 6). Calcium ions thus enhance the affinity of I91L protein for the *Eco*RV site on DNA so that it can bind specifically to this site, but the degree of enhancement is lower than that for wild-type *Eco*RV. In reactions containing Ca^{2+} and high concentrations of either I91L or wild-type *Eco*RV, binding occurred at additional nonspecific sites on the DNA, thus converting the initial singly-bound species to multiply-bound forms. As in the absence of Ca^{2+} (Figure 5b), the same concentrations of I91L and wild-type *Eco*RV were needed to form these nonspecific complexes (Figure 6). The lower yield of the specific complex with I91L can thus be accounted for by a weakened Ca^{2+}-dependent binding to the recognition site coupled to no change in the subsequent nonspecific binding elsewhere on the DNA, the latter chasing the singly-bound species through to the multiply-bound forms.

Mutagenesis of the Metal-Binding Sites. The *Eco*RV endonuclease is thought to require two metal ions per active site in order to catalyse phosphodiester hydrolysis (Kostrewa & Winkler, 1995; Vipond et al., 1995; Baldwin et al., 1995). One Mg^{2+} (or Mn^{2+}) ion is located between Asp90, Asp74, and the scissile phosphodiester bond (the 90/74 site), but the occupancy of this site is insufficient to initiate the chemical reaction. The reaction appears to need the binding of a second ion of Mg^{2+} (or Mn^{2+}) between Asp74 and Glu45 (the 74/45 site). However, there is a possibility that I91L differs from wild-type *Eco*RV by needing only one metal ion per active site. This could explain why I91L has its optimal activity at low concentrations of $MnCl_2$ and is inhibited by higher concentrations (Figure 3): perhaps the binding of one Mn^{2+} ion at low concentrations of $MnCl_2$ creates the active complex while the binding of a second

Mn^{2+} ion at high concentrations of $MnCl_2$ disrupts the organization of the active site. To test this idea, secondary mutations were introduced into the I91L protein by SDM at either Asp90 or Glu45 in order to perturb each of the two metal-binding sites in turn. Previous studies on wild-type *Eco*RV had shown that the conservative substitution, Glu→Asp at position 45, causes a major drop in activity while the analogous change at position 90, Asp→Glu, has no effect, but that non-conservative substitutions at position 90 have greater effects than those at position 45 (Selent et al., 1992). Consequently, in this study, the replacements at positions 45 and 90 in I91L were made with amino acids that retained the overall size and shape of the wild-type residue but lacked the carboxylate groups: Glu45→Met and Asp90→Asn.

The double mutants were purified to 90% homogeneity by chromatography on phosphocellulose, and serial dilutions of these enzymes were then assayed for DNA cleavage activity in buffers that contained either Mg^{2+} or Mn^{2+} ions as described previously (Vipond & Halford, 1996). The I91L+E45M double mutant showed no detectable DNA cleavage activity with Mg^{2+} as the cofactor, while, in the presence of Mn^{2+}, trace amounts of DNA cleavage were observed at the highest enzyme concentration tested (Table 1). The Mn^{2+}-dependent activity of the I91L+E45M double mutant was about 10 000 times lower than the I91L single mutant. The I91L+D90N was completely inactive in both Mg^{2+} and Mn^{2+} buffers: no DNA cleavages were detected at the highest enzyme concentrations tested (Table 1). The mutations at Glu45 and Asp90 on the I91L protein have similar effects to mutations at these positions in wild-type *Eco*RV (Selent et al., 1992). The I91L enzyme thus needs the right side chains at positions 45 and 90 as much as the wild-type enzyme, presumably because it also needs to bind metal ions at both the 90/74 and the 74/45 sites for catalytic activity. The I91L mutant and wild-type *Eco*RV seem to employ the same chemical mechanism for phosphodiester hydrolysis.

DISCUSSION

Random mutagenesis targeted to the residues that lie close to the active site of the *Eco*RV endonuclease yielded several mutants in which Ile91 had been replaced by Leu (Vipond & Halford, 1996). The function of Ile91 in *Eco*RV has not been analyzed previously by mutagenesis. On the basis of the crystal structures of *Eco*RV (Winkler et al., 1993; Kostrewa & Winkler, 1995), there would have been very little reason to select this residue for an analysis by SDM, and, even if it had been selected, it is unlikely that the chosen mutation would have been Ile→Leu. In many instances, Ile→Leu substitutions have no repercussions on the structure or function of the protein (Schulz & Schirmer, 1979; Matthews, 1993). Ile residues are mainly located in the hydrophobic cores of proteins, at sites that can accommodate alternate packing arrangements (Sauer & Lim, 1992). In contrast, this Ile→Leu substitution in *Eco*RV has a profound effect on the interactions of the protein with metal ions, severely reducing its activity with Mg^{2+} as the cofactor yet enhancing both its activity and its specificity with Mn^{2+}. The properties of the mutant protein with Mn^{2+} are in many ways similar to wild-type *Eco*RV with Mg^{2+} so the mutation effectively switches *Eco*RV from being a Mg^{2+}-dependent enzyme to one demanding Mn^{2+}.

I91L with Mg^{2+}. The Ile91→Leu mutation clearly disables the protein for binding Mg^{2+} ions. When bound to its recognition site, wild-type *Eco*RV has a high affinity for Mg^{2+}, and likewise for I91V and I91A, but the affinity of I91L for this metal ion was too low to measure (Figure 1). This may be the sole reason why the DNA cleavage rates for I91L with Mg^{2+} are so much lower than wild-type *Eco*RV. In principle, this idea could be tested by measuring DNA cleavage rates for I91L at ever increasing concentrations of $MgCl_2$: the rates should eventually reach the level of the wild-type enzyme, but this may need a concentration that is 1000 times higher than the maximum used in Figure 1 (i.e., 10 M $MgCl_2$). Consequently, it is impossible to determine whether or not the intrinsic Mg^{2+}-dependent activity of I91L, at a saturating concentration of $MgCl_2$, differs from wild-type *Eco*RV. The mutant was also impaired for binding Ca^{2+} ions since it failed to produce the specific *Eco*RV−metal−DNA complex with Ca^{2+} as readily as wild-type *Eco*RV (Figure 6), though its defect in this respect was less marked than that for Mg^{2+} binding.

The low affinity of I91L for Mg^{2+} ions can, however, account for why this enzyme produces nicked intermediates during its reactions while nicked forms are not liberated in wild-type reactions (Halford & Goodall, 1988). In order to cleave both strands of the DNA, both subunits of the dimeric protein must bind the metal ions before the enzyme dissociates from the DNA, and this is only likely if the enzyme has a high affinity for the metal. The nicking activity of I91L with Mg^{2+} accounts in turn for the viability of *E. coli* cells that carry the I91L endonuclease but lack the *Eco*RV methyltransferase. The I91L enzyme had been isolated as a null mutant of *Eco*RV (Vipond & Halford, 1996), but it possesses a low level of DNA cleavage activity rather than zero activity. However, mutants of the *Eco*RI endonuclease with low activities can be maintained in cells that lack the *Eco*RI methyltransferase provided that the cells have an active DNA ligase (Heitman et al., 1989). The survival of the I91L mutant of *Eco*RV without a methyltransferase is thus likely to be due to DNA ligase repairing nicks before any double-strand breaks are made. In cells containing the wild-type *Eco*RV restriction−modification system, the methyltransferase protects the cellular DNA at the *Eco*RV recognition site, yet the endonuclease still nicks the cellular DNA at noncognate sites but these are also repaired by DNA ligase (Taylor et al., 1990). In vitro, the I91L protein retains its high Mn^{2+}-dependent activity in reactions that contain 20 times the concentration of $MgCl_2$ over $MnCl_2$ (data not shown), so the fact that it was isolated as a null mutant in vivo shows that the concentration of free Mn^{2+} in *E. coli* must be extremely low.

I91L with Mn^{2+}. Mutants of *Bam*HI that are more active with Mn^{2+} than Mg^{2+} have been described before (Xu & Schildkraut, 1991) and likewise for *Eco*RV (Vermote et al., 1992; Selent et al., 1992). However, all previous mutants that were more active with Mn^{2+} than Mg^{2+} still had Mn^{2+}-dependent activities that were inferior (or at best equal) to the wild-type protein with Mn^{2+} and their Mn^{2+}-dependent activities were very much lower than the wild-type with Mg^{2+}. Similarly, a ribozyme that had no activity when Mg^{2+} was replaced with Ca^{2+} has been converted by in vitro selection into one that can utilize Ca^{2+} ions, but the Ca^{2+}-dependent activity of the mutant was still far below the Mg^{2+}-dependent activities of either mutant or wild-type RNA

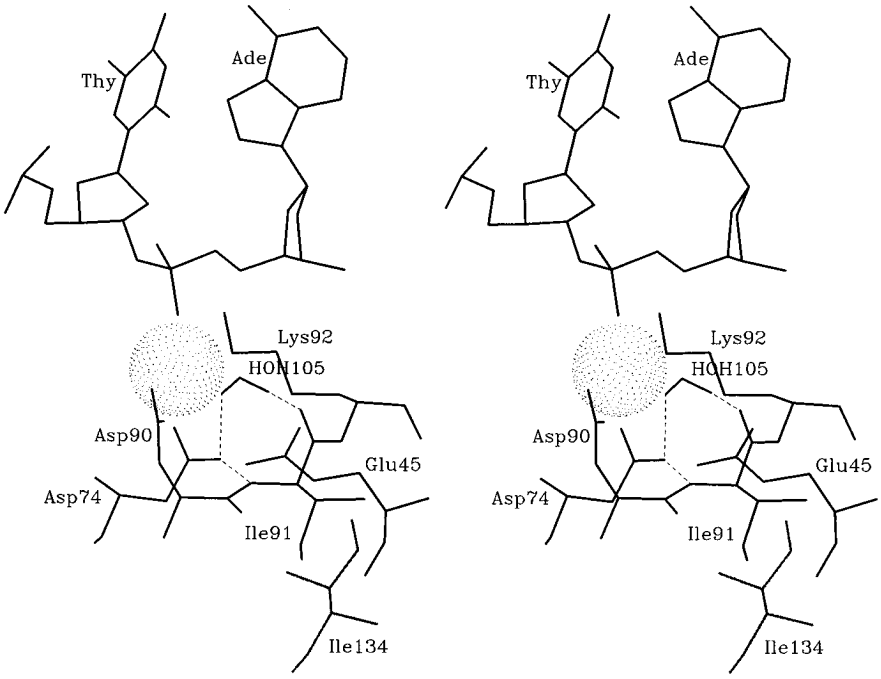

FIGURE 7: Ile91 in *Eco*RV. The stereodiagram shows the location of Ile91 in the crystal structure of the *Eco*RV restriction endonuclease bound to the duplex form of AAAGATATCTT and to Mg^{2+} (Kostrewa & Winkler, 1995). Several amino acids in the immediate vicinity of Ile91 are shown as is also a water molecule (HOH105) and the TpA step in the DNA that is cleaved by *Eco*RV. The Mg^{2+} ion is depicted as a dotted sphere, and hydrogen bonds are shown by dashed lines. Data were taken from PDB file 1RVB.

(Lehman & Joyce, 1993). In contrast, the Ile91→Leu mutation in *Eco*RV achieves virtually a complete switch-over in metal ion requirement from Mg^{2+} to Mn^{2+}. An earlier mutant of *Eco*RV, D90E, failed to distinguish between Mg^{2+} and Mn^{2+} in that it had high activities with both ions (Selent et al., 1992), but I91L is completely specific for Mn^{2+}. The fact that such a switch in the specificity of a protein for a metal ion can be achieved by an Ile→Leu substitution illustrates the power of random genetic methods in generating novel specificities for proteins. It is doubtful whether the current strategies for the design of metal-binding sites in proteins (Berg, 1993) could have engineered a site that is as specific for a novel metal ion as the active site of I91L for Mn^{2+}.

A mutant restriction enzyme that recognizes a novel DNA sequence would, however, be more useful than one recognizing a novel metal ion, but this has yet to be achieved (Fisher et al., 1995). The conversion of a restriction enzyme to a new sequence specificity in vivo demands the parallel conversion of the modification methyltransferase to the same sequence (Roberts & Halford, 1993), but the properties of I91L suggest a strategy that circumvents the need for a methyltransferase, inactivity in vivo coupled to high activity under non-physiological conditions in vitro. The I91L mutant was isolated by its survival in cells lacking the *Eco*RV methyltransferase (Vipond & Halford, 1996) so it could in principle be mutated in vivo to recognize a new sequence without destroying the cell. The resultant enzyme would necessarily lack activity in vivo, but it might still possess the high activity of I91L in vitro with Mn^{2+}. Perhaps the other restriction enzymes that possess the P-D-...-(D/E)-X-K motif at their active sites (Anderson, 1993) could be mutated at residue "X" (Ile91 in *Eco*RV) to become specific for Mn^{2+} ions.

Phosphodiester hydrolysis by the *Taq*I endonuclease is faster with Mn^{2+} than with Mg^{2+}, though Mn^{2+} gives a slower rate for the subsequent dissociation of the product and thus a lower k_{cat} (Cao et al., 1995). For wild-type *Eco*RV, the type of stopped-flow kinetics previously applied to its Mg^{2+}-dependent reaction (Baldwin et al., 1995) has also shown rapid phosphodiester hydrolysis with Mn^{2+} followed by slow product dissociation (G. S. Baldwin and S. E. H., manuscript in preparation). The I91L mutant of *Eco*RV behaves similarly. In the presence of Mn^{2+}, it rapidly cleaved both strands of the DNA and its turnover rate (k_{cat}) was subsequently limited by the dissociation of the cleaved DNA (Figure 2). Nevertheless, I91L differed from wild-type in that, instead of cleaving DNA at a uniform rate at varied concentrations of $MnCl_2$ (Vermote & Halford, 1992), it had its maximal activity at low concentrations of $MnCl_2$ and was inhibited at higher concentrations (Figure 3). The decline in k_{cat} at high levels of Mn^{2+} must be due to a reduced rate for the slowest step in the reaction pathway, so it seems that the binding of Mn^{2+} ions to I91L can attenuate product release. Since the double mutants, I91L+E45M and I91L+D90N, were virtually inactive (Table 1), the attenuation cannot come from the binding of Mn^{2+} to either the 90/74 or the 74/45 sites for metals at the catalytic center of *Eco*RV: these two sites would need to be filled for phosphodiester hydrolysis, and this is achieved by low concentrations of Mn^{2+}. However, the crystal structure of the enzyme−product complex for *Eco*RV shows a metal ion that is absent from the enzyme−substrate complex, bridging the main chain carbonyl group of Gln69 and the 5′-phosphate of the cleaved DNA (Kostrewa & Winkler, 1995). A metal at this site could delay the dissociation of the cleaved DNA. Perhaps this site has a low affinity for Mn^{2+} in the I91L protein but a higher affinity in the native protein, so that the delay with I91L is observed only at high concentrations of $MnCl_2$ while the native protein yields its low k_{cat} with Mn^{2+} at all concentrations of $MnCl_2$. Other sites for additional metal ions are located elsewhere in the DNA-binding cleft

(Jeltsch et al., 1995), and these may also influence the rate of product dissociation.

In the presence of Mn^{2+}, wild-type *Eco*RV has similar activities against both cognate and noncognate sites on DNA (Vermote & Halford, 1992), yet the high activity of I91L with Mn^{2+} at the *Eco*RV recognition site is not accompanied by high activity against noncognate sites (Figure 4). The I91L protein, like the wild-type, binds to DNA in the absence of divalent metal ions without any discernible preference for the recognition site (Figure 5), so it must generate its specificity for DNA cleavage at the recognition site upon the binding of Mn^{2+} to the enzyme−DNA complex. For wild-type *Eco*RV, the ligands that coordinate Mg^{2+} appear to possess the correct octahedral geometry only in the specific complex when the protein is at the recognition site. The nonspecific complex lacks this precise geometry, but, because the coordination complexes for Mn^{2+} are generally more stable than those for Mg^{2+}, Mn^{2+} can perhaps tolerate the nonideal geometry in this complex (Winkler et al., 1993). Many other enzymes involved in DNA metabolism catalyze noncognate reactions better with Mn^{2+} than with Mg^{2+} (Tabor & Richardson, 1989; Baker et al., 1991; Shah et al., 1994), and these might also be due to Mn^{2+} tolerating nonideal coordination geometries. However, the ability of I91L to discriminate accurately between cognate and noncognate DNA sequences in the presence of Mn^{2+} shows that Mn^{2+} can be just as fastidious as Mg^{2+} about binding to a particular coordination geometry. Even though Mn^{2+} can bind readily to both specific and nonspecific complexes with wild-type *Eco*RV, the protein framework in I91L creates a binding site for Mn^{2+} only in the specific complex.

Correlation to Structure. In the crystal structure of wild-type *Eco*RV bound to its recognition sequence (Kostrewa & Winkler, 1995), Ile91 is located on the protein−DNA interface but its side chain points away from the DNA and into the hydrophobic core of the protein (Figure 7). The perturbation to *Eco*RV activity caused by the I91L mutation is uniquely due to Leu at position 91. When this site was occupied by either Val or Ala, the proteins were similar to wild-type *Eco*RV (Table 1, Figure 5) while Gly at position 91 appeared to misfold the protein.[3] The unique feature of Leu is that its side chain is branched at the Cγ position while both Ile and Val are branched at Cβ and Ala is unbranched. The effects of Leu at position 91 are thus likely to be due to the methyl group on Cγ instead of Cβ. Given the role of hydration effects on DNA recognition by restriction enzymes (Robinson & Sligar, 1995), one possibility would have been that this mutation alters the hydrophobicity of the surface of the protein facing the DNA. However, this is unlikely because both the Cβ and Cγ loci in Ile91 are in hydrophobic environments away from the interface (Kostrewa & Winkler, 1995).

Another possible explanation of the phenotype of I91L involves the peptidyl amino and carbonyl groups on Ile91. The main chain amino group forms a hydrogen bond to the carboxylate in the side chain of Asp74 in both the presence and absence of Mg^{2+} ions (Kostrewa & Winkler, 1995). In the enzyme−substrate complex without Mg^{2+}, the main chain carbonyl group forms a hydrogen bond to a molecule of water (HOH105 in Figure 7), and, upon the addition of Mg^{2+}, this water becomes part of the coordination shell around the ion at the 90/74 site (Kostrewa & Winkler, 1995). The mutation might alter these interactions and thus affect the binding of the metal at the 90/74 site in a way that creates a binding site for Mn^{2+} to the exclusion of Mg^{2+}. If so, Leu at position 91 would have to disturb the path of the peptide backbone differently from either Val or Ala at this position. We have attempted to model the perturbation in structure due to these mutations by energy minimization, but the variations in backbone conformation from the wild-type structure caused by Leu, Val, or Ala at position 91 were similar to each other and were very small, typically 0.2 Å, though Gly at position 91 gave a larger displacement (calculations not shown).

The switch from Ile to Leu will, however, alter the packing of the side chain against its immediate neighbors in the structure, in particular the methylene groups in the side chain of Glu45 which wrap around the Cβ methyl group on Ile91 (Figure 7). Leu at position 91 will perturb the configuration of Glu45, while Val or Ala might retain the wild-type configuration. Glu45 is not coordinated to the metal ion in the structure of *Eco*RV bound to its recognition site in the presence of Mg^{2+}, but it is coordinated to a metal in the enzyme−product complex (Kostrewa & Winkler, 1995). For its catalytic reaction, the *Eco*RV endonuclease may need to bind one metal ion between Asp90 and Asp74 (as shown in Figure 7) and another between Asp74 and Glu45 (Vipond et al., 1995; Baldwin et al., 1995). Thus the most likely explanation for the phenotype of I91L is that the mutation alters the position of Glu45 so that the distance and/or angle between the carboxylate groups of Glu45 and Asp74 become inappropriate for the coordination of Mg^{2+} but are now ideal for the coordination of Mn^{2+}, at least in the specific complex of I91L bound to its recognition site. This view is supported by preliminary crystallographic studies on I91L: the addition of Mn^{2+} to the cocrystals of wild-type *Eco*RV bound to its recognition sequence leads to the binding of the metal ion only at the 90/74 site (Kostrewa & Winkler, 1995) while the same addition to cocrystals with I91L results in Mn^{2+}-binding to both the 90/74 and the 74/45 sites (F. K. Winkler, personal communication). In the nonspecific complex for I91L, this distance is presumably inappropriate for Mn^{2+} in the same way that the nonspecific complex for wild-type *Eco*RV lacks the ability to bind Mg^{2+} ions.

The I91L mutant thus illustrates how the *Eco*RV endonuclease manages to cleave DNA specifically at its recognition sequence. It produces the correct coordination shells for the metal ions only when bound to its recognition site even though the metals in question can be either Mg^{2+} for the wild-type enzyme or Mn^{2+} for I91L.

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[3] The packing of the hydrophobic core may be unable to adjust to a change in the size of the side chain at position 91 as large as Ile→Gly. However, since the core can accommodate Ile→Ala perfectly well, an alternative explanation for misfolding by Gly could be the propensity of this residue to take up unusual ϕ/ψ angles (Schulz & Schirmer, 1979).

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