Kinetic Analysis of a Mutational Hot Spot in the *Eco*RV Restriction Endonuclease†

E. Louise Hancox and Stephen E. Halford*

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

Received January 23, 1997; Revised Manuscript Received April 7, 1997⊗

ABSTRACT: The *Eco*RV endonuclease contacts the minor groove of DNA through a peptide loop encompassing residues 67−72. This loop adapts to distorted DNA in the specific complex and to regular DNA in the nonspecific complex. Random mutagenesis had previously identified glutamine 69 as the key component of the loop and this study reports on mutants with glutamate (Q69E), lysine (Q69K), or leucine (Q69L) at this position. The mutants bound DNA specifically at the *Eco*RV recognition site in the presence of Ca^{2+}, in the same manner as wild-type *Eco*RV. In the absence of divalent metals, Q69K and Q69L showed the same nonspecific binding as native *Eco*RV while Q69E failed to bind DNA. Glutamate at position 69 presumably repels nonspecific DNA whilst allowing the adaptations to specific DNA. Both Q69E and Q69K had severely impaired DNA cleavage activities, while Q69L had a steady-state k_{cat} within an order of magnitude of wild-type *Eco*RV though its primary product was nicked DNA, in contrast to double strand breaks by wild-type *Eco*RV. The activity of Q69L required higher concentrations of Mg^{2+} than the wild-type and showed a sigmoidal dependence upon the Mg^{2+} concentration, indicating two metal ions per strand scission. Transient kinetics on Q69L gave lower rate constants for phosphodiester hydrolysis than wild-type *Eco*RV and its reaction also involved a slow conformational change preceding DNA cleavage that had no equivalent with the wild-type. Gln69 in *Eco*RV thus plays key roles in the adjustments of the protein to varied DNA structures and in the alignment of the catalytic functions for DNA cleavage.

In the crystal structure of the *Eco*RV restriction endonuclease bound to its recognition sequence, GATATC, the DNA is located in a deep cleft between the two subunits of the dimeric protein (Winkler et al., 1993; Kostrewa & Winkler, 1995). Each subunit makes its primary contacts to the DNA through two peptide loops though several other segments of the polypeptide chain contact the DNA phosphates (Wenz et al., 1996). One loop, encompassing residues 182−188, is called the R (for recognition) loop: this lies in the major groove of the DNA, and several of its amino acids make direct hydrogen bonds to bases in the recognition sequence. The second loop, encompassing residues 67−72, is called the Q loop since it contains two glutamine residues. The Q loop approaches the minor groove of the DNA and interacts extensively with the sugar−phosphate backbone of the DNA: Asn70 is the only residue on the Q loop that makes a minor-groove contact to a base but this interaction is not maintained in the different crystal structures of *Eco*RV bound to specific DNA (Winkler et al., 1993; Kostrewa & Winkler, 1995). The GATATC sequence bound to *Eco*RV is sharply distorted from B-form DNA, with a marked bend at the center of the site (Stöver et al., 1993; Vipond & Halford, 1995; Cal & Connolly, 1996). This distortion inserts the scissile phosphodiester bond into the active site of the enzyme, simultaneously creating the binding sites for Mg^{2+} between the DNA and the key catalytic residues in the protein, Asp90, Asp74, and Glu45 (Vipond et al., 1995; Baldwin et al., 1995).

In the crystal structure of *Eco*RV bound to nonspecific DNA, the DNA is again located in the cleft between the protein subunits but it retains a B-like structure and lies distant from the active site (Winkler et al., 1993). The failure to create the metal-binding sites between the protein and the DNA can account for why the noncognate complex has a lower affinity for Mg^{2+} than the cognate complex and thus a lower activity (Halford et al., 1993). The R loop in the nonspecific complex is located too far from the major groove for any direct contacts to the DNA. The absence of the hydrogen bonds between amino acids on the R loop and the DNA bases has, however, no impact upon the $\Delta G°$ for DNA binding by *Eco*RV in the absence of Mg^{2+} (Taylor et al., 1991; Stöver et al., 1993; Waters & Connolly, 1994). The energy from the contacts to specific DNA is presumably utilized in distorting the DNA. In contrast, even though the DNA is positioned differently in the specific and nonspecific complexes, the Q loop still makes a series of intimate contacts to the sugar−phosphate backbone of the nonspecific DNA. In the structure of the free protein without DNA, both R and Q loops are largely disordered (Winkler et al., 1993,). The Q loop is thus a conformationally mobile segment of polypeptide chain that seems to be able to mould itself to a variety of DNA structures.

The roles of several of the amino acids in the R loop of *Eco*RV have been examined by site-directed mutagenesis (Thielking et al., 1991; Vermote et al., 1992). Only one site-directed mutant has been reported for the Q loop, N70Q: this had a smaller effect on enzyme activity than most R loop mutants (Thielking et al., 1991). Nevertheless, random mutagenesis targeted to the Q loop, followed by selection for null mutants on the basis of viability in cells lacking the *Eco*RV methyltransferase, showed that almost all of the amino acids in the Q loop could be mutated to yield the null

† This work was supported by the Biotechnology and Biological Sciences Research Council.

* Address all correspondence to this author at the 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. E-mail: s.halford@bristol.ac.uk.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1997.

S0006-2960(97)00156-6 CCC: $14.00

phenotype (Vipond & Halford, 1996). Many of the mutants contained substitutions at Gln69, while other positions in the Q loop yielded mutants at lower frequencies. The collection of mutants at Gln69 included every amino acid that could be accessed by a single base change in its codon. However, inspection of the crystal structures of *Eco*RV failed to reveal any obvious explanation for why Gln69 should be such a hot-spot for null mutants. In the complexes of *Eco*RV bound to either specific or nonspecific DNA (Winkler et al., 1993), the side chain of Gln69 lies parallel to the sugar−phosphate backbone of the DNA and its methylene groups make extensive van der Waals contacts to both the DNA and to other regions of the protein, but the carbonyl and amino groups at the end of the side chain do not make hydrogen bonds to the DNA. Neither the main chain amino nor the carbonyl groups of Gln69 seem to have any specific function in the enzyme−substrate complex though the structure of *Eco*RV bound to the product from its DNA cleavage reaction revealed that the peptidyl carbonyl group is coordinated to one of the two Mg^{2+} ions liganded to the 5′-phosphate of the cleaved DNA (Kostrewa & Winkler, 1995). The other Mg^{2+} ion in the enzyme−product complex is coordinated to both Asp90 and Glu45 and this may play a key role in phosphodiester hydrolysis. In contrast, the coordination shell for the Mg^{2+} between the main chain carbonyl of Gln69 and the 5′-phosphate is completed by four water molecules. The Mg^{2+} on Gln69 is unlikely to have a role in catalysis since this site will exist only after the phosphodiester is converted to a 5′-phosphomonoester.

The objective of this study was thus to explain why the *Eco*RV endonuclease is so sensitive to perturbations at Gln69. A preliminary analysis of one of the random mutants, Q69E, had revealed <0.1% of wild-type activity with Mg^{2+} as the cofactor and a similar activity to wild-type with Mn^{2+} (Vipond & Halford, 1996). This study extends the previous analysis of Q69E and also reports on two other proteins from the random mutagenesis, Q69K and Q69L. Instead of glutamine at position 69, the three mutants carry, respectively, glutamate (Q69E) to introduce an additional negative charge while retaining all of the other properties of the wild-type side chain; lysine (Q69K) to introduce an additional positive charge; leucine (Q69L) to lose both hydrogen-bond acceptor and donor functions of the side chain. All three proteins were tested for DNA binding and DNA cleavage, the latter by both steady-state and single-turnover kinetics. The kinetics of these mutants are compared with those in the preceding paper for the wild-type enzyme (Erskine et al., 1997).

EXPERIMENTAL PROCEDURES

Proteins. The derivatives of pBSKSRVD encoding the Q69E, Q69K, and Q69L mutants of *Eco*RV were from Vipond and Halford (1996): the genes for the *Eco*RV endonuclease on these plasmids are identical to that for wild-type *Eco*RV except for single base substitutions in the codon for Gln69. The plasmids were used to transform *Escherichia coli* CSH50 [pMetB] and the transformants cultured, with induction of *Eco*RV expression, as described by Vermote et al. (1992). The mutant proteins were purified from induced cultures by the same procedure used for wild-type *Eco*RV (Luke et al., 1987) except for the omission of the blue agarose column. All three mutants behaved the same as the wild-type during purification. Fractions were analysed by SDS−PAGE and those containing the protein at ≥90% homogeneity were pooled and stored at −20 °C in 50% glycerol. Protein concentrations were determined from OD_{280} reading by using the extinction coefficient for wild-type *Eco*RV, 1.04×10^5 M^{-1} cm^{-1} (D'Arcy et al., 1985) where M refers to the molarity of the dimeric protein.

DNA Cleavage and Binding Assays. The monomeric form of pAT153 was labeled with [*methyl*-^{3}H]thymidine and the supercoiled DNA purified as before (Taylor & Halford, 1989). To measure rates of DNA cleavage in the presence of $MgCl_2$, samples were removed from the reaction at timed intervals and immediately added to a stop-mix containing 0.1 M EDTA:[1] the supercoiled, open-circle, and linear forms of the DNA were separated from each other by electrophoresis through agarose, and the amount of each form was determined by scintillation counting (Halford & Goodall, 1988; Taylor & Halford, 1989). Computer analysis of kinetic data followed the same procedures as Erskine et al. (1997). For DNA binding studies, the 381 and 389 bp *Eco*RI−*Bam*HI fragments were isolated from pAT153 and pAT153b respectively and end-labeled by Klenow reactions using [α-^{32}P]dATP (Taylor et al, 1991). The binding reactions contained one of these fragments, typically at ∼20 pM, and varied concentrations of *Eco*RV endonuclease in buffers containing either EDTA or $CaCl_2$ (Vipond & Halford, 1995). After electrophoresis of the DNA through polyacrylamide, the radioactivity on the gel was recorded in a PhosphorImager and evaluated with ImageQuant software (both from Molecular Dynamics). Unless noted otherwise, both cleavage and binding reactions were performed in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM β-mercaptoethanol, and 100 μg/mL bovine serum albumin (*Eco*RV buffer; Vipond et al., 1995), supplemented with $MgCl_2$, $CaCl_2$, or EDTA as described.

RESULTS AND DISCUSSION

DNA Binding. The gel-shift method was used to measure DNA binding by the Q69E, Q69K and Q69L mutants of *Eco*RV. Gel-shift experiments with wild-type *Eco*RV and DNA carrying the *Eco*RV site give a ladder of DNA−protein complexes in the absence of divalent metal ions, due to the binding of 1, 2, 3, ..., *n* molecules of protein per molecule of DNA (Taylor et al., 1991). The same series of complexes are observed with DNA lacking an *Eco*RV site, and an analysis of the amounts of each complex formed at varied protein concentrations showed that the first, second, ..., n^{th} binding events all have the same equilibrium constants, regardless of whether the DNA has an *Eco*RV site. Most mutants of *Eco*RV bind to DNA in the absence of divalent cations with the same lack of sequence specificity and with similar affinities to the wild-type protein (Vermote et al., 1992; Selent et al., 1992; Jeltsch et al., 1995; Vipond et al., 1996). In the presence of Ca^{2+}, an ion that does not support DNA cleavage, wild-type *Eco*RV gives initially a single complex with DNA containing an *Eco*RV site but no complexes with DNA lacking the site: additional binding to nonspecific sites occurs only at much higher protein concentrations (Vipond & Halford, 1995). With Ca^{2+}

[1] Abbreviations: bp, base pair(s); EDTA, ethylenedinitrolotetraacetic acid; k_1 and k_2, rate constants for first and second strand cleavages; k_c, rate constant for conformational change; K_D, equilibrium dissociation constant.

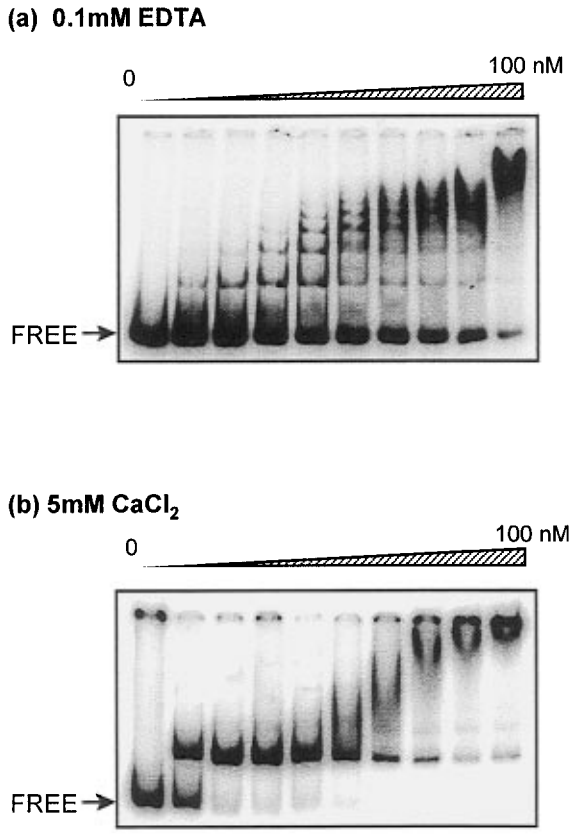

FIGURE 1: Q69L binding to specific DNA. The binding reactions contained ~20 pM DNA (the 381 bp *Eco*RI-*Bam*HI fragment from pAT153, ^{32}P-labeled) and either no *Eco*RV protein (left-hand lanes) or one of the following concentrations of the Q69L mutant of *Eco*RV: 0.4, 0.8, 1.6, 3.1, 6.3, 13, 25, 50, 100 nM (from left to right). The reactions were done in *Eco*RV buffer supplemented with either 0.1 mM EDTA (panel a) or 5 mM $CaCl_2$ (panel b). After electrophoresis through polyacrylamide, the gels were analyzed on a phosphorimager and the phosphorescence records are shown here. An arrow on the left of each gel marks the position of free DNA.

present, the majority of *Eco*RV mutants with altered DNA−protein contacts still bind DNA specifically though the equilibrium constants for the binding to the recognition site can differ considerably from the wild-type value (Jeltsch et al., 1995; Vipond et al., 1996; Wenz et al., 1996).

The DNA substrate used in the binding studies was a 381 bp molecule with one recognition site for *Eco*RV, the *Eco*RI−*Bam*HI fragment from pAT153 that had been used before with both wild-type and mutant *Eco*RV proteins (Taylor et al., 1991; Vermote et al., 1992; Vipond et al., 1996). Control experiments employed the equivalent 389 bp *Eco*RI−*Bam*HI fragment from pAT153b, that lacks an *Eco*RV site due to its disruption by an 8 bp insertion (Taylor & Halford, 1989). In the absence of divalent metal ions, the incubation of Q69L with the 381 bp DNA generated the same ladder of DNA−protein complexes (Figure 1a) as seen with wild-type *Eco*RV (Taylor et al., 1991). The concentration of the Q69L protein required to convert half of this DNA to DNA−protein complexes was the same as that for the wild-type protein. An identical ladder was also observed under these experimental conditions when Q69L was incubated with the 389 bp DNA lacking an *Eco*RV site: the concentration of Q69L required to convert half of this DNA to complexes was the same as that for the DNA with the recognition site (data not shown). The Q69L mutation in *Eco*RV thus affects neither the overall affinity for DNA in the absence of metal ions nor its lack of specificity for the recognition site. In the presence of $CaCl_2$, the addition of increasing concentrations of Q69L to the DNA with an *Eco*RV site initially produced a single DNA−protein complex (Figure 1b), in the same yield as that obtained with wild-type *Eco*RV (Vipond & Halford, 1995), while the DNA lacking an *Eco*RV site failed to produce an equivalent complex (data not shown). The initial binding of Q69L in the presence of Ca^{2+} must therefore be at the *Eco*RV recognition site though nonspecific binding was noted at higher protein concentrations.

The DNA-binding properties of the Q69K mutant (data not shown) were identical to those for Q69L in the absence of metal ions (Figure 1a). However, while the results for Q69K in the presence of Ca^{2+} were qualitatively similar to those for Q69L (Figure 1b), again giving rise to a single DNA−protein complex, the concentration of Q69K required to convert half of the 381 bp DNA to the specific complex was about 100 times higher than that for wild-type *Eco*RV. Thus, while the general pattern of DNA binding by Q69K and Q69L are similar to wild-type, with both being activated for specific DNA recognition by Ca^{2+} as a mimic for Mg^{2+}, Q69K has a lower affinity for the recognition site than wild-type while Q69L has the same affinity as wild-type.

The Q69E protein, in contrast, failed to give the ladder of DNA−protein complexes seen in binding studies with wild-type *Eco*RV in the absence of divalent cations. Instead, at protein concentrations that give multiple complexes with either wild-type *Eco*RV or Q69L, no complexes were detected with Q69E (Figure 2a). Only the very highest concentration of Q69E tested gave any reduction in the amount of free DNA. Under these conditions, Q69E appears to have virtually no affinity for any sequence on the 381 bp DNA. However, the addition of $CaCl_2$ to the binding reactions between Q69E and the DNA carrying the *Eco*RV site resulted in the formation of a single DNA−protein complex (Figure 2b). No equivalent complex was seen with Q69E on the 389 bp DNA lacking the *Eco*RV site (data not shown), so the single complex on the 381 bp DNA must again be at the recognition site. The concentration of Q69E required to recruit half of the 381 bp DNA into the specific complex (0.8 nM) was somewhat higher than that for the wild-type enzyme (0.2 nM) but, while the initial complex with wild-type *Eco*RV gave rise to multiply bound forms at higher protein concentrations, Q69E was severely depressed for multiple binding (Figure 2b).

The observation that the Gln → Leu substitution at position 69 made no difference to the binding of *Eco*RV to either specific or nonspecific DNA (Figure 1) concurs with the crystal structures of the wild-type protein (Winkler et al., 1993; Kostrewa & Winkler, 1995). Neither the amide nor the carbonyl group on the side chain of Gln69 is involved in hydrogen-bonding interactions with the DNA. Nevertheless, lysine at this position weakened the specific binding to the recognition site in the presence of metal ions while having essentially no effect on nonspecific DNA binding in the absence of metal ions. This behavior is similar to many other mutants of *Eco*RV, almost all of which show the same pattern of nonspecific DNA binding in the absence of divalent cations as the wild-type enzyme: the exceptions, such as P73G or I91G, are generally cases where the mutation has a gross effect upon protein structure, and these show weakened binding (Vermote et al., 1992; Selent et al., 1992;

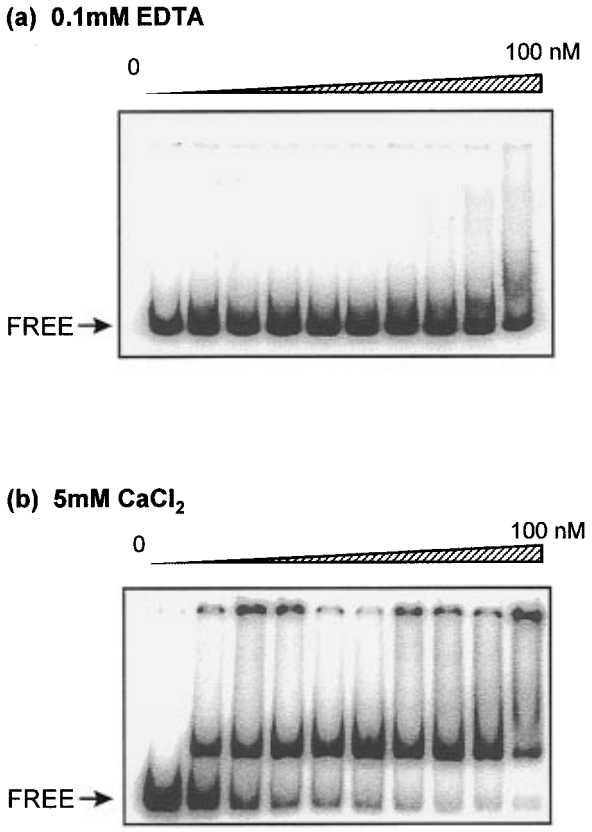

FIGURE 2: Q69E binding to specific DNA. The binding reactions contained ~20 pM DNA (the 381 bp *Eco*RI-*Bam*HI fragment from pAT153, ^{32}P-labeled) and either no *Eco*RV protein (left-hand lanes) or one of the following concentrations of the Q69E mutant of *Eco*RV: 0.4, 0.8, 1.6, 3.1, 6.3, 13, 25, 50, 100 nM (from left to right). The reactions were done in *Eco*RV buffer supplemented with either 0.1 mM EDTA (panel a) or 5 mM $CaCl_2$ (panel b). After electrophoresis through polyacrylamide, the gels were analyzed on a phosphorimager and the phosphorescence records are shown here. An arrow on the left of each gel marks the position of free DNA.

Jeltsch et al., 1995; Vipond et al., 1996). Many of these mutants also show lower affinities than the wild-type enzyme in the Ca^{2+}-induced specific binding to the recognition site (Jeltsch et al., 1995; Vipond et al., 1996; Wenz et al., 1996). In contrast, glutamate at position 69 had the opposite effect to lysine: it incapacitated the protein for binding to nonspecific DNA sequences whilst having only a minor effect on the formation of a specific protein−metal-DNA complex at the recognition site (Figure 2). Amongst the many mutants of *Eco*RV characterized to date, the change of Gln69 to Glu is the only substitution that has a greater impact upon nonspecific binding over specific binding. The opposite results with Glu or Lys at this position may reflect the ability of the Q loop to adapt to different DNA structures. Perhaps the conformation of the Q loop in the nonspecific complex results in an unfavorable juxtaposition of the negative charge on Glu at 69 against another negative charge from DNA or protein while its conformation in the specific complex avoids this juxtaposition. The converse, with an unfavorable juxtaposition of positive charges in the specific complex, might apply to Lys at position 69.

Steady-State Kinetics. The plasmid pAT153 has been used before as a substrate for wild-type and mutant *Eco*RV proteins (Halford & Goodall, 1988; Taylor & Halford, 1989; Vermote et al., 1992; Vipond et al., 1996). In the standard steady-state reaction with the wild-type enzyme, employing

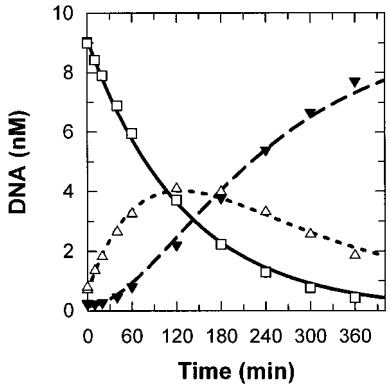

FIGURE 3: Steady-state reaction with Q69L. The reaction at 25 °C contained 10 nM pAT153 (^{3}H-labeled, 90% supercoiled) and 0.5 nM Q69L in *Eco*RV buffer with 10 mM $MgCl_2$. Aliquots were withdrawn from the solutions at timed intervals after the start of the reaction, indicated on the *x*-axis, and mixed immediately with an EDTA stop-mix. 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, and the concentrations of each form were determined by scintillation counting: □, supercoiled DNA; △, open-circle DNA; ▼, linear DNA.

10 nM pAT153 and 0.5 nM enzyme in the optimal buffer for *Eco*RV with 10 mM $MgCl_2$, virtually all of the supercoiled substrate is converted within 20 min to the linear product cleaved in both strands: no accumulation of the open-circle DNA, cleaved in one strand, occurs during the steady-state phase. The absence of open-circle DNA from wild-type reactions is due to the rate constants for both first and second strand scissions being much faster than that for product dissociation, so the dissociation occurs only after both DNA strands are cut (Erskine et al., 1997).

When the Q69E or Q69K mutants of *Eco*RV were tested under the standard conditions for steady-state reactions, the extent of DNA cleavage was too low to measure even when the reactions were monitored for 6 h (data not shown). However, 6 h reactions with Q69L under standard steady-state conditions gave extensive DNA cleavage (Figure 3) though the reaction profile with this mutant differed markedly from that with wild-type *Eco*RV. Instead of the direct conversion of supercoiled substrate to linear product (Halford & Goodall, 1988; Taylor & Halford, 1989), the steady-state reaction with Q69L initially yielded the open-circle form of pAT153, due to DNA cleavage in one strand, and only later the linear form due to double-strand breaks at the recognition site. The maximal concentration of open-circle DNA reached during the course of this reaction, ~4 nM, exceeded the concentration of the Q69L protein, 0.5 nM (Figure 3). The DNA cleaved in only one strand therefore cannot be an enzyme-bound intermediate but rather it must be liberated into free solution during the catalytic turnover. For the reaction shown in Figure 3, the initial rate for the utilization of supercoiled DNA yielded a reaction velocity of 0.09 min^{-1}, 10 times lower than that for wild-type *Eco*RV under the same conditions (0.9 min^{-1}: Halford & Goodall, 1988), but the rate for Q69L is for single strand breaks while that for wild-type *Eco*RV denotes double-strand breaks. On account of the liberation and subsequent utilization of the open-circle DNA by Q69L, the overall time for the complete conversion of supercoiled substrate to linear product by the mutant exceeds that for wild-type *Eco*RV by a factor of >10.

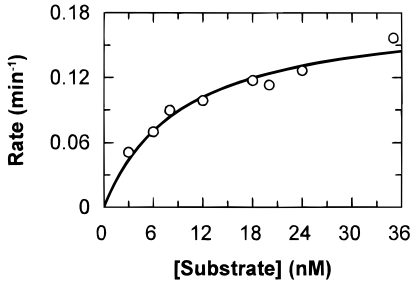

FIGURE 4: Steady-state kinetics on Q69L at varied DNA concentrations. Reactions in *Eco*RV-buffer with 10 mM $MgCl_2$ at 25 °C contained the concentration of pAT153 (≥90% supercoiled DNA) indicated on the *x*-axis and the Q69L mutant of *Eco*RV at one-tenth of the DNA concentration. Initial rates were measured from the decrease in the concentration of supercoiled DNA with time and were normalized against the concentration of enzyme to produce the values for the rates (mol of S/mol of E/min) given on the *y*-axis: each value is the average from duplicate or triplicate experiments. The line drawn is the Michaelis−Menten curve that gives the optimal fit to the experimental data: the best fit was with values of 9.5 nM for K_m and 0.18 min^{-1} for k_{cat}.

Table 1: Kinetic Parameters for DNA Cleavage by Gln69 Mutants

	k_{cat}[a] (min^{-1})	K_m[a] (nM)	k_1[b] (s^{-1})	k_2[b] (s^{-1})
WT	0.9[c]	0.5[c]	3.4[d]	2.8[d]
Q69L	0.18	9.5	0.028	0.023
Q69E	nd[e]	nd	2.15×10^{-4}	nd
Q69K	nd	nd	1.05×10^{-4}	nd

[a] From steady-state reactions at 25 °C in *Eco*RV buffer with 10 mM $MgCl_2$, using varied concentrations of pAT153 and with *Eco*RV protein at one-tenth of the DNA concentration. [b] From single-turnover reactions at 25 °C in *Eco*RV buffer with 10 mM $MgCl_2$, using 10 nM pAT153 and 100 nM *Eco*RV protein. [c] From Taylor and Halford (1989). [d] From Erskine et al. (1997). [e] Not determined.

To determine whether the reduced reaction velocity with Q69L was due to an increase in its K_m for pAT153 or a reduction in k_{cat}, or both, DNA cleavage rates were measured in reactions with varied concentrations of pAT153 in the presence of a fixed concentration of $MgCl_2$: steady-state conditions were maintained by using the mutant enzyme at one-tenth of the concentration of the DNA substrate (Figure 4). The reaction velocities increased with increasing concentrations of pAT153 in hyperbolic fashion and were fitted to the Michaelis−Menten equation to yield values for both k_{cat} and K_m (Table 1). The k_{cat} for single-strand breaks by Q69L, from its maximal velocity at an infinite substrate concentration, was about 5 times lower than that for double-strand breaks by the wild-type enzyme. In addition, despite the almost identical behavior of Q69L and native *Eco*RV in DNA-binding assays (Figure 1), the K_m of Q69L for supercoiled pAT153, 9.5 nM, was about 20 times higher than that for wild-type *Eco*RV (Table 1). The K_m for Q69L is, however, similar to the K_D for the binding of wild-type *Eco*RV to its recognition site on pAT153 under these reaction conditions, for which values of 10−14 nM were obtained by Erskine et al. (1997). Whenever the rate-limiting step in an enzyme mechanism is the dissociation of the product at the end of the pathway, as is the case with wild-type *Eco*RV (Erskine et al., 1997), the K_m is bound to be smaller than the K_D (Gutfreund, 1995) but the pathway for Q69L contains a slow step before phosphodiester hydrolysis (see below) so, in this case, the K_m should be similar to the K_D.

The Gln → Leu mutation causes a 100-fold reduction in k_{cat}/K_m while the changes to either Glu or Lys result in enzymes whose activities are too low to permit evaluations of k_{cat}/K_m. All three of these proteins had originally been identified as null mutants of *Eco*RV, by selecting for viable colonies after transforming the mutagenized plasmids into an *E. coli* strain that lacks the *Eco*RV methyltransferase (Vipond & Halford, 1996). In this situation, it might have been expected that any DNA cleavage activity at the unmodified *Eco*RV sites on the chromosome of the cell would have resulted in cell death. The very low in vitro activities of Q69E and Q69K meet this expectation. The higher activity of Q69L might appear to be sufficient to destroy the unmethylated chromosome, but this protein initially nicks the DNA and only later cuts the second strand (Figure 3). In vivo, the nicks may well be repaired by the cellular DNA ligase before Q69L can process them to double-strand breaks. It has been shown before that the viability of cells carrying mutants of the *Eco*RI endonuclease in the absence of the *Eco*RI methyltransferase requires an active DNA ligase (Heitman et al., 1989). Similarly, the viability of cells containing the wild-type *Eco*RV restriction−modification system depends on DNA ligase: the methyltransferase protects the *Eco*RV sites on the chromosome, but single-strand breaks made by the nuclease at other unprotected sites are repaired by ligase (Taylor et al., 1990).

Magnesium Dependencies. A possible reason for why steady-state reactions with Q69L liberate open-circle DNA, while wild-type reactions convert the supercoiled substrate directly to linear product, is that the Q69L protein has a lower affinity for Mg^{2+} ions. This could result in only one subunit of the protein dimer binding the Mg^{2+} ions necessary for phosphodiester hydrolysis so that only one DNA strand can be cut at a time. For native *Eco*RV, 1 mM $MgCl_2$ is sufficient for maximal velocity and no increase in steady-state rate is observed at higher concentrations (Halford & Goodall, 1988), though very low levels of $MgCl_2$ result in both reduced velocities and also the production of open-circle DNA during the course of the reaction (Luke et al., 1987). However, to observe these effects with wild-type *Eco*RV, the concentration of $MgCl_2$ has to be reduced to 100 μM, a level that approaches the concentration of phosphate moieties on 10 nM pAT153 (i.e., 75 μM), so that is it is no longer possible to give a value for the concentration of free Mg^{2+} ions in solution. Steady-state rates of DNA cleavage by Q69L were therefore measured at a fixed concentration of pAT153 across the range of $MgCl_2$ concentrations from 1−10 mM.[2] At all levels of $MgCl_2$ tested, Q69L gave reaction profiles similar to that shown in Figure 3, with the supercoiled substrate being transformed first to open-circles and only later to linear DNA, but the reaction velocities, measured from the conversion of supercoiled to open-circle DNA, showed a sigmoidal dependence on the concentration of $MgCl_2$ (Figure 5).

[2] These experiments were limited to the range from 1 to 10 mM $MgCl_2$. Concentrations of $MgCl_2$ <1 mM were precluded because a significant fraction of the Mg^{2+} ions would then have been bound by other ligands in the solutions: the total concentration of Mg^{2+}-binding ligands in these reactions, from DNA phosphates and from the EDTA in both enzyme and DNA preparations, is ∼0.1 mM. Concentrations of $MgCl_2$ >10 mM were also precluded because, even though a constant ionic strength of 0.1 M was maintained by adjusting the concentration of the NaCl, the differences between Mg^{2+} and Na^+ in their interactions with DNA will not give precisely the same level of charge neutralization (Record et al., 1991).

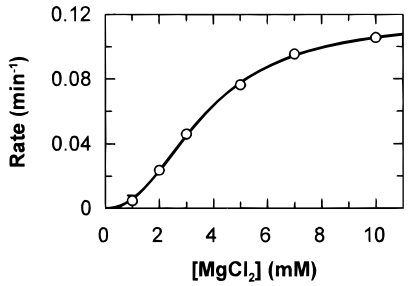

FIGURE 5: Steady-state kinetics on Q69L at varied $MgCl_2$ concentrations. Reactions at 25 °C contained 10 nM pAT153 (≥90% supercoiled DNA), 1.0 nM Q69L, and the concentration of $MgCl_2$ indicated on the *x*-axis. The reaction buffer was the same as *Eco*RV buffer except that the concentration of NaCl was adjusted for each experiment so that the total ionic strength from both the NaCl and the $MgCl_2$ was always 0.1 M. Initial rates were measured from the decrease in the concentration of supercoiled DNA with time and were normalized against the concentration of enzyme to produce the values for the rates (mol of S/mol of E/min) given on the *y*-axis: each value is the average from triplicate experiments. The line drawn is the Adair curve (eq 2) that gives the optimal fit to the experimental data: the best fit was with values of 36 mM for K_A and 0.42 mM for K_B.

A sigmoidal dependence of a reaction velocity against ligand concentration can be accounted for by the Adair equation (Gutfreund, 1995). For a mechanism of the type

$$E + M + M \leftrightarrow E{-}M_A + M \leftrightarrow E{-}M_A{-}M_B \qquad (1)$$

in which the enzyme is only active after sequentially binding metals (M) to two sites, A and B, it can be shown that

$$v/V_{max} = [M]^2/\{([M] + K_A)([M] + K_B)\} \qquad (2)$$

where K_A is the K_D for binding metal to A in the free protein and K_B the K_D for binding metal to B in the $E{-}M_A$ complex. When eq 2 was tested against the experimental data, it accounted accurately for how the reaction velocities vary with the concentration of $MgCl_2$ and the nonlinear regression fits yielded unique values for both K_A and K_B, 36 and 0.4 mM, respectively (Figure 5). Alternatively, for the mechanism

$$E + nM \leftrightarrow E{-}M_n \qquad (3)$$

the Hill equation

$$v/V_{max} = [M]^n/([M]^n + K_D) \qquad (4)$$

can account for a sigmoidal dependence of reaction velocity on ligand concentration (Gutfreund, 1995). Equation 4 gave as close a match to the experimental data as that obtained with eq 2 (analysis not shown): the optimal fit was with values of 2.25 for *n*, the Hill coefficient, and 19 mM for the K_D. However, the Hill equation describes the simultaneous binding of ligands to a series of intrinsically identical sites in the protein. Hence, it might have been appropriate if the activity of Q69L had required the binding of one Mg^{2+} ion to each of the two active sites in the dimeric protein but the reaction velocities measured here refer to the conversion of the intact duplex to product cleaved in just one strand. Other mechanisms that might account for the sigmoidal curve include schemes with $Mg^{2+}{-}$DNA complexes as the true substrate for *Eco*RV. Even so, the simplest interpretation of the sigmoidal curve is that it is due to an Adair mechanism involving the consecutive binding of two Mg^{2+} ions to two separate loci within one of the two active sites of the dimer.

Alternate proposals have suggested that phosphodiester hydrolysis by *Eco*RV requires either two metal ions per active site (Kostrewa & Winkler, 1995; Vipond et al., 1995; Baldwin et al., 1995) or just one metal ion (Jeltsch et al., 1993, 1995). Previously, it had not been possible to distinguish between these proposals by analysing the reaction velocity for *Eco*RV on macromolecular DNA at varied concentrations of $MgCl_2$. For wild-type *Eco*RV, the velocities are at their maximum throughout the accessible range of Mg^{2+} concentrations:[2] the apparent affinity for Mg^{2+} is thus too high to measure (Taylor & Halford, 1989). For most mutants of *Eco*RV, the velocities increase linearly with increasing concentrations of Mg^{2+}: the apparent affinity for Mg^{2+} is then too low to measure (Vermote et al., 1992; Vipond et al., 1996). Fortuitously, the Q69L mutation lessens the affinity of *Eco*RV for Mg^{2+} by just the right degree to bring the curve for velocity against Mg^{2+} concentration into the accessible range of $MgCl_2$ concentrations (Figure 5). The data with this mutant support two-metal schemes for phosphodiester hydrolysis by *Eco*RV, though the results are consistent with either both metals having catalytic functions (Vipond et al., 1995) or with one metal playing purely a structural role (Jeltsch et al., 1995). Sigmoidal curves for *Eco*RV activity against Mg^{2+} concentration have also been observed with the native enzyme on 16 bp duplexes (A. Pingoud, personal communication): these substrates allow the reaction to be studied at lower metal ion concentrations than is the case with macromolecular DNA.

Single-Turnover Kinetics. Quench-flow experiments on the reaction of wild-type *Eco*RV with pAT153 under single-turnover conditions (with a molar excess of endonuclease over plasmid, typically 100 nM enzyme and 10 nM DNA) had revealed the consecutive cleavage of the two DNA strands, with both processes being completed in <5 s (Erskine et al., 1997). Under these reaction conditions, the Q69E and Q69K mutants, which had failed to produce any DNA cleavage under steady-state conditions, both gave detectable amounts of cleavage though over very much longer reaction times than that for wild-type *Eco*RV (Figure 6). The reactions of Q69E followed a sequential pathway in which the enzyme first cut one DNA strand, converting the supercoiled substrate to open-circle DNA, and then the second strand to yield the linear product (Figure 6a). The Q69K mutant was the poorest of the mutants tested here and, even after 140 min, its reaction had yielded virtually none of the linear product (Figure 6b). For both Q69E and Q69K, the decline in the concentration of the supercoiled substrate with time was fitted to a single exponential to give a rate (k_1) for the cleavage of the first strand (Table 1). For Q69E, the value for k_1 was 15 000 times lower than that with the same concentration of wild-type *Eco*RV while the value for Q69K was 30 000 times lower than wild-type. However, these are relative rates measured at one particular enzyme concentration. While 100 nM wild-type *Eco*RV is almost sufficient for its maximal rate (Erskine et al., 1997), this will not be the case for Q69K: this mutant is also defective in DNA binding (see above).

Single-turnover reactions with Q69L were carried out by either adding the enzyme to a solution containing both the DNA and Mg^{2+} or by adding $MgCl_2$ to a premix of enzyme

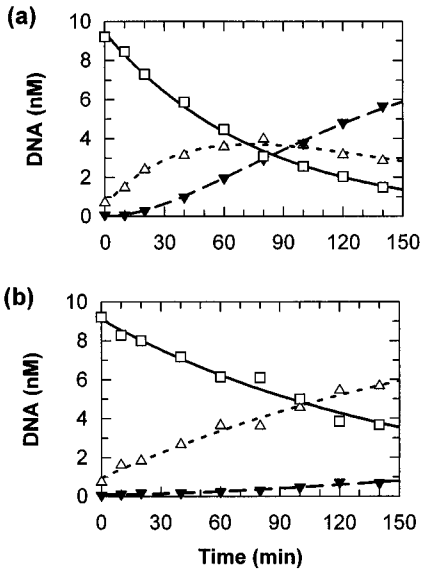

FIGURE 6: DNA cleavage by Q69E and Q69K. The reactions at 25 °C contained 10 nM pAT153 (92% supercoiled DNA) and 100 nM *Eco*RV protein, either the Q69E mutant (panel a) or the Q69K mutant (panel b), in *Eco*RV buffer with 10 mM $MgCl_2$. Aliquots were withdrawn from the reactions at the time points indicated on the *x*-axis and mixed immediately with an EDTA quench. The amounts of supercoiled (□), open-circle (△), and linear DNA (▼) were determined as in Figure 3. The lines drawn through the data-points for the utilization of supercoiled DNA are the single-exponential decays that give the optimal fits to the experimental data: the best fits were with values of 2.15×10^{-4} s^{-1} for Q69E (panel a) and 1.05×10^{-4} s^{-1} for Q69K (panel b).

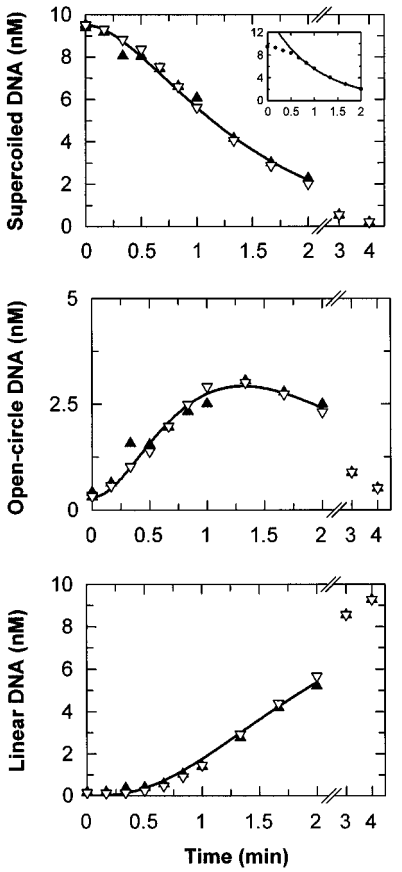

FIGURE 7: Single-turnover reactions with Q69L. The reactions at 25 °C contained 10 nM pAT153 (95% supercoiled DNA) and 100 nM Q69L protein in *Eco*RV buffer with 10 mM $MgCl_2$. Aliquots were withdrawn from the reactions at the time points indicated on the *x*-axis and mixed immediately with an EDTA quench. The amounts of supercoiled DNA (panel a), open-circle DNA (panel b), and linear DNA (panel c) were determined as in Figure 3. In all three panels, unfilled data points (△) refer to reactions where the above composition was obtained by adding the enzyme to a solution containing both the DNA and the $MgCl_2$ and filled data points (▼) refer to reactions in which the above composition was obtained by adding the $MgCl_2$ to a solution containing both enzyme and DNA. The lines drawn are the theoretical lines from eq 5 that give the optimal fits to the experimental data: for [ES + E*S] in panel a; for [E*O] in panel b; for [E*L] in panel c. For both data sets, the best fits were obtained with values of 0.025 s^{-1} for k_c, 0.028 s^{-1} for k_1, and 0.023 s^{-1} for k_2. For the insert to panel a, a single-exponential decay was fitted to all of the data points for the concentration of supercoiled DNA taken after 1 min: the single exponential is an inadequate description of the reaction since it deviates from all of the data points taken during the first minute of the reaction.

and DNA. Despite the Gln → Leu substitution causing only a 5-fold reduction in k_{cat}, the single-turnover kinetics for this mutant were monitored without recourse to the quench-flow apparatus. Instead, aliquots were withdrawn from the reactions by hand at 10 s intervals and mixed immediately with EDTA: the quenched samples were then analyzed by electrophoresis through agarose, and the amounts of the supercoiled, open-circle, and linear forms of pAT153 determined (Figures 7a, 7b, and 7c respectively). The reaction profiles for Q69L differed in only two respects from the single-turnover reactions of wild-type *Eco*RV starting with enzyme and DNA in separate solutions (see Figure 1 in the preceding paper in this issue). Firstly, the single-turnover of Q69L took ~5 min to reach completion while the wild-type reaction had taken <5 s. Secondly, wild-type *Eco*RV gave different kinetics depending on whether the reactions started from enzyme and DNA in separate solutions or from the premix of enzyme with DNA: the reactions from separate solutions had shown an initial lag phase prior to the cleavage of the supercoiled substrate while the utilization of supercoiled DNA in the premix reactions had followed a single exponential starting from zero time (Erskine et al., 1997). In contrast, Q69L gave the same kinetics regardless of whether or not the enzyme was premixed with the DNA: in both sorts of reactions, an initial lag phase was observed prior to any utilization of the supercoiled substrate (insert to Figure 7a) and an equivalent lag phase was also observed in the production of open-circle DNA (Figure 7b).

For wild-type *Eco*RV, the lag phase preceding first strand scission in the reactions starting from enzyme and DNA in separate solutions is due to the binding of the protein to the DNA, while the premix reactions start with the enzyme bound to random nonspecific sites on the DNA from which the transfer to the specific site is very rapid (Erskine et al., 1997). The Q69L mutant shows the same nonspecific DNA binding activity as wild-type *Eco*RV in the absence of divalent metal ions (Figure 1a), so its premix reaction will also start with the enzyme bound to random nonspecific sites. Consequently, the lag phase seen with Q69L in both premix reactions and in those starting from separate solutions cannot be due to the binding of the protein to the DNA. One possible explanation for the lag phase with Q69L is that the transfer from nonspecific to specific sites is much slower with the mutant than the wild-type, as has been noted with other mutants of *Eco*RV (Jeltsch et al., 1996). To test for this possibility, a series of single-turnover reactions were carried out with varied concentrations of the Q69L protein, from 50 to 400 nM: all other components were as in Figure

7. The distance along the DNA between the *Eco*RV recognition site on pAT153 and the nearest molecule of nonspecifically bound protein will be progressively reduced by increasing the protein concentration [see Table 1 in Erskine et al. (1997)], so, if the transfer determines the duration of the lag phase, then increasing concentrations of Q69L ought to result in shorter lag phases. However, the time span of the lag phase showed virtually no change across the range of concentrations tested (data not shown).

The simplest model for this additional lag phase is that it reflects a slow conformational change in the enzyme−DNA complex prior to DNA cleavage. If so, the single-turnover reaction for Q69L follows the mechanism,

$$\mathrm{ES} \xrightarrow{k_c} \mathrm{E^*S} \xrightarrow{k_1} \mathrm{E^*O} \xrightarrow{k_2} \mathrm{E^*L} \qquad (5)$$

where S, O, and L refer to supercoiled, open-circle, and linear DNA, respectively, and * denotes a conformationally altered state of the enzyme, E, and/or the DNA: k_c is the rate constant for the conformational change, and k_1 and k_2 are the constants for first and second strand scissions. The experimentally determined values for the concentration of supercoiled DNA (Figure 7a) can be equated to [ES + E*S], so that the decline in these values at increasing time points is equivalent to the increase in [C] in a two-step A → B → C scheme: the increase in [C] with time displays a lag phase whenever the rate constants for the A → B and the B → C stages are similar to each other (Gutfreund, 1995). The way in which the concentrations of all three forms of the DNA varied with time matched the expectations from eq 5 (Figure 7) and values for k_c, k_1, and k_2, were determined by the same curve fitting procedure used by Erskine et al. (1997) for the single-turnover reactions of wild-type *Eco*RV starting with enzyme and DNA in separate solutions. The rate constants for first and second strand cleavages, 0.028 and 0.023 s^{-1} (Table 1), were both about 100 times slower than those for the wild-type enzyme, 3.4 and 2.8 s^{-1} (Erskine et al., 1997). In contrast, the rate constant for the conformational change with Q69L, 0.025 s^{-1}, is >100 times smaller than that for wild-type *Eco*RV. For the wild-type enzyme, the conformational changes seen by X-ray crystallography (Winkler et al., 1993) must have rate constants of >3 s^{-1}, since they do not limit the rates for phosphodiester hydrolysis.

While eq 5 accounts for the single turnovers of Q69L (Figure 7), it indicates that the mutant cleaves the two DNA strands without an intervening dissociation from the DNA, in contradiction to the steady-state kinetics for Q69L which show clearly that the enzyme must dissociate from the nicked DNA before cutting the second strand (Figure 3). However, under single-turnover conditions with high concentrations of enzyme, the plasmid will bind many molecules of the enzyme and the first and second strand cleavages will not necessarily be carried out by the same molecule. One molecule of enzyme, with Mg^{2+} ions at only one active site, might cut one strand and then move away from the recognition site by linear diffusion. Another molecule already bound to the DNA could then transfer to the recognition site to cleave the second strand. The substrate for the second enzyme is nicked DNA, so its kinetics may well differ from those for the first enzyme on the intact duplex. In contrast, under steady-state conditions with the enzyme at a lower concentration than the DNA, the DNA will not be multiply bound by enzyme and the cleavage of the second strand will require a new association of protein onto the DNA.

Correlation to Structure. Random mutagenesis, followed by a genetic screen for loss of activity, makes no preconceptions about the roles of individual amino acids in protein function. It thus has the potential to demonstrate that certain residues are crucial to the function of the protein even when no particular role can be assigned to that amino acid from the crystal structure. Gln69, on the Q loop of the *Eco*RV restriction endonuclease, illustrates this point. The Q loop approaches the minor groove of the DNA and, as a result of its conformational plasticity, manages to make numerous contacts to the sugar−phosphate backbone with both specific and nonspecific DNA (Winkler et al., 1993). Random mutagenesis covering all of the amino acids in the Q loop gave more mutants at Gln69 than anywhere else, so this is likely to be the key residue on this loop. However, the crystal structures of *Eco*RV solved to date (Winkler et al., 1993; Kostrewa & Winkler, 1995) provide no obvious explanation for why Gln69 should be so crucial for *Eco*RV activity. From the collection of null mutants isolated by Vipond and Halford (1996), the proteins selected for further study included one with an acidic residue, Q69E; another with a basic residue, Q69K; the third with a neutral residue, Q69L. The proteins showed distinct properties, depending on the nature of the amino acid at this position.

The substitutions with either acidic or basic residues virtually abolished catalytic activity (Table 1). It was therefore impossible to identify which step(s) in the reaction pathway for *Eco*RV are affected by these mutations. Q69K binds to DNA specifically at the *Eco*RV recognition site in the presence of Ca^{2+} but with a lower affinity than wild-type *Eco*RV. While this may contribute to the low activity for Q69K, its binding affinity is reduced by a much smaller factor than its rate for phosphodiester hydrolysis. In contrast, Q69E has a severely reduced affinity for nonspecific DNA while showing virtually unaltered binding to the *Eco*RV site in the presence of Ca^{2+} ions, a behavior not seen before with any other mutant of *Eco*RV. For wild-type *Eco*RV, the binding to nonspecific DNA is an essential step on the route to the recognition site (Taylor et al., 1991; Jeltsch et al., 1996; Erskine et al., 1997). However, the impaired binding of Q69E to nonspecific DNA cannot be solely responsible for its low DNA cleavage activity in the presence of Mg^{2+} ions, since the activity of this mutant with Mn^{2+} is similar to wild-type *Eco*RV (Vipond & Halford, 1996).

The mutant carrying Leu in place of Gln69 was sufficiently active to allow for an analysis of its mechanism of action and this revealed differences from wild-type *Eco*RV at several stages in the reaction pathway. In terms of reaction mechanism, the most striking property of Q69L is perhaps the slow conformational change that precedes DNA cleavage (Figure 7). Wild-type *Eco*RV undergoes extensive conformational changes as it binds to nonspecific DNA and again to specific DNA, as indicated by the crystal structures of the free protein and its complexes with either sort of DNA (Winkler et al., 1993). Discrete changes also occur upon the binding of Mg^{2+} to the specific complex (Kostrewa & Winkler, 1995). Yet all of the conformational changes in the wild-type system must be rapid events since they do not limit the rates for the subsequent DNA cleavage reactions (Baldwin et al., 1995; Erskine et al., 1997). The activation energy barrier for one or more of these conformational

changes must be much higher with Q69L than with wild-type *Eco*RV.

Other differences between Q69L and native *Eco*RV include its reduced affinity for Mg^{2+}, as judged by the minimal concentration of $MgCl_2$ for maximal activity (Figure 5), and its severely reduced rate constants for phosphodiester hydrolysis (Figure 7, Table 1). One possible explanation for these alterations is that the Gln → Leu substitution prevents the Q loop from being able to tuck into the minor groove and that this displacement alters the position of the segment of polypeptide chain that follows directly after the Q loop. The latter includes Asp74, a key ligand for both Mg^{2+} ions at the active site of *Eco*RV (Kostrewa & Winkler, 1995) that is essential for *Eco*RV activity (Selent et al., 1992; Vipond et al., 1996). The displacement of the Q loop from its position in the wild-type enzyme could therefore account for both the reduced affinity for Mg^{2+} and for the reduced rates for phosphodiester hydrolysis. The requirement for precise positioning of the Q loop had been noted previously in studies with a DNA analog where the thymidine adjacent to the scissile bond had been replaced with 4′-thiothymidine, a derivative with sulfur in place of the oxygen in the furanose ring. Oligonucleotides containing this derivative are not cleaved by *Eco*RV (Hancox et al., 1993), and X-ray crystallography on *Eco*RV bound to such an oligonucleotide in the presence of $MgCl_2$ showed that the Mg^{2+} ions seen at the active site of wild-type *Eco*RV were not present with this derivative (D. Kostrewa and F. Winkler, personal communication). However, the Q loop had been displaced as a result of the sulfur atom in the 4′-thiothymidine occupying part of the position for Asn70.

An alternative explanation for the behavior of the mutants at Gln69 comes from the juxtaposition of Gln69 in one subunit of the dimeric protein against Thr37 in the other subunit. Thr37 forms a hydrogen bond to the phosphate two nucleotides downstream of the cleavage site (Winkler et al., 1993), but the substitution of Thr37 with Ala reduces DNA cleavage activity by much more than the equivalent substitutions at any other residue in *Eco*RV that contacts a DNA phosphate (Wenz et al., 1996). Hence, it was suggested that the extraordinarily large effect of the T37A mutation was due to the displacement of Gln69 in the other subunit of the protein dimer and that this in turn affected the position of Asp74 in that second subunit (Wenz et al., 1996). Thr37 is part of a long α-helix at the bottom of the DNA-binding cleft in *Eco*RV. However, this α-helix carries several residues that play key roles in catalysis, including Asp36 (N. Stanford and S. E. Halford, unpublished) and Glu45 (Selent et al., 1992; Vipond et al., 1995, 1996). Consequently, the loss of activity upon disrupting the Gln69−Thr37 interface is not necessarily due to a movement of the Q loop. Instead, mutations at either Gln69 (this study) or Thr37 (Wenz et al., 1996) might affect the position of the α-helix that carries Thr37 and thus displace both Asp36 and Glu45. Since Glu45 is a ligand for one of the Mg^{2+} ions at the active site (Kostrewa & Winkler, 1995; Baldwin et al., 1995), its displacement is also likely to weaken the affinity for Mg^{2+}. The distinction between this proposal for the effect of mutations at Gln69 and that given above, the displacement of the Q loop, requires crystal structures for the mutant proteins. Such studies are currently underway (M. Thomas and R. L. Brady, personal communication). Preliminary results from X-ray crystallography on Q69L indicate that the Gln → Leu mutation has virtually no effect on the position of the Q loop while the α-helix carrying Asp36, Thr37, and Glu45 is displaced from its position in wild-type *Eco*RV.

ACKNOWLEDGMENT

We thank Leo Brady, Bernard Connolly, Mark Thomas, Alfred Pingoud, Barry Vipond, Fritz Winkler, and everyone in this laboratory for advice, discussions, and unpublished data.

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BI970156K