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Determinants of *Eco*RI endonuclease sequence discrimination

(hydrogen bonds/cassette mutagenesis/restriction/kinked DNA)

M. C. Needels*†, S. R. Fried*, R. Love‡, J. M. Rosenberg‡, H. W. Boyer*, and P. J. Greene*§

*Department of Biochemistry and Biophysics, Unversity of California at San Francisco, San Francisco, CA 94143; and ‡Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260

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ABSTRACT **The arginine at position 200 of *Eco*RI endonuclease is thought to make two hydrogen bonds to the guanine of the sequence GAATTC and thus be an important determinant of sequence discrimination. Arg-200 was replaced by each of the other 19 naturally occurring amino acids, and the mutant endonucleases were assessed for activities *in vivo* and *in vitro*. The mutant endonuclease with lysine at position 200 exhibits the most *in vivo* activity of all the position 200 mutants, although the *in vitro* activity is less than 1/100th of wild-type activity. Five other mutants show more drastically reduced levels of *in vivo* activity (Cys, Pro, Val, Ser, and Trp). The Cys, Val, and Ser mutant enzymes appear to have *in vivo* activity which is specific for the wild-type canonical site despite the loss of hydrogen bonding potential at position 200. The Pro and Trp mutants retain *in vivo* activity which is independent of the presence of the *Eco*RI methylase. In crude cell lysates, only the Cys mutant shows a very low level of *in vitro* activity. None of the mutant enzymes show a preference for alternative sites in assays *in vitro*. The implications of these results are discussed.**

To elucidate the molecular basis of one example of the interaction of proteins with specific sequences of DNA, we are investigating the DNA binding and cleavage mechanisms of the *Eco*RI endonuclease. The catalytically active form of the endonuclease is a highly stable dimer composed of identical 31-kDa subunits of known amino acid sequence. The enzyme recognizes the double-stranded DNA sequence 5′-GAATTC-3′ and cleaves the phosphodiester bonds on both strands between the G and A residues. Mg^{2+} is required for cleavage; in its absence the enzyme forms a stable sequence-specific complex with DNA containing the substrate site ($K_d \approx 10$ pM) (1, 2). The endonuclease is found in *Escherichia coli* with a companion methylase which transfers a methyl group to the N6 of the central adenine of the substrate sequence, rendering it resistant to cleavage (3).

The 3.0-Å-resolution structure of *Eco*RI endonuclease bound to its canonical sequence (GAATTC) implicates hydrogen bonding of three amino acids on each subunit (Glu-144, Arg-145, and Arg-200) to the purine bases in the major groove as the primary determinants of sequence specificity (4). Glu-144 and Arg-145 from opposite subunits form four hydrogen bonds with the two adjacent adenines of a half site (GAA), while Arg-200 forms two hydrogen bonds with the terminal guanine. This is consistent with the proposal that sequence discrimination can be provided by the protein making two hydrogen bonds per base pair in the sequence GAATTC (reviewed in ref. 4).

The protein–DNA interface of the enzyme–substrate complex contains an alternating array of positive and negative charges which are thought to be important for maintaining the local structure of this interface (4). The amino acids forming sequence-specific hydrogen bonds with bases are part of this array, as is Arg-203 and phosphates of the DNA backbone. Our initial model for sequence discrimination predicted that some substitutions of the three residues forming hydrogen bonds to bases would alter specificity at the base (or bases) contacted by that residue, provided that the charge array were not perturbed. Substitution of Glu-144 by the basic side chain of lysine (5) or the neutral side chain of glutamine (6), replacements which might alter hydrogen bonding, also affects the charge array, and these mutant enzymes are devoid of all apparent activity *in vivo* and *in vitro*. Glu-144 and Arg-145 are located at the subunit interface, and the lysine replacement at position 144 prevents dimerization (5). Mutant endonucleases with conservative substitutions which would preserve the charge array and thus the local structure of the protein and the protein–DNA interface (Glu-144 to Asp, Arg-145 and Arg-200 to Lys) retain catalytic activity at -GAATTC- (6). In the case of Lys-200, catalytic activity is decreased ≈100-fold. The most striking effect of these conservative substitutions is an alteration in the kinetic pathway. The reaction of the wild-type endonuclease at its canonical site is balanced so that doubly cleaved DNA is the predominant product of a single binding event. This pathway is influenced by temperature and flanking sequence; however, optimum conditions favor double strand cleavage (reviewed in ref. 1). For the Lys-200 mutant, dissociation rates from DNA are faster than those of the wild-type enzyme, and the release of the nicked intermediate is especially enhanced, so that double-strand cleavage is no longer the predominant outcome of a single binding event.

A dual function of Arg-200 has been proposed (4): (*i*) it discriminates guanine from adenine, thymidine, and cytosine in the sequence GAATTC through two hydrogen bonds, and (*ii*) it is a crucial part of the charge array (see above). To understand more fully the role of the arginine at position 200, we have replaced the wild-type residue with each of the other 19 naturally occurring amino acids and have evaluated the functionality of the resultant mutant endonucleases.

MATERIALS AND METHODS

Materials. All restriction enzymes, polynucleotide kinase, and DNA ligase were obtained from New England Biolabs. The large fragment of DNA polymerase I (the Klenow fragment) was purchased from Bethesda Research Laboratories. Enzymes were used according to the suppliers' instructions. Oligonucleotides were supplied by the Biomolecular Resource Center at the University of California, San Francisco.

Strains and Plasmids. *E. coli* D12.10 (*hsdM, hsdR, recA13, ara14, proA2, lacY1, lacI^Q, galK2, supE44*) was used for preparation of plasmid DNA and *Eco*RI endonuclease. The plasmid pCasII was utilized for cassette mutagenesis and expression of the *Eco*RI endonuclease. pCasII was derived

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Abbreviation: IPTG, isopropyl β-D-thiogalactoside
†Present address: Roche Diagnostic Systems, Nutley, NJ 07110.
§To whom reprint requests should be addressed.

from pKGS (7) by engineering several unique restriction sites into the *Eco*RI endonuclease gene by mismatched primer mutagenesis (8). This plasmid contains the endonuclease gene under control of the *lac* promoter so that the enzyme is inducible by isopropyl β-D-thiogalactoside (IPTG). The presence of the wild-type *Eco*RI endonuclease in a cell is lethal unless the cognate methylase is also present. Therefore, unless otherwise noted, strains harbored a derivative of pSC101 which contains the gene for the *Eco*RI methylase (9).

pBRUC19 (a gift from James Hartley, Bethesda Research Laboratories) is a high copy number derivative of pBR322 and was used as a substrate for enzyme assays.

Cassette Mutagenesis. Site saturation at amino acid position 200 of *Eco*RI endonuclease followed the procedure of Schultz and Richards (10).

Mutations at the codon for Arg-200 were identified by determining the sequence spanning the cassette oligonucleotide. DNA sequence was analyzed as described by Yanofsky *et al.* (5). Plasmids encoding each of the 20 amino acids at codon 200 were isolated and purified by retransformation into *E. coli* D12.10. The sequence spanning the cassette oligonucleotide was resequenced to verify the identity of the mutation.

***In Vivo* Assays.** Intracellular cleavage of DNA in *E. coli* by the *Eco*RI endonuclease is lethal, and the reduced activity of mutant endonucleases has a deleterious effect on growth. This property provides a sensitive *in vivo* assay for the activity of mutant endonucleases.

Plating phenotypes. Relative colony size was assessed in the presence or absence of IPTG, which induces high expression of the endonuclease.

Differential viability curves. Growth in liquid cultures was compared in media containing or lacking IPTG.

***In Vitro* Assays.** Cleavage of DNA and *Eco*RI protein levels were assayed in crude cell lysates of wild type and the 19 mutant strains.

Preparation of lysates for in vitro *analysis. E. coli* D12.10 strains harboring a plasmid encoding mutant endonuclease were grown to an OD_{600} of ≈0.5 in liquid medium and induced with 1 mM IPTG for 45 min. Cell lysates were prepared by sonication of these cells (2). Total protein in lysates was measured by the method of Bradford (11), using bovine gamma globulin as a standard.

Immunological quantitation of Eco*RI endonuclease mutants.* Levels of mutant or wild-type endonuclease in crude cell lysates were determined by densitometric scanning of slot-blots which were prepared as described (12). Western blotting was performed as described by Yanofsky *et al.* (5).

Enzyme assays. Three types of reaction conditions were used for *in vitro* enzyme activity assays. (*i*) "Physiological" salt: 6 mM Tris·HCl, pH 7.4/6 mM $MgCl_2$/6 mM 2-mercaptoethanol/110 mM NaCl is the standard reaction buffer for the wild-type enzyme. (*ii*) Low salt: low salt is identical to "physiological" salt except NaCl is 10 mM. (*iii*) "Star": this buffer was higher pH, lower $MgCl_2$, and glycerol [20 mM 1,3-bis [tris(hydroxymethyl)methylamino]propane·HCl, pH 8.5/2 mM $MgCl_2$/20% (vol/vol) glycerol/20 mM NaCl].

For reactions with crude cell lysates, the substrate was linear pBRUC19 (1 nM), and the total volume was 20 μl. An appropriate dilution of cell lysate was added directly to the reaction mixture, so that mutant *Eco*RI endonuclease concentrations were between 2 and 4 nM (as judged by the immunological quantitation). The DNA cleavage activity observed in cell lysates of mutant strains was due to the endonuclease protein, since this activity was sensitive to immunological inactivation by polyclonal antibody raised against wild-type *Eco*RI endonuclease.

Purification and assay of [Pro²⁰⁰]Endonuclease. [Pro²⁰⁰]-Endonuclease was purified to near homogeneity following standard *Eco*RI endonuclease purification procedures (2). Nicking activity was assayed by utilizing supercoiled pBRUC19 as a substrate. Cleavage at sites other than -GAATTC- was assayed by utilizing λ phage DNA as an alternative high molecular weight substrate.

RESULTS

Isolation of 19 Mutants with Substitutions at Position 200. Plasmids containing single inserts of the mutagenic oligonucleotide were identified by restriction analysis. The cassette region of 119 of these plasmids was sequenced. The first round of cassette mutagenesis yielded plasmids with codons for 18 amino acids. The last two mutants (His and Tyr) were isolated from a second round of cassette mutagenesis employing oligonucleotides coding for only His and Tyr at position 200.

Determination of the Level of Endonuclease Protein in the Mutant Strains. Immunological quantitation of *Eco*RI endonuclease mutants showed that all mutant proteins are present at a level equal to or greater than that of wild type. A single band of a species the same size as the wild-type endonuclease monomer was observed after electrophoresis of mutant lysates on denaturing gels, indicating that the mutant enzymes were not subject to degradation.

Assay of Endonuclease Activity by Plating Phenotype. Plasmids encoding the mutations at position 200 were used to transform *E. coli* D12.10 strains with or without the *Eco*RI methylase gene (in trans). The effect of the mutant genes on cell growth was evaluated by comparing colony size on medium containing or lacking IPTG (Table 1). Thirteen of the 19 mutant strains survive in the absence of methylase, and growth is unaffected by IPTG induction of the endonuclease. This plating phenotype is indicative of no *in vivo* endonuclease activity and is defined as null. The Lys-200 mutant exhibits the greatest *in vivo* activity among the remaining mutants, and *Eco*RI methylase is required for survival. *E. coli* strains harboring the remaining 5 mutants survive in the absence of methylase, but IPTG induction of these endonucleases decreases growth. These 5 endonuclease mutants fall

Table 1. Plating phenotypes

	With *Eco*RI methylase						Without *Eco*RI methylase					
	30°C		37°C		41°C		30°C		37°C		41°C	
Mutant	K	KI	K	KI	K	KI	K	KI	K	KI	K	KI
	Wild type											
Arg	±	−	±	−	±	−	−	−	−	−	−	−
	Lys-200											
Lys	+	+	+	+	+	+	−	−	−	−	−	−
	Null											
Leu	+	+	+	+	+	+	+	+	+	+	+	+
	Methylase-protected											
Cys	+	+	+	+	+	+	+	−	+	−	+	±
Val	+	+	+	+	+	+	+	−	+	∓	+	+
Ser	+	+	+	+	+	+	+	±	+	+	+	+
	Methylase-neutral											
Pro	+	±	+	−	+	−	+	±	+	−	+	−
Trp	+	+	+	±	+	∓	+	+	+	+	+	±

Plating phenotypes were determined by suspending individual colonies in 100 μl of liquid medium and spreading onto agar plates lacking or containing 1 mM IPTG, to induce high endonuclease expression. Mutants were tested in parallel in *E. coli* D12.10 containing or lacking the *Eco*RI methylase gene. Plates were incubated at 30°C, 37°C, or 41°C. All mutants not shown exhibit the null phenotype. K, medium containing kanamycin sulfate at 75 μg/ml; KI, medium containing both kanamycin sulfate and 1 mM IPTG; +, "normal" colony size (the largest size seen); ±, slightly reduced colony size relative to +; ∓, greatly reduced colony size relative to +; −, no colonies.

into two classes. The Cys, Val, and Ser mutant strains are protected by *Eco*RI methylase and are designated methylase-protected. The methylase-protected phenotype is most pronounced at 30°C, indicating some temperature sensitivity of the mutant endonucleases. The relative *in vivo* activity of these mutants is Cys > Val > Ser, with the Cys mutant resulting in lethality while the Ser mutant exhibits minimal but consistent colony size reduction. The Pro and Trp mutant strains are not protected by the presence of the methylase and are designated methylase-neutral. The methylase-neutral phenotype is most pronounced at 41°C. The Pro mutant results in lethality and the Trp mutant results in minimal colony size reduction. The lack of protection by the *Eco*RI methylase is what one would expect if a mutant endonuclease cleaved sites other than the canonical sequence. However, other considerations (see below) suggest an alternative explanation.

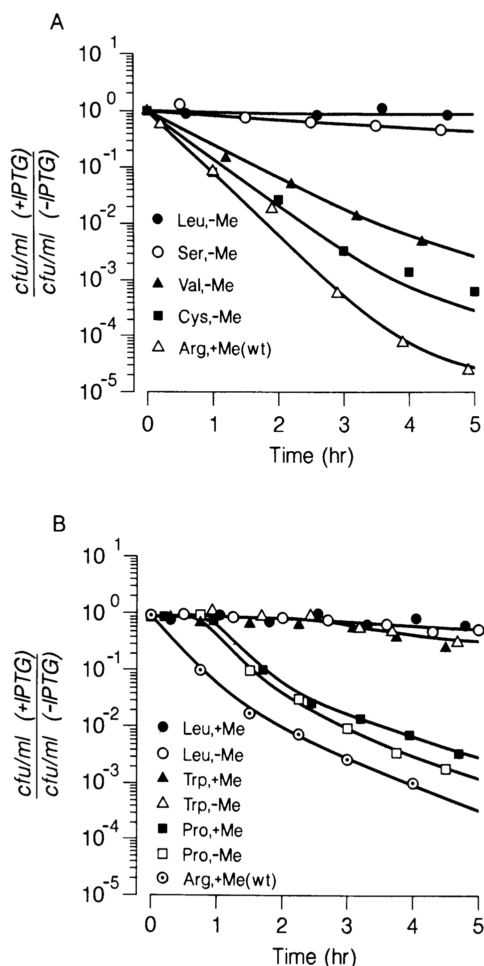

FIG. 1. Differential viability curves. (*A*) Survival curves of mutants eliciting a methylase-protected phenotype. (*B*) Survival curves of mutants eliciting a methylase-neutral phenotype. −Me and +Me indicate that the cells lack or contain, respectively, the gene for the *Eco*RI methylase. wt, Wild type. Twenty-five milliliters of liquid medium was incubated at the temperature of the experiment (30°C or 41°C) and inoculated from a logarithmic-phase culture to an OD_{600} of ≈0.015. Duplicate cultures were grown in parallel for each mutant in a shaking water bath; one was made 1 mM in IPTG after growing to an OD_{600} of 0.04. Aliquots were withdrawn periodically and appropriate dilutions were spread onto agar medium. After overnight incubation at 37°C, individual colonies were counted and the number of colony-forming units (cfu) per ml as a function of time was determined. The fraction of survivors after induction was determined by dividing the number of cfu/ml in an induced culture by the number in an uninduced culture. Two determinations of cfu/ml were performed for each time point. The time indicated is the time after induction.

Assay of Endonuclease Activity by Differential Viability in Liquid Culture. Liquid cultures of D12.10 cells harboring mutant endonuclease genes (except Lys-200) were analyzed for viable cells at various times after induction. A representative null mutant (Leu-200) was employed as a control. Viability was assayed at the temperature where the plating phenotype for a given mutant was most pronounced (see Table 1). The viability curves are shown in Fig. 1.

The differential viability curves of the various mutant strains corroborate results of the plating phenotype analysis. In addition, these experiments indicate that for the methylase-protected mutants the effect on cell growth begins immediately after induction, whereas methylase-neutral mutants show a lag (Fig. 1). Viable counts plotted as a function of time demonstrate that the Cys, Pro, and Val mutant strains experience net cell killing upon induction. However, for the Trp and Ser mutant strains, it cannot be determined whether the cultures experience net cell killing or merely a reduction in growth rate. In this assay, as in the plating assay, the Trp and Ser mutants exhibit only marginal activity.

DNA Cleavage *in Vitro* by the *Eco*RI Endonuclease Mutants. Crude extracts of strains containing all mutant and wild-type endonucleases were assayed for their ability to cleave linear double-stranded DNA (Table 2). pBRUC19 digested with *Ava* I was employed as the substrate. Cleavage of the single *Eco*RI site of this substrate results in the formation of diagnostic fragments. Three reaction conditions were tested: "physiological" salt, low salt, and "star" (see *Materials and Methods*). None of the mutants studied here showed double-strand DNA cleavage activity under conditions of "physiological" salt. Under low-salt conditions, the Lys-200 mutant showed wild-type site-specific double-strand cleavage activity (ca. 10% of wild-type activity under the same conditions). The low-salt buffer enhances electrostatic interactions between the endonuclease and DNA, and several mutant enzymes show increased activity in this buffer (ref. 2; unpublished observations). Under "star" conditions, the Lys mutant exhibits greater cleavage activity than wild type, and double-strand cleavage by the Cys mutant is apparent at a very low level. "Star" conditions enhance the cleavage of noncanonical (star) sites by the wild-type enzyme (4, 13). These conditions also increase the activity of several mutants at the canonical site. The lower ionic strength and added glycerol enhance the electrostatic component of DNA binding, and can thereby partially compensate for lower binding energy at noncanonical sites or by mutant enzymes at the canonical site; however, since both the substrate and the enzyme could be separately affected by these buffer changes, factors in addition to enhanced electrostatic energy could be contributing to increased activity in this buffer. Double-strand cleavage at sites other than GAATTC was not ob-

Table 2. Relative *in vitro* activities of mutant and wild-type *Eco*RI endonuclease

Reaction conditions	Relative endonuclease activity		
	Arg-200 (wt)	Lys-200	Cys-200
Physiological salt	1.0	<0.005*	<0.003*
Low salt	0.3	0.03	<0.003*
Star	0.2	3	0.003

Reaction buffers are as described in the text. All activities are shown relative to the wild-type (wt) activity in "physiological" salt. Reactions were at 30°C, 37°C, and 41°C for times ranging from 1 min to 2 hr, depending on the dilution and the mutant used. Reaction products were separated by electrophoresis on 1% agarose gels. Gels were stained with ethidium bromide and photographed, and the reactions were quantitated by scanning the negatives with a Joyce-Loebl Ephortec densitometer.

*No activity was detectable in these extracts under the conditions assayed, so the detection limit of the assay is indicated.

served for wild type or any of the mutants. Non-canonical-site double-strand cleavage by wild-type *Eco*RI endonuclease requires a high concentration of enzyme as well as "star" conditions. Single-strand cleavage could not be assayed because of endogenous nucleases in the cell lysates. The endonuclease activities were all sensitive to preincubation of cell lysates with *Eco*RI antibody.

The [Pro^{200}]endonuclease was purified to eliminate nonspecific nucleases and assayed on supercoiled pBRUC19 to detect possible nicking activity. λ phage DNA was used as a substrate to search for double-strand cleavage at noncanonical sites. No DNA cleavage by the [Pro^{200}] endonuclease was detectable on either substrate.

DISCUSSION

The phenotype of D12.10 expressing mutant endonucleases provides a sensitive assessment of *in vivo* DNA cleavage and was used to evaluate the mutants at position 200. Lethality or reduction in colony size upon induction of mutant endonucleases appears to be a reasonable assessment of *in vivo* DNA cleavage, since the *in vivo* activity of the two mutants with the most pronounced effects on phenotype (Lys, Cys) correlates with the level of *in vitro* DNA cleavage. The lack of *in vivo* activity by 13 of 19 mutants at position 200 and the dramatic reduction of activity in the remaining mutants highlight the critical role of arginine at this position for enzyme function.

The Lys mutant has the greatest activity *in vivo* and *in vitro*, although the latter is approximately two orders of magnitude less than wild-type activity under standard reaction conditions. A Lys residue at position 200 could form two hydrogen bonds to guanine in a manner analogous to Arg, although the Lys hydrogen bonds would probably be weaker (14). The conservation of positive charge maintains electrostatic interactions similar to those of the wild-type enzyme–DNA complex, and sequence discrimination by the Lys mutant is not compromised. The Cys mutant has greatly reduced *in vitro* cleavage activity for the canonical sequence (0.3% of wild type). The observed activity is just above the detection limits of the assay (see Table 2); hence, we could not determine whether or not additional sites could be cleaved at lower rates. The Val and Ser mutants have no detectable *in vitro* activity in crude cell extracts; however, the phenotype of these mutant strains (i.e., methylase-protected) indicates that there may be DNA cleavage *in vivo* at the canonical sequence.

Why do Cys, Ser, and Val have residual activity while 13 other substitutions, having both larger and smaller chemically diverse side chains, are nulls? Without further information one can only rationalize an explanation with the aid of the following considerations. All of the large neutral and negatively charged residues result in null enzymes. Arg has a bulky side chain, and substitution with the large neutral, acidic, or hydrophobic side chains into the structural model does not result in obvious steric problems. Since Arg-200 is a critical member of the charge array (described above and in ref. 4), it is likely that these substitutions would result in perturbation of the local structure of the protein in a manner analogous to that proposed for the Lys and Gln substitutions at position 144 (5, 6). In addition, the large hydrophobic and negatively charged residues might present an unfavorable interaction at the substrate interface (e.g., charge incompatibility or water exclusion).

If Cys, Val, or Ser were substituted directly into the *Eco*RI–DNA complex, the side chain of each would extend to the Cγ of arginine. The proximal end of the Arg-200 side chain makes extensive contacts with the side chain of Arg-203; in addition, it appears to be partially sandwiched between residues in the region of 141–142 and in the region of 197. Some of these relationships are shown in Fig. 2. Cys, Val, and Ser might be able to maintain the overall protein structure via similar packing arrangements in this region, while smaller side chains (Gly and Ala) might not provide the necessary van der Waals contacts to support the structure. The temperature sensitivity of the Cys, Ser, and Val mutants is consistent with the proposal that packing is significant for the stability of the protein. Because it is structurally and/or chemically similar, Thr might be expected to be a part of this series. If so, its activity is undetectable.

The *Eco*RI endonuclease is thought to undergo conformational changes upon binding to substrate DNA (2, 4), and recent crystallographic results show that when Mg^{2+} is introduced into the *Eco*RI–DNA complex and catalysis occurs, there are further conformational changes in the protein (J.M.R., unpublished data). It is possible that Cys, Val, and Ser perturb this series of changes to a lesser degree than other substitutions.

The wild-type sequence discrimination of the Cys, Val, and Ser mutants is maintained even though none of these residues has the ability to form hydrogen bonds to guanine; therefore other factors must be contributing to sequence discrimination. For example, sequence-dependent structure of the

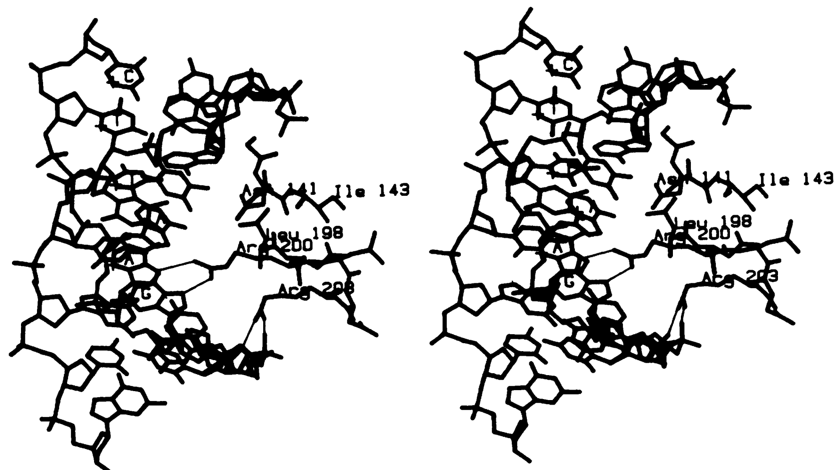

FIG. 2. Stereoview of a portion of the *Eco*RI–DNA complex in the vicinity of Arg-200. Nine base pairs of DNA are shown, including the cognate guanine. The bases in one strand of the substrate site are labeled, GAATTC. Amino acids 140–143 (Gly-Asn-Ala-Ile) and 198–203 (Leu-Asn-Arg-Leu-Asp-Arg) are shown. Hydrogen bonds between Arg-200 and guanine and ion pairs between Arg-203 and the phosphate backbone are represented by light lines. Leu-198, Arg-203, Asn-141, and Ala-142 form the immediate neighbors of Arg-200 as discussed in the text.

DNA substrate could provide redundancy of discrimination. Sequence-dependent variations in DNA structure have been demonstrated in crystalline and solution structures for several short synthetic oligonucleotides, and it has been postulated that these variations could contribute to sequence, discrimination by proteins (15, 16). The DNA in the *Eco*RI complex is distorted by the presence of neokinks (4). It has been suggested that the energy required to drive the DNA into the kinked conformation could depend on base sequence, thereby providing an indirect mechanism of sequence discrimination (17). The discrimination mechanism of the wild-type endonuclease would involve two components, (*i*) direct protein–base hydrogen bonds and (*ii*) utilization of sequence-dependent conformation in the DNA—i.e., the entire recognition is overdetermined. This model postulates that the sequence GAATTC is the sequence that most easily adopts the observed kinked conformation. The Cys-200 mutant therefore preferentially cleaves GAATTC in spite of the loss of hydrogen bonds to the G·C base pair. Recent results with repressors suggest that indirect recognition mechanisms may be general features of sequence specific DNA–protein interactions (18–20). DNase I (21) also utilizes DNA conformation in sequence discrimination.

Two other mutants, Pro-200 and Trp-200, exhibit a reduction in cell growth which is not reversed by the presence of the *Eco*RI methylase. If these mutants cleave DNA, the lack of protection by the methylase suggests cleavage at sequences other than the canonical site. The Pro-200 mutant has *in vivo* activity as pronounced as that of the Cys mutant, but DNA cleavage activity has not been detected in crude cell lysates or partially purified enzyme. Also, a lag is observed in the differential growth curve, and this result suggests that a mechanism other than DNA cleavage may be responsible for the methylase-independent lethality (22). For example, the detrimental effect on cell growth might result from extensive aggregation of mutant protein. The cold sensitivity of the methylase-neutral phenotype could be due to inhibition of aggregation (23), and the time lag in the differential viability curve might result from the need to build up to a threshold intracellular concentration of mutant endonuclease before aggregation occurs. Because of the failure to detect DNA cleavage by purified protein and the lag in the differential growth curve, it is likely that the phenotype exhibited by the Pro and Trp substitutions does not result from cleavage of noncanonical sites.

Saturation mutagenesis of position 200 highlights the difficulty of altering the specificity of the *Eco*RI endonuclease through single amino acid replacement. This could be a general feature of restriction enzymes that distinguishes them from repressors (24–26). It should be noted that a specificity change would be lethal in a restriction enzyme since the companion methylase would no longer protect chromosomal DNA. The resistance of *Eco*RI endonuclease to mutationally induced specificity changes could have arisen from this evolutionary pressure.

In summary, Arg-200 is a crucial residue in the function of the *Eco*RI endonuclease. The activity of the most conservative replacement, Lys, is reduced two orders of magnitude, and most replacements result in totally inactive proteins. With some substitutions (i.e., Cys and Val) removing the hydrogen bonding potential at position 200 significantly reduces but does not eliminate the ability of *Eco*RI endonuclease to specifically cleave the sequence GAATTC. Since cleavage specificity is determined by factors in addition to direct hydrogen bonding between Arg-200 and the cognate guanine, we propose that sequence discrimination by this potentially lethal enzyme is redundant and overdetermined.

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