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Mode of Action on *Eco*RI Restriction Endonuclease: *Eco*RI and *Eco*RI Variant N199H have Active Monomeric Forms

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The N199H variant of the *Eco*RI endonuclease has about twice the catalytic activity of the wild-type. A comparison of their biochemical characteristics, using synthetic oligonucleotides 5′-dAAAACTTAAGAA-AAAAAAAAA-3′ (KA) and 5′-dTTTTTGAATTCT-TTTTTTTTT-3′ (KT), helps to define the cleavage reaction pathway of these enzymes. Both *Eco*RI and *Eco*RI variant N199H were found to cleave single-stranded KA or KT about three times faster than the double-stranded forms, although the KT oligonucleotide was more susceptible. Using the ssDNA substrate in kinetic analyses, lower K_m values were obtained for the N199H variant than for the wild-type at low (50 mM), as well as high (200 mM), sodium chloride concentrations. This difference between the endonucleases is attributed to a greater accessibility for the substrate by the variant, and also a higher affinity for the DNA backbone. It also appears that the relative activities of the two enzymes, particularly at high ionic strength, are proportional to their populations in the monomeric enzyme form. That is, according to gel filtration data, half of the N199H molecules exist as monomers in 200 mM NaCl, whereas those of the wild-type are mainly dimeric. Consequently, the Asp199 residue of the *Eco*RI endonuclease may be implicated in the protein–protein interaction leading to dimerization, as well as in coupling to DNA substrates. In summary, it is proposed that active monomeric endonuclease molecules, derived from the dimeric enzyme, recognize and form a complex with a single stranded form of the DNA substrate, which then undergoes nucleophilic substitution and cleavage.

Keywords: *Eco*RI, *Eco*RI variant N199H, Mode of action, Monomeric form.

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Introduction

The *Eco*RI restriction endonuclease binds DNA in a nonspecific manner and diffuses linearly to find its target site (Terry *et al.*, 1983; Terry *et al.*, 1985; Von Hippel and Berg, 1986). A specific DNA-protein interaction at the target site induces a conformational change in the *Eco*RI endonuclease that allosterically binds and cleaves DNA substrate (Kim *et al.*, 1984; King *et al.*, 1989). Recently, based on structural and mechanistic studies (Jeltsh *et al.*, 1992), a mechanism proposed for DNA cleavage by *Eco*RI endonuclease has suggested that nucleophilic substitution is activated by the 3′-phosphate group for hydrolysis of phosphodiester linkages. However, the individual reaction steps involving the binding and recognition of DNA by the *Eco*RI endonuclease have not be resolved.

Electrostatic interactions between the acidic phosphodiester backbone of DNA and basic amino acids on the protein surface (Goppelt *et al.*, 1980; Terry *et al.*, 1983; Jen-Jacobson *et al.*, 1983) hold the enzyme in place so that it effectively slides, or scans, along an isopotential surface on the DNA. This electrostatic attraction is sensitive to changes in the ionic strength. At low salt concentration, the protein has a reduced dissociation rate for nonspecific interaction with the substrate and, therefore, is able to diffuse a great distance along the DNA strand. When, at high ionic strength, the attraction of the protein for the target DNA is diminished, the dissociation rate of the protein increases, and the target sequence becomes a three-dimensional search (Leirmo *et al.*, 1987)

At a salt concentration above 0.2 M, the wild-type *Eco*RI endonuclease forms a dimer or tetramer, depending on the protein concentration (Modrich and Zabel, 1976; Langowsiki *et al.*, 1981). When the latter is about 0.1–0.2 M, the dimer is the more stable species, although the monomer becomes the dominant form in NaCl solutions below 0.2 M (Geiger *et al.*, 1989). Therefore, the ionic strength is a significant factor in determining the

scanning mechanism and sequence-specific interactions, as well as protein–protein dimerization of *Eco*RI.

A number of restriction endonucleases cleave ssDNA as well as dsDNA (Blakesley and Wells, 1975; Horiuchi *et al.*, 1975; Yoo and Agawal, 1980), and the ssDNA-cleaving activity of *Eco*RI endonuclease using immobilized oligomer has also been reported (Norbert *et al.*, 1987). In general, DNA cleavage catalyzed by a restriction enzyme entails two separate cleavage reactions that lead to the hydrolysis of a phosphodiester bond in each of the two individual strands of the DNA duplex. Under optimum reaction conditions, their rates of cleavage can become marginally different (Pingoud *et al.*, 1993) and give interesting information about the enzyme-catalyzed reaction itself (Keith, 1992). Here, we report a mechanistic study employing *Eco*RI and its highly active variant N199H, which augments our understanding of structure–function relationships involved in protein–DNA binding and protein–protein interactions.

Materials and Methods

Recombinant DNA and electrophoretic techniques Preparation, restriction analysis, modification, and ligation of plasmid DNA were carried out following standard methods (Sambrook *et al.*, 1989). DNA preparation and electrophoresis of DNA fragments were carried out according to standard methods (Sambrook *et al.* 1989). DNA fragments were isolated using Geneclean BIO101 (Dianova, Hambrug, FRG).

Enzyme preparation and activity assay Wild-type and N199H mutant *Eco*RI restriction enzymes were purified from each transformed *E. coli* (Kim *et al.* 1996). For the activity assay, oligonucleotides substrates were designed corresponding to the recognition sequence and oligo (dA) or oligo (dT) side chains. Measurements of salt effect on the catalytic activity of wild-type *Eco*RI and *Eco*RI variant N199H were preincubated for 30 min at 25°C in the reaction buffer (20 mM Tris-HCl, pH 7.2, and 10 mM $MgCl_2$). Then, oligonucleotide 5′-dTATAGAATTCTAT-3′ (2 μM) and each concentration of salt were added. Aliquots were taken out at 5, 10, 15, 20, and 25 min, and the reaction was then quenched by adding EDTA (20 mM, final concentration). The reaction mixtures were analyzed by HPLC.

The assays for the concentration effect of wild-type *Eco*RI and *Eco*RI variant N199H in the reaction buffer were preincubated at concentrations of 0.035, 0.05, and 0.07 μM. Then, oligonucleotide 5′-dTATAGAATTCTAT-3′ (2 μM) and salt (50 mM) were added. Aliquots were taken at 5, 10, 15, 20, 25, and 30 min and the reaction mixtures were analyzed by HPLC after reaction quenching. The oligonucleotides were analyzed by HPLC on a 4.6 mm × 4.5 cm ULTRASPHER ODS column (Beckman, Fullerton, USA) at a flow rate of 2.5 ml/min with a 7 min gradient from 6% to 15% acetonitrile in 0.1 M TEAB, pH 7.3, using a Beckman System Gold.

Autoradiography of PAGE for the cleavage of single-stranded M13mp18 DNA by wild-type *Eco*RI was done by the standard method. Single-stranded M13mp18 DNA was first cleaved *Hae*III, followed by digestion with wild-type *Eco*RI endonuclease and by *Eco*RI variant N199H. After the cleavage reaction, the fragments were labeled by an exchange reaction with T4 polynucleotide kinase in the presence of v-^{32}P-ATP at 37°C for 30 min, and loaded for polyacrylamide gel electrophoresis.

Analytical gel permeation chromatography of the *Eco*RI variant N199H was studied using the general methods. 1 μM *Eco*RI (100 μl) was dialyzed against a column of buffer containing 20 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, and 50 mM NaCl (or 200 mM NaCl). Each dialysate was analyzed on a Superose 12 HR 10/30 column (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer containing 50 mM NaCl or 200 mM NaCl, as previously described (Geiger *et al.*, 1989). Enzyme fractions were identified with SDS-PAGE.

The time course of the cleavage reaction was analyzed as per standard reaction methods with 50 mM NaCl without enzyme preincubation. The enzyme was preincubated at 25°C for 30 min in 200 mM NaCl or in 50 mM NaCl. After the enzyme preincubation, KT (1 μM) was added, and the final salt concentrations were adjusted to 200 mM.

Results and Discussion

Cleavage reactions of synthetic dsDNA by wild-type *Eco*RI and *Eco*RI variant N199H We have shown that the *Eco*RI variant N199H has superior activity to the wild-type enzyme in a previous report (Kim *et al.* 1996). Its higher activity was attributed mainly to its high affinity and accessibility for the DNA substrate, as reflected in its low K_M value.

We know that the polarity of the 199th amino acid residue in *Eco*RI endonuclease influences the cleavage rates of DNA substrates (Kim *et al.* 1996), which indicates that hydrolysis of the phosphate backbone in a DNA substrate is sensitive to the enzyme reaction environment, particularly the ionic strength of the medium. Hence, as salt effects should provide mechanistic information about enzyme–DNA substrate interactions, we examined their influence on the cleavage reactions of duplex KS–KS [KS, 5′-dTATAGAATTCTAT-3′] by the wild-type *Eco*RI endonuclease and its variant N199H. At first, the wild-type *Eco*RI endonuclease, which was preincubated in reaction buffer for 30 min followed by KS substrate and salt addition, showed the higher catalytic activity at 150 mM NaCl concentration (Fig. 1a). At a 200 mM salt concentration, its catalytic activity almost disappeared, whereas the *Eco*RI variant N199H exhibited its highest activities (Fig. 1b). This means that the reaction centers of both enzymes were affected by ionic strength in their protein–DNA interactions. Another noteworthy characteristic of the wild-type enzyme was the nonlinearity in the course of its cleavage reaction of the duplex DNA substrate. As shown in Fig. 1a, the repeated experiments showed that its rate was shortly lower at 15 min at the lower salt concentrations. This suggested that the active form of the enzyme interacts separately with each strand of the duplex DNA substrate, and catalyzes the cleavage of each at its own reaction rate. Accordingly, it was inferred

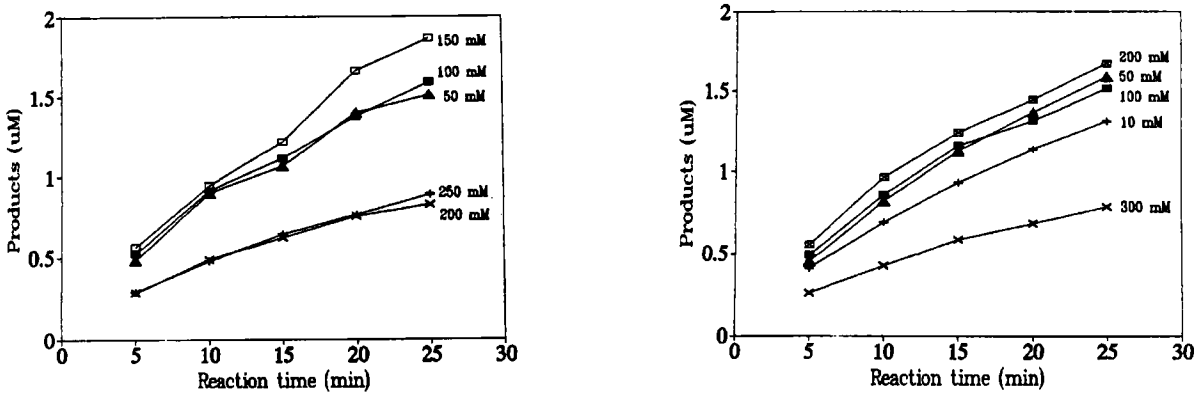

Fig. 1. Salt effect on the catalytic activity of wild-type *Eco*RI and *Eco*RI variant N199H. (a) Wild-type enzyme (0.07 μM) and (b) *Eco*RI variant N199H (0.05 μM) were preincubated for 30 min at 25°C in the reaction buffer (20 mM Tris-HCl, pH 7.2, and 10 mM $MgCl_2$). Then, oligonucleotide 5′-dTATAGAATTCTAT-3′ (2 μM) and salt were added. Final salt concentrations for wild-type *Eco*RI were 50 (▲), 100 (■), 150 (□), 200 (×), and 250 mM (+) NaCl, and for *Eco*RI variant N199H were 10 (+), 50 (▲), 100 (■), 200 (□), and 300 mM (×) NaCl. Aliquots were taken out at 5, 10, 15, 20, and 25 min, and then the reaction was quenched by adding EDTA (20 mM, final concentration). The reaction mixtures were analyzed by HPLC.

that the *Eco*RI endonuclease molecule binds a single-stranded DNA substrate, recognizes the 5′-dGAATTC-3′ sequence, and cleaves it. Therefore, we examined the cleavage reactions of synthetic single-stranded oligonucleotides by *Eco*RI and its variant, as described in the next section.

In experiments to determine the optimum enzyme concentration with a given concentration of oligonucleotide on cleavage, it was found that cleavage occurred readily when the concentration of the wild-type *Eco*RI endonuclease was 0.07 μM, although its overall catalytic activity was far lower than expected (Fig. 2a). By contrast, the *Eco*RI variant N199H showed comparable activities at 0.05 and 0.07 μM enzyme concentrations (Fig. 2b) than at concentrations of 0.05 μM and 0.035 μM. This strongly indicates that the cleavage reaction rate is proportional to the population of active enzyme forms. Therefore, the wild-type *Eco*RI endonuclease needs a higher enzyme concentration than its N199H variant in order to generate its optimum concentration of the active form, i.e., perhaps the monomeric rather than dimeric enzyme.

Enzyme reactions with single-stranded oligonucleotides, KA and KT In order to understand the nonlinearity of the cleavage reaction, and to examine the mechanism of the cleavage reaction of *Eco*RI endonuclease, we designed two oligonucleotides, KA (21 mer) and KT (21 mer), complementary to each other, in which each incorporates one kind of nucleotide aside from the recognition sequence to minimize self-complementarity with itself. Each oligonucleotide existed as a single strand under the reaction conditions because of its low melting point. Also, in order to facilitate HPLC

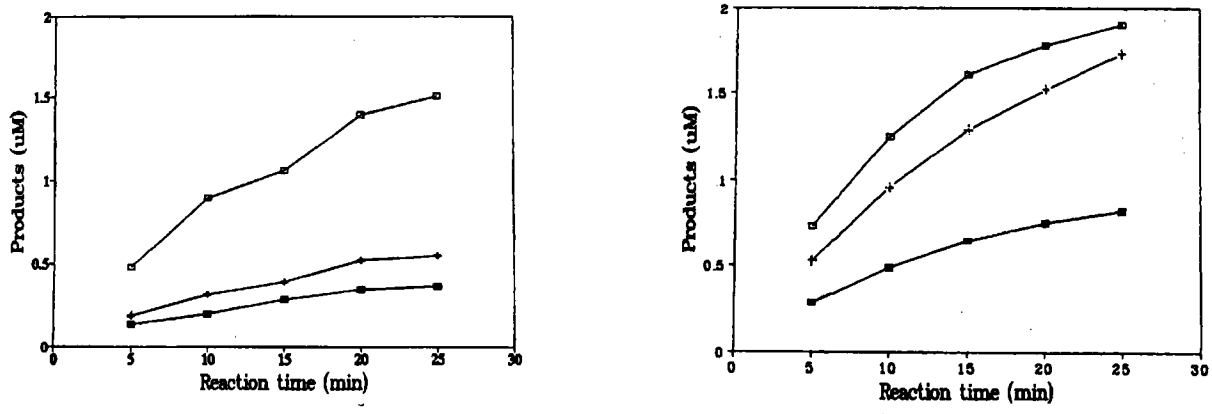

Fig. 2. Concentration effect of wild-type *Eco*RI and *Eco*RI variant N199H. (a) Wild-type *Eco*RI and (b) *Eco*RI variant N199H in the reaction buffer were preincubated as described in Fig. 1, at concentrations of 0.035 (■), 0.05 (+), and 0.07 μM (□). Then, oligonucleotide 5′-dTATAGAATTCTAT-3′ (2 μM) and salt (50 mM) were added. Aliquots were taken at 5, 10, 15, 20, 25, and 30 min and after quenching the reaction mixtures were analyzed by HPLC.

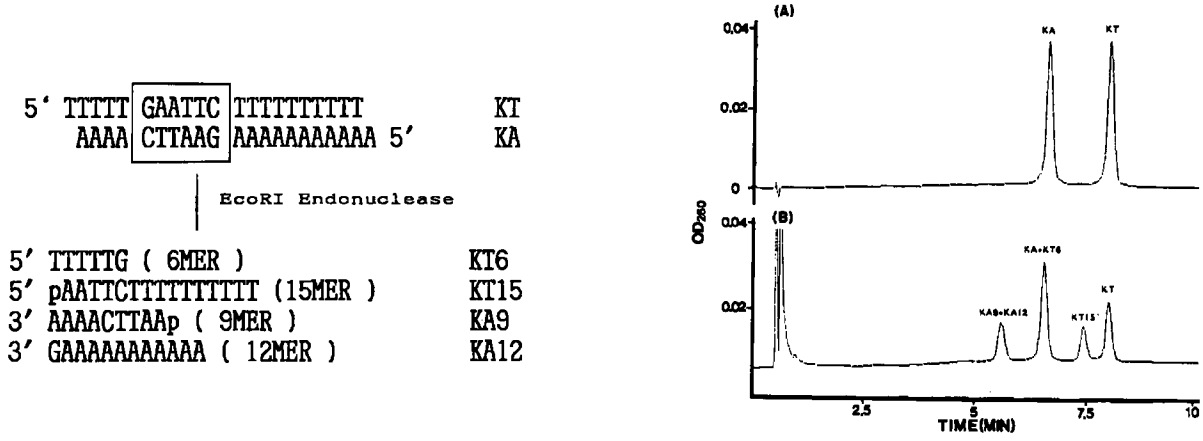

Fig. 3. (a) Nucleotide sequence of synthetic oligonucleotides, KT and KA, and the cleavage products by *Eco*RI endonuclease. (b) HPLC chromatograms before and after *Eco*RI cleavage of KT–KA. Equimolar amounts of KT and KA (ea. 1 μM) in reaction buffer containing 20 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, and 50 mM NaCl were dissolved and denatured at 90°C, and the digestion at 37°C by *Eco*RI (0.1 μM) endonuclease was monitored. The oligonucleotides were analyzed by HPLC on a 4.6 mm × 4.5 cm ULTRASPHER ODS column (Beckman) at a flow rate of 2.5 ml/min with a 7 min gradient from 6% to 15% acetonitrile in 0.1 M TEAB, pH 7.3, using a Beckman System Gold.

analysis of the cleavage reactions, the internal recognition sites were positioned to give a different size of cleavage product from each (Fig. 3).

The cleavage reactions of single-stranded DNA substrates, KA and KT, by *Eco*RI and its N199H variant were examined and monitored by HPLC, as shown in Fig. 4. Unexpectedly, both enzymes cleaved KA or KT at almost 3-times the rate as duplex KA–KT. This means that the enzyme recognizes the nucleotide sequence, 5′-dGAATTC-3′ in a single-stranded DNA substrate, and cleaves it. A plausible explanation is the ease of formation of the enzyme–DNA complex due to omission of the melting step of dsDNA. In other words, melting of the dsDNA substrate to form the transient complex may be regarded as a rate-determining step in the cleavage of duplex DNA. Furthermore, KT was found to be cleaved slightly faster than KA. This implies not only that the enzyme interacts with the recognition nucleotides of ssDNA, but also that its neighboring nucleotides influence the formation of the transient enzyme–DNA complex. Hence, it is safe to say that recognition and cleavage of duplex DNA substrates by *Eco*RI endonuclease do not occur in a concerted manner.

In additional experiments, a single-stranded M13mp18 DNA was employed as a substrate for double cleavage reactions by both *Eco*RI and *Hae*III endonucleases, in order to confirm the formation of such enzyme–DNA complexes. The *Hae*III endonuclease, which was known to cleave single-stranded DNA, was first reacted with M13mp18 DNA, and the result was compared with that

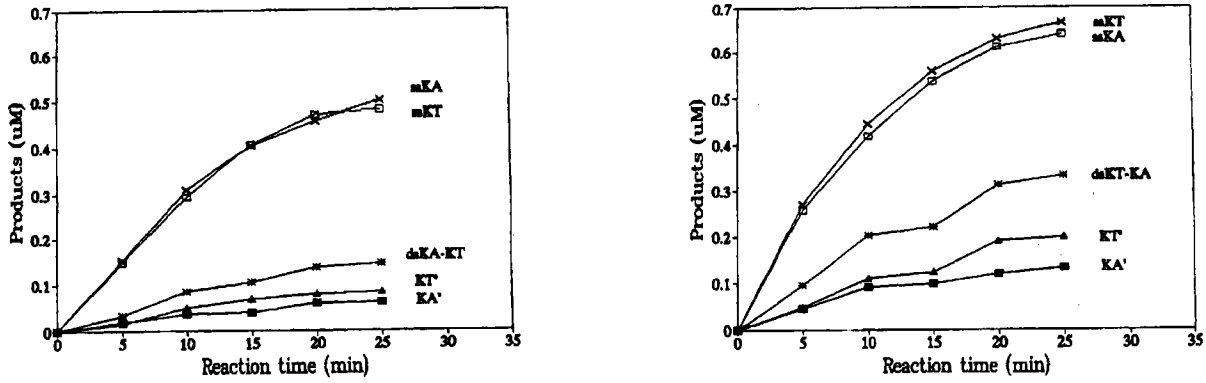

Fig. 4. Time course of cleavage reactions of single-stranded KT and KA, and double-stranded KT–KA, by wild-type *Eco*RI (a) and *Eco*RI variant N199H (b). ssKT, ssKA, and dsKT-KA in reaction buffer containing 20 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$ and 50 mM NaCl were denatured and then the enzymes (0.1 μM) were added and incubated for 5 min at 37°C. Each aliquot at 5, 10, 15, 20, and 25 min were analyzed by HPLC as described in Fig. 1 to measure the reaction progress. KT′ and KA′ indicate the time course of each ssKT and ssKA deduced from the dsKT–KA reaction.

obtained from a double digestion by both *Hae*III and *Eco*RI endonucleases of the DNA substrate. As expected, the single-stranded DNA fragment sizes were identical to those estimated from the restriction map of M13mp18 (Fig. 5). From these results, it was concluded that the enzyme itself recognizes the nucleotide sequence, 5′-dGAATTC-3′, in single-stranded DNA, and then forms the transient complex for the hydrolysis of the phosphodiester linkage.

Dimerization of *Eco*RI and its variant, N199H Geiger *et al.* (1989) reported that at an enzyme concentration of 0.1–0.2 μM, the *Eco*RI endonuclease exists as the dimeric form at high ionic strength (0.2 M NaCl) whereas the monomeric enzyme is more stable below 0.2 M NaCl. As the dimeric form has been regarded as the catalytically active form (Geiger *et al.*, 1989), we have compared here the dimerization tendencies of *Eco*RI and its variant N199H in relation to their catalytic activities.

The results of gel filtration using Superose 12 HR10/30 column indicated that *Eco*RI and its N199H variant had different ratios of the monomeric to dimeric form at 50 mM and 200 mM NaCl (Fig. 6). As summarized in Table 1, about 70% of the variant was monomeric in 200 mM NaCl, whereas the wild-type *Eco*RI was about 51% under the same conditions. As a result, we suggest that the Asn199 residue of the *Eco*RI endonuclease is implicated in protein–protein interactions that lead to dimerization of the enzyme.

The fact that variant N199H had a higher population of the monomeric form, and also a higher activity at 200 mM

Table 1. Analytical gel filtration of WT and His199 mutnat *Eco*RI enzyme at 50 mM and 200 mM NaCl.

enzyme	% of monomeric enzyme[a]	
	50 mM NaCl	200 mM NaCl
Wild-type	79	51
His199	81	70

[a] area of monomeric form ÷ Total area (monomer + dimer) ×100

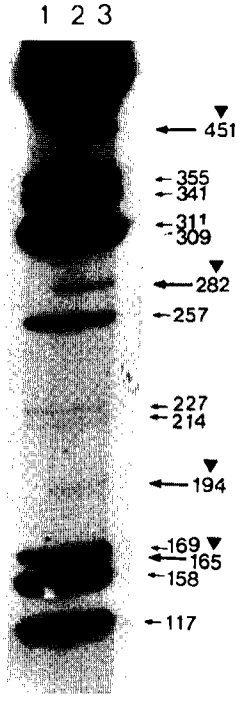

Fig. 5. Autoradiograph of PAGE for the cleavage of single-stranded M13mp18 DNA by wild-type *Eco*RI. Single-stranded M13mp18 DNA was first cleaved by *Hae*III, and subsequently digested with wild-type *Eco*RI endonuclease (2) and *Eco*RI variant N199H (3). After the cleavage reaction, the fragments were labeled by an exchange reaction with T4 polynucleotide kinase in the presence of γ-^{32}P-ATP at 37°C for 30 min, and loaded for polyacrylamide gel electrophoresis. The bands (solid triangle) were derived from cleavage of M13mp18 by *Eco*RI and the variant, whereas the other bands accounted for *Hae*III fragments of M13mp18.

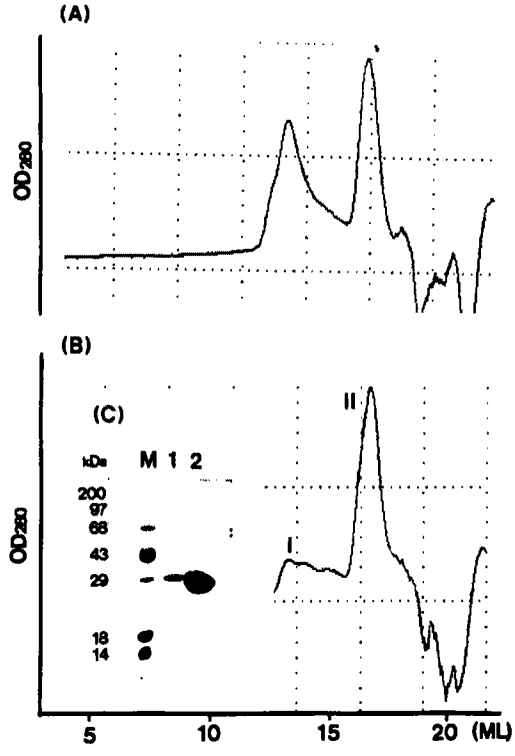

Fig. 6. Analytical gel permeation chromatography of *Eco*RI variant N199H. 1 μM *Eco*RI (100 μl) was dialyzed against a column of buffer containing 20 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$ and 50 mM NaCl (or 200 mM NaCl). Each dialysate was analyzed on a Superose 12 HR 10/30 column (Pharmacia) equilibrated with the same buffer containing 50 mM NaCl (A) and 200 mM NaCl (B) as described previously (Geiger *et al*, 1989). Peak I, responsible for the dimer form, and peak II, for the monomer form, were identified with SDS-PAGE (C): lane 1, peak I; lane 2, peak II, and lane M, molecular size markers.

salt concentration than the wild-type enzyme, indicates that the monomeric form of the *Eco*RI endonuclease is the active form. Consequently, the introduction of a His199 residue is partly responsible for inhibiting dimerization so that a higher population of active monomeric enzyme occurs, as well as for affecting the catalytic domain of the variant N199H so as to facilitate nucleophilic substitution by a water molecule.

In support of this conclusion, an experiment was designed to determine the effect on the *Eco*RI enzyme of preincubation without DNA substrate, before the cleavage reactions were performed at different salt concentrations, because preincubation should allow for dimerization to proceed without interference by the DNA. Three reaction conditions were selected as follows: (a) the standard reaction in 50 mM NaCl without enzyme preincubation; (b) the enzyme was preincubated for 30 min in 200 mM NaCl before adding oligonucleotide substrate; and (c) the enzyme was preincubated for 30 min in 50 mM NaCl and then the oligonucleotide was introduced at a final salt concentration of 200 mM. As shown in Fig. 7, the catalytic activity of the wild-type enzyme in reaction (c) was about 4 times higher than that of reaction (b). In reaction (c), as the ionic strength was 50 mM NaCl, the monomeric enzymes were well situated for DNA–protein or protein–protein interactions. In reaction (b), however, the enzymes were largely dimerized, so that enzyme activity was low. These findings indicate that the monomeric form of *Eco*RI endonuclease itself participates in the cleavage reaction of single-stranded DNA. Consequently the enzyme–substrate binding affinity of *Eco*RI at high salt concentration was affected negatively, whereas there was a positive effect on active dimerization of the protein.

Although two identical subunits of the *Eco*RI dimer are involved in the interacting two halves of palindromic recognition sequences in double-stranded DNA, *Eco*RI itself does not require a two-fold enzyme symmetry in order to act on single-stranded DNA. That is, the monomeric form of *Eco*RI endonuclease may be sufficient for binding and cleaving ssDNA, as has been shown for a few restriction enzymes, *viz.*, *Bsu*RI (Kiss *et al.*, 1985), *Bgl*I (Lee and Chirikjian, 1979), and *Bsp*RI (Koncz *et al.*, 1978).

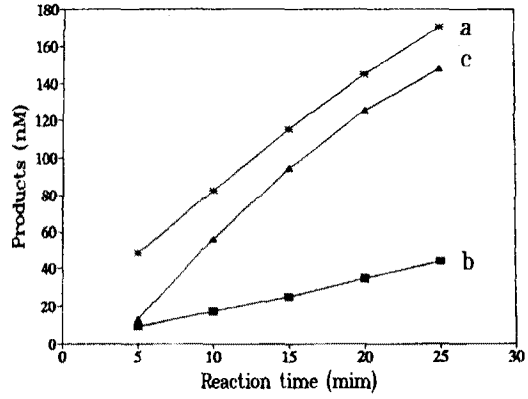

Fig. 7. Time course of the cleavage reaction of KT by the preincubated *Eco*RI (0.1 μM). (a) Standard reaction with 50 mM NaCl without enzyme preincubation. (b) Preincubation of enzyme at 25°C for 30 min in 200 mM NaCl, and (c) in 50 mM NaCl. After the enzyme preincubation, KT (1 μM) was added, and the final salt concentrations were adjusted to 200 mM for (b) and (c).

Steady-state kinetics of *Eco*RI and its variant, N199H Reaction rates of *Eco*RI and its N199H variant with single-stranded oligonucleotides, KA and KT, were measured at low and high salt concentration. As shown in Table 2, the variant was more active than the wild-type enzyme at both salt levels. K_m values of the variant calculated from the reaction kinetics were 2.5-fold lower in 50 mM NaCl and 24-fold lower in 200 mM NaCl, as compared with those of the wild-type enzyme. This again demonstrates that the decreased dimerization of the variant at the high salt concentration leads to a high affinity and accessibility to a ssDNA substrate. However, there was no significant difference in K_{cat} between the wild-type *Eco*RI and its variant under standard reaction conditions. These results support the view that the monomeric form of *Eco*RI endonuclease binds and acts on ssDNA.

Using a mutagenic approach, Geiger *et al.*(1989) reported that *Eco*RI (Gln144Lys145) and *Eco*RI (Gln144Lys145Lys200) mutants have an apparent decreased propensity to form the dimer or tetramer. A detailed analysis of the steady-state kinetics of cleavage of double-stranded oligonuceltides showed that the reduction in activity for the mutants is due mainly to a decrease in

Table 2. Michaelis–Menten parameters for the cleavage of ssDNA (KT and KA) with wild-type and His199 mutant *Eco*RI at low and high salt concentrations.

enzyme	substrate	50 mM NaCl			200 mM NaCl		
		K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m
Wild-type	KA	1.68	0.34	0.2	24	0.36	0.015
	KT	0.63	0.14	0.22	nd	nd	nd
His199	KA	0.63	0.21	0.44	1.0	0.07	0.07
	KT	0.27	0.13	0.48	0.31	0.03	0.1

nd = not detected

K_{cat} (Alves *et al.*, 1989). Also, as mentioned in a previous report (Kim *et al.*, 1996), *Eco*RI variant N199H also showed a low K_{cat} against synthetic double-stranded oligonucleotides, whereas there were no detectable differences between K_{cat} values of *Eco*RI and its N199H variant in reactions with single-stranded DNA (Table 2), indicating that protein–protein dimerization partly affects the dsDNA cleavage reaction in terms of accessibility to the DNA substrate.

Overall, we conclude that the substitution of Asn199 of *Eco*RI by a His residue increases the enzyme's binding affinity and accessibility to DNA substrates, by impairing the dimerization potential of the enzyme. Due to these two alterations in characteristics, *Eco*RI variant N199H possesses an increased activity for the cleavage of ssDNA, although, for the cleavage of dsDNA, the monomeric enzyme acts in a stepwise manner to induce the substrate to unwind and be cleaved.

In conclusion, *Eco*RI variant N199H, which was generated by a protein engineering approach, contributed information that supplements our understanding of the mode of action of the *Eco*RI endonuclease. Clearly, as each amino acid residue of the enzyme plays a cooperative role in its activity, the variant endonuclease, having a single mutation, allowed us to monitor subtle changes in the molecular action of the enzyme. In particular, the Asn199 residue in the *Eco*RI endonuclease not only plays its unique role as a component contributing to the molecular environment for catalytic activity, but also as an amino acid residue essential for enzyme dimerization. The question of enzyme degeneracy in nucleotide recognition remains to be studied by mutagenesis.

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