Engineering of Variants of the Restriction Endonuclease *Eco*RV That Depend in Their Cleavage Activity on the Flexibility of Sequences Flanking the Recognition Site†

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ABSTRACT: The present work describes mutants of the restriction enzyme *Eco*RV that discriminate very efficiently between oligodeoxynucleotide substrates with an *Eco*RV recognition sequence in different sequence context. All of these *Eco*RV variants harbor substitutions at position 226, where in the cocrystal structure of the specific *Eco*RV/DNA complex an arginine contacts the backbone of the DNA substrate upstream of the recognition sequence, and cleave an oligodeoxynucleotide with an *Eco*RV site in a nonflexible sequence context (the recognition site being flanked by runs of A and T) with much higher catalytic efficiency (k_{cat}/K_m) than an oligodeoxynucleotide with an *Eco*RV site in a flexible sequence context (the recognition site being flanked by runs of AT), in contrast to the wild-type enzyme, that cleaves both substrates with the same catalytic efficiency. Steady-state and single-turnover kinetics indicate that the enhanced selectivity of the mutants is due to the catalytic step of the reaction. It is possible to enhance the discriminatory power of these *Eco*RV variants through the choice of appropriate reaction conditions, in particular low salt concentration and low reaction temperatures. It must be emphasized that the enhanced selectivity of these *Eco*RV variants toward *Eco*RV sites in a flexible and nonflexible sequence context, respectively, is not only seen with oligodeoxynucleotides, but also with plasmid substrates.

Type II restriction endonucleases are components of bacterial restriction/modification systems that serve as defense systems against bacteriophage infections (reviews: *1*−*3*). These homodimeric enzymes cleave double-stranded DNA at defined palindromic sequences 4−6 base pairs in length with very high specificity. Typically, the canonical sequence is cleaved by several orders of magnitude more quickly than a sequence that deviates by only one base pair (*4*−*6*). Due to this remarkable property, restriction endonucleases have become widely used tools in molecular biology and biotechnology. Although over 2000 enzymes with 210 different specificities are known (*7*), there is still considerable demand for new specificities, especially for enzymes that recognize longer sequences, 8 or even 10 bp[1] in length, which may be useful for the analysis and manipulation of eucaryotic genomes. An alternative for the screening of bacterial isolates for naturally occurring restriction endonucleases with new specificities is the alteration of specificities of known enzymes by protein engineering methods. The basis for rational protein engineering was set by the solution of cocrystal structures of restriction endonuclease/DNA complexes (*8*−*11*). The first extensive site-directed mutagenesis studies (review: *12*) focused on amino acid residues that form direct contacts to the bases of the corresponding recognition sequences. However, contrary to optimistic expectations, no variants with new specificities and reasonable activities were obtained. Rather, enzyme variants produced were nearly inactive. From an evolutionary point of view, this is reasonable because a restriction endonuclease that cleaves at sites other than the recognition sequence, which are no targets for the corresponding DNA methyltransferase, is toxic for the bacterial host. It can be assumed that during evolution these enzymes were the subject of a very efficient selection to make them discriminate reliably between their recognition site and all other naturally occurring DNA sequences. In principle, there are two possibilities to "teach" an enzyme to do something it has "learnt" to avoid during evolution. First, one can try to change the selectivity of restriction endonucleases toward not naturally occurring, modified substrates, for example, substrates in which thymidine residues within the recognition site are replaced by uracil residues (*13*−*15*). A second, from the biotechnological point of view more interesting, but also more demanding approach is to expand the recognition sequence of a given enzyme. A variant of a restriction endonuclease that cleaves its recognition site only in a special sequence context would not be harmful to the bacterial host, because the DNA methyltransferase still modifies this sequence. It could, on the other hand, affect the ability of the cell to defend itself against foreign DNA, because it cleaves fewer sites (or even none) on the DNA of invading bacteriophages. Recently we have demonstrated for the restriction endonuclease *Eco*RV that several mutants defec-

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[1] Abbreviations: bp, base pair(s); T_m, melting temperature; *k*, initial rate; k_{st}, first-order rate constant; PCR, polymerase chain reaction.

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tive in contacts to the phosphodiester backbone of the DNA—in contrast to the wild-type enzyme—show different activities toward two recognition sites with different flanking sequences on a plasmid substrate without a drastic loss of specific activity (*16*).

In the present work, we will describe variants of the restriction endonuclease *Eco*RV that discriminate very efficiently between substrates with different sequences flanking the recognitions site. All of these mutants harbor amino acid substitutions at position 226, where in the case of wild-type *Eco*RV an arginine residue contacts the backbone of the DNA substrate. It is possible to enhance the discriminatory power of these enzymes through the choice of appropriate reaction conditions, in particular low salt concentration and low reaction temperatures.

EXPERIMENTAL PROCEDURES

EcoRV and EcoRV Variants. Site-directed mutagenesis and the purification of *Eco*RV variants were carried out essentially as described previously (*16*).

Oligodeoxynucleotide Substrates. The oligodeoxynucleotides employed as substrates for *Eco*RV, viz., d(AAAAAAA-*GATATC*TTTTTTT)$_2$ (I) and d(TATATAT*GATATC*ATATA-TA)$_2$ (II), were purchased from Interactiva (Ulm, Germany); for the purpose of fluorescence detection (see below, under *Single-Turnover Cleavage Experiments with Oligodeoxynucleotides*), they carried a fluorescein group on their 5′-terminus.

For the determination of T_m values, 2 μM solutions of oligodeoxynucleotide in standard reaction buffer (20 mM Tris/HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) were used. Melting curves were recorded at 260 nm in a U-3000 spectrophotometer (Hitachi, Tokyo, Japan) between 30 and 80 °C with a heating rate of 12 °C/h. The T_m values were calculated from the first-order derivatives of the absorption versus temperature profiles.

Plasmid Substrates. To compare the cleavage of oligodeoxynucleotide substrates by *Eco*RV variants with the cleavage of *Eco*RV sites in an identical sequence context on a plasmid substrate, oligodeoxynucleotides I and II (see above, without a fluorescein group on the 5'-terminus) were ligated into the *Eco*RI site of pUC8. After transformation in *Escherichia coli* DH5α cells, positive clones were identified by *Eco*RV digest and verified by sequencing. The new plasmids, pUC8 I and pUC8 II, harbor a single *Eco*RV site with the flanking sequences of oligodeoxynucleotides I and II, respectively. Both plasmids were prepared using the QIAGEN Plasmid Midi Kit (Qiagen, Hilden, Germany). Analysis of the preparations by agarose gel electrophoresis showed that at least 90% of the DNA was covalently closed supercoiled material.

Steady-State Cleavage Experiments with Oligodeoxynucleotides. Prior to their use in steady-state cleavage experiments, the oligodeoxynucleotides were radioactively labeled on their 3'-end with [α-^{32}P]ddATP and terminal deoxynucleotidyl transferase (MBI Fermentas) according to the protocol of the supplier. To ensure annealing of the self-complementary oligodeoxynucleotides, the labeling mixtures were heated for 10 min to 75 °C and then cooled slowly to room temperature. For the determination of k_{cat} and K_m values, the rate of the *Eco*RV-catalyzed DNA cleavage reaction was investigated as a function of oligodeoxynucleotide concentration. The reaction mixtures contained 1 nM to 30 μM radioactively labeled oligodeoxynucleotides in standard reaction buffer supplemented with 100 μg/mL bovine serum albumin in a total volume of 10−40 μL; the concentration of double-stranded oligodeoxynucleotide was always at least 10 times higher than the concentration of *Eco*RV dimer. The reaction was started by the addition of 0.1 pM to 1.5 μM *Eco*RV (diluted in 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 100 μg/mL bovine serum albumin), and the reaction mixture was incubated at 23 °C. After defined time intervals, 1−4 μL aliquots were withdrawn from the reaction mixture, spotted onto a DEAE-cellulose plate (Macherey-Nagel, Düren, Germany), and subjected to homochromatography (*17*). The detection and quantification of the separated substrates and products were achieved using an InstantImager system (Canberra Packard). Initial rates (k) were calculated from the linear part of the progress curves obtained at six or more different substrate concentrations, which were chosen to cover at least a range from 0.2 to 5 × K_m. The K_m and k_{cat} values were obtained by a best fit of the cleavage data to the Michaelis−Menten equation: $k = k_{cat}S/(S + K_m)$ (k, initial rate; S, concentration of substrate).

Initial rates for the cleavage of oligodeoxynucleotides under nonstandard conditions were determined in the same way as under standard conditions, the only difference being various concentrations of NaCl (5−205 mM) in the reaction buffer and different reaction temperatures (15, 20, 25, and 30 °C), respectively. These experiments were performed only at single substrate concentrations of 1 μM for the wild-type enzyme and 10 μM for the R226V variant. Apparent activation energies (E_a) were calculated from the slope of plots of ln(k) against 1/T according to the Arrhenius equation: $k = A \exp(-E_a/RT)$ (k, initial rate; A, frequency factor; R, gas constant; T, absolute temperature).

Single-Turnover Cleavage Experiments with Oligodeoxynucleotides. In the case of the single-turnover reactions, the fluorescence label of the oligodeoxynucleotides was used directly to monitor the *Eco*RV-catalyzed cleavage reaction. Prior to their use in single-turnover cleavage experiments, the oligodeoxynucleotide substrates were heated for 10 min in 10 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 200 mM NaCl to 75 °C and cooled slowly to room temperature to ensure the annealing of the self-complementary strands.

Since most of the single-turnover reactions were too fast for conventional mixing, a quenched-flow apparatus, the SFM-3 (Bio-logic, Claix, France), was used instead. The reactions were started by mixing equal volumes of 0.2 or 1 μM oligodeoxynucleotide and a 2-fold molar excess of *Eco*RV dimer in standard reaction buffer supplemented with 1 mM EDTA and 100 μg/mL BSA (syringe 1) and 21 mM $MgCl_2$ (syringe 2) and stopped after defined time intervals between 0.05 and 12.8 s by adding one-third volume of 100 mM EDTA (syringe 3) to the reaction mixture. Aliquots of the reaction mixtures (containing ca. 5 fmol of oligodeoxynucleotide) were heated in 12 μL of Template Suppression Reagent (Perkin-Elmer) for 5 min to 95 °C, cooled on ice, and analyzed using an ABI PRISM 310 Genetic analyzer (Perkin-Elmer) equipped with a 47 cm capillary (inner diameter: 50 μm) containing the POP-4 polymer supplemented with 8 M urea (Perkin-Elmer). The probes were electroinjected into the capillary for 5 s at 15 000 V, and

the run was performed at 15 000 V and 60 °C for 13 min with Genetic Analysis Buffer supplemented with 1 mM EDTA (Perkin-Elmer) as electrode buffer. The quantification of substrates and products of the reaction was done with GENESCAN 2.01 software (Perkin-Elmer). Time constants for the single-turnover reactions (k_{st}) were obtained by a best fit of the progress curves to a function with a single exponential.

Cleavage Assays with λ- and Plasmid-DNA. A 25 μg/mL sample of λ-DNA (Boehringer Mannheim, Germany) or plasmid-DNA was incubated at 37 °C (unless otherwise stated) in standard reaction buffer supplemented with 100 μg/mL bovine serum albumin with varying amounts of *Eco*RV (diluted in 20 mM Tris/HCl, 50 mM NaCl, 100 μg/mL BSA). After defined time intervals, 10 μL aliquots were withdrawn, and the reaction was stopped by adding 3 μL of agarose gel loading buffer containing 250 mM EDTA. The reaction products were separated by electrophoresis on 1.2% agarose gels, which were subsequently stained with ethidium bromide and photographed using a video system (Intas, Göttingen, Germany). For the digestion of λ-DNA and linear pATRV (*16*), *Eco*RV activity is given in relative units defined by the amount of enzyme needed to completly digest the DNA. In the case of covalently closed supercoiled plasmid substrates, initial velocities (k) for the linearization by *Eco*RV were determined. The quantitation of the intensities of substrate and product bands was carried out using the computer program TINA 2.07d (Raytest, Straubenhardt, Germany), and the initial rates were obtained from the linear part of the progress curves from plots of percentage of the intensity of the linear band versus time.

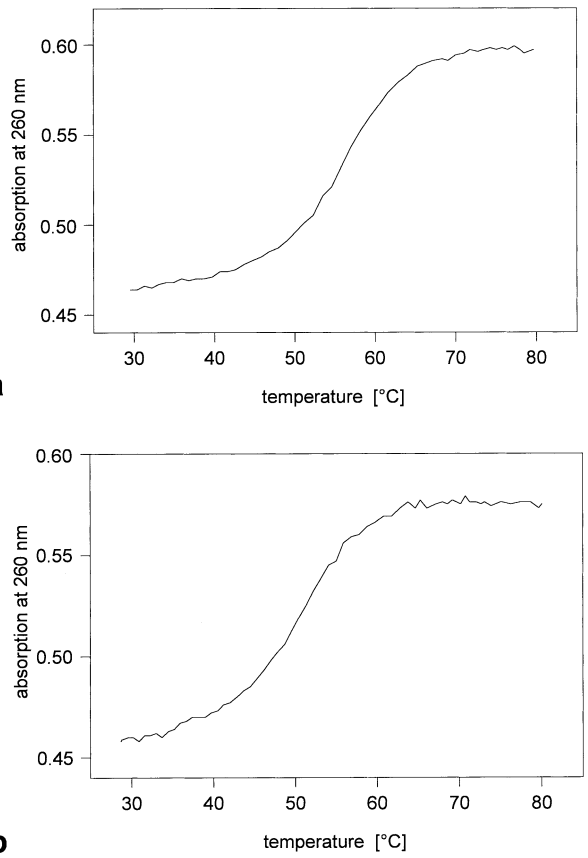

FIGURE 1: Melting curves of oligodeoxynucleotides I, d(AAAAAAAGATATCTTTTTTT)$_2$ (a), and II, d(TATATATGATATCATATATA)$_2$ (b), used as substrates for *Eco*RV.

RESULTS

Selection of EcoRV Variants. In the course of screening of several alanine mutants, three *Eco*RV variants turned out to have an enhanced sensitivity toward sequences flanking the recognition sequence, namely, the *Eco*RV variants T93A, R140A, and R226A (*16*). To reinforce this property, we pursued two different strategies: first, the combination of these mutations to yield all possible double mutants (T93A/R140A, T93A/R226A, and R140A/R226A); and second, the generation of new single mutants at positions 140 or 226. The arginine residues at these positions were exchanged to Asp, Gln, Glu, His, Leu, and Val, respectively.

We introduced the mutations into the *Eco*RV gene by PCR mutagenesis, overexpressed the resulting *Eco*RV variants as N-terminally His$_6$-tagged proteins, and purified them to near-homogeneity (better than 95%) by chromatography on nickel chelate columns.

All new single mutants at positions 140 or 226 with the exception of R140D and R140E displayed specific *Eco*RV activity in standard reaction buffer with λ-DNA and pATRV in the range of 1−10% of the wild-type activity, whereas all double mutants were essentially inactive (data not shown). Due to their low activity, the double mutants were not further investigated.

Steady-State Cleavage Experiments under Standard Conditions. Two oligodeoxynucleotides were used as substrates for wild-type *Eco*RV and *Eco*RV variants in cleavage experiments: d(AAAAAAAGATATCTTTTTTT)$_2$ (I) and d(TATATATGATATCATATATA)$_2$ (II). Both oligodeoxynucleotides are self-complementary 20-mers of identical AT content with a central *Eco*RV site (underlined). Under the experimental conditions used, both oligodeoxynucleotides form stable duplexes. Melting of oligodeoxynucleotides I and II is reversible; the T_m values are 57 and 52 °C, respectively (Figure 1).

Preliminary experiments with substrates I and II indicated that *Eco*RV with single amino acid exchanges at positions 140 and 226, respectively, cleaves both oligodeoxynucleotides, unlike the wild-type enzyme, with different rates. However, the differences were much more pronounced with the mutations at position 226. Consequently, only the wild-type enzyme and the seven *Eco*RV variants at position 226 were included in a detailed kinetic analysis under steady-state conditions. Table 1 shows the steady-state kinetic parameters k_{cat}, K_m, and k_{cat}/K_m for the cleavage of oligodeoxynucleotides I and II with wild-type *Eco*RV and the *Eco*RV variants R226A, -D, -E, -H, -L, -Q, and -V. As examples, Michaelis−Menten diagrams for wild-type *Eco*RV and the R226V variant with both substrates are shown in Figure 2. The wild-type enzyme cleaves both substrates with roughly the same catalytic efficiency; the k_{cat}/K_m values differ only by a factor of 2. Accordingly, the selectivity σ, which is defined here as the ratio of k_{cat}/K_m for oligodeoxynucleotide I and k_{cat}/K_m for oligodeoxynucleotide II, is low. However, the various mutants at position 226, with the exception of the R226E mutant, hydrolyze oligodeoxynucleotide I with a much higher catalytic efficiency than oligodeoxynucleotide II. In comparison to the wild-type enzyme, the selectivity of these mutants is enhanced by a factor of 60−170 (Table

Table 1: Steady-State Kinetic Parameters and Selectivities (σ) for Cleavage of Oligodeoxynucleotides I, d(AAAAAAAGATATCTTTTTTT)$_2$, and II, d(TATATATGATATCATATATA)$_2$, by Wild-Type *Eco*RV and *Eco*RV Variants[a]

	oligodeoxynucleotide						
	I			II			I/II
mutant	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	σ
wild-type	63 ± 16	0.053 ± 0.009	(2.0 ± 0.6) × 10^7	34 ± 7	0.015 ± 0.004	(3.8 ± 1.4) × 10^7	0.53 ± 0.25
R226E	1.2 ± 0.6	3.0 ± 1.2	(6.5 ± 4.3) × 10^3	0.43 ± 0.11	7.4 ± 1.2	(9.7 ± 2.9) × 10^2	6.6 ± 4.9
R226L	19 ± 1	3.9 ± 1.8	(8.4 ± 3.9) × 10^4	0.14 ± 0.07	0.86 ± 0.55	(2.8 ± 2.2) × 10^3	31 ± 29
R226A	16 ± 5	0.90 ± 0.24	(2.9 ± 1.2) × 10^5	0.15 ± 0.05	0.48 ± 0.18	(5.4 ± 2.7) × 10^3	54 ± 37
R226D	21 ± 6	1.6 ± 0.7	(2.1 ± 1.1) × 10^5	0.17 ± 0.05	0.79 ± 0.33	(3.5 ± 1.9) × 10^3	60 ± 43
R226Q	20 ± 7	0.77 ± 0.15	(4.4 ± 1.7) × 10^5	0.19 ± 0.04	0.44 ± 0.06	(7.0 ± 1.9) × 10^3	62 ± 28
R226H	15 ± 4	0.84 ± 0.18	(2.9 ± 0.9) × 10^5	0.11 ± 0.04	0.49 ± 0.22	(3.8 ± 2.1) × 10^3	76 ± 49
R226V	19 ± 1	1.5 ± 0.4	(2.2 ± 0.5) × 10^5	0.15 ± 0.04	1.0 ± 0.2	(2.4 ± 0.8) × 10^3	90 ± 34

[a] Selectivities (σ) were determined according to the equation: $\sigma = [k_{cat}/K_m(I)]/[k_{cat}/K_m(II)]$; standard errors of at least three independent determinations are given.

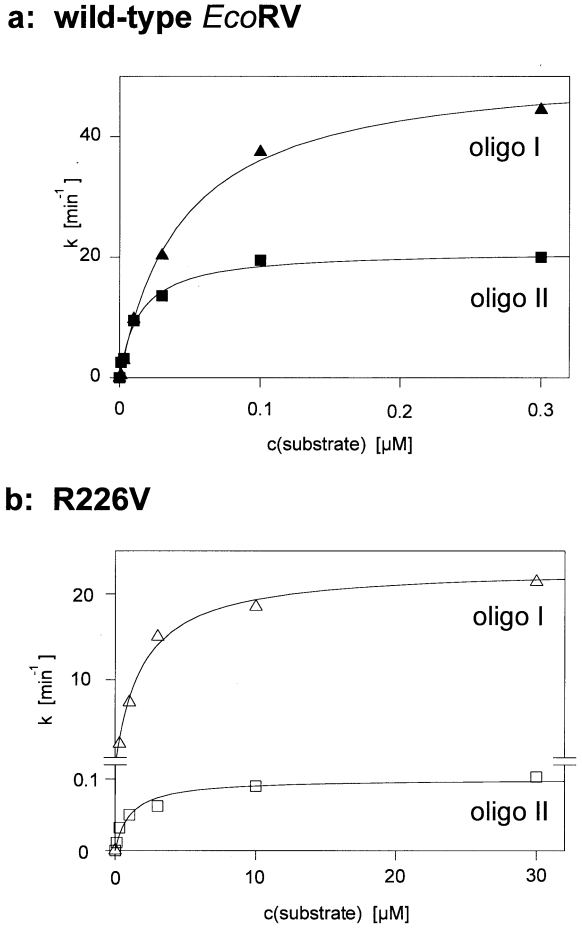

FIGURE 2: Michaelis−Menten diagrams for the cleavage of oligodeoxynucleotides I, d(AAAAAAAGATATCTTTTTTT)$_2$ (triangles), and II, d(TATATATGATATCATATATA)$_2$ (squares), by wild-type *Eco*RV (a, filled symbols) and the R226V variant (b, open symbols). The symbols represent experimental data and the solid lines a best fit to the Michaelis−Menten equation with the k_{cat} and K_m values as given in Table 1. Note the change in scale in the ordinate of graph (b).

1). The variations in σ are mainly due to differences in k_{cat}. Whereas the wild-type enzyme cleaves oligodeoxynucleotide I under saturating substrate concentrations only by a factor of 2 faster than oligodeoxynucleotide II, for all variants at position 226 except R226E this factor is 100 or more. The variations in K_m, on the other hand, are smaller and point in the opposite direction; i.e., all *Eco*RV variants with the exception of the R226E variant have a 2−5-fold higher affinity for substrate II than for substrate I. It should be noted that the gain in selectivity is accompanied by a decrease in catalytic efficiency (k_{cat}/K_m); the variants R226L, -A, -D, -Q, -H, and -V for the "good" substrate retain 1−2% of the catalytic efficiency of the wild-type enzyme (Table 1).

The variant R226E is a special case. It cleaves both substrates with much lower activity than all other *Eco*RV variants analyzed; the gain in selectivity is, in comparison to wild-type *Eco*RV, only small.

Pre-Steady-State Cleavage Experiments under Standard Conditions. In addition to the kinetic analysis under steady-state conditions, we performed single-turnover measurements with enzyme in excess over substrate with the wild-type *Eco*RV and the R226V variant, the most "selective" of all *Eco*RV variants analyzed (Table 1). The concentrations of enzyme and substrate were chosen to be at least equal to or to exceed the corresponding K_m values. The rate of the reactions required the use of a quenched-flow apparatus. Different from the reactions under steady-state conditions, we used for the analysis and quantification of substrates and products of the single-turnover reactions a capillary electrophoresis system (Figure 3). We obtained first-order cleavage rate constants, k_{st}, by a fit of the entire progress curves to a function with a single exponential (Figure 4, Table 2). The k_{st} values determined for the wild-type enzyme and the R226V variant with oligodeoxynucleotide II are − within the limit of experimental error − identical to the corresponding k_{cat} values (Table 2). This implies that the rates measured with saturating concentrations of substrate under steady-state conditions are limited by the catalytic step of the reaction, i.e., the hydrolytic cleavage of the phosphodiester bond, or reaction steps preceding it. This is not the case for oligodeoxynucleotide I. The rate measured under single-turnover conditions with this substrate for either *Eco*RV variant exceeds the corresponding k_{cat} by a factor of 2−3. Consequently, not only phosphodiester bond hydrolysis accounts for the steady-state k_{cat}, as is the case for oligodeoxynucleotide II, but also another step in the catalytic cycle of *Eco*RV, most probably product dissociation, as shown recently for the cleavage of a 12-mer oligodeoxynucleotide with wild-type *Eco*RV (*18*). However, the ratio of the relative cleavage rate constants with oligodeoxynucleotides I and II for R226V and the wild-type enzyme under single-turnover and steady-state conditions with saturating concentrations of substrate, respectively, which are critical for the

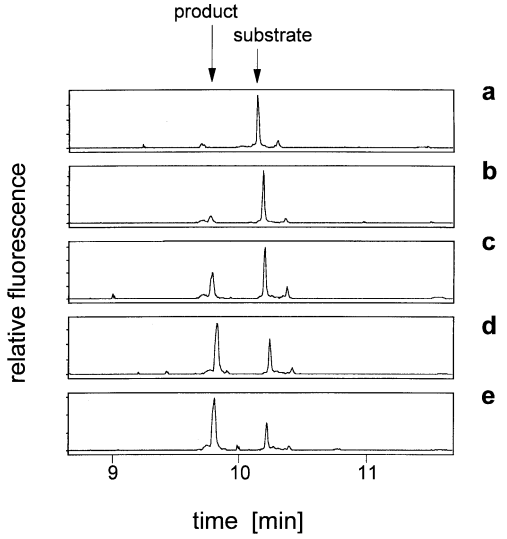

FIGURE 3: Example of the time course of a restriction enzyme catalyzed cleavage reaction carried out in a quenched-flow apparatus and analyzed by a capillary electrophoresis system. Shown are the chromatograms of the product mixtures obtained after cleavage of 0.1 μM oligodeoxynucleotide I, d(AAAAAAAGATATCTTTTTTT)$_2$, with 0.2 μM wild-type *Eco*RV for 0 (a), 100 (b), 400 (c), 1600 (d), and 6400 ms (e).

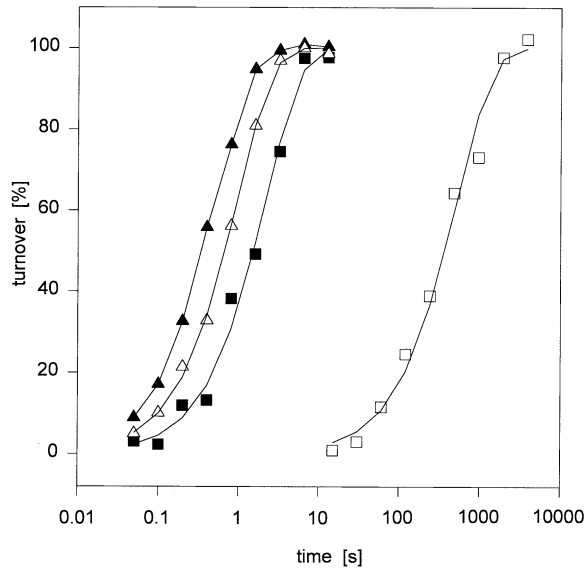

FIGURE 4: Cleavage of oligodeoxynucleotides I, d(AAAAAAAGATATCTTTTTTT)$_2$ (triangles), and II, d(TATATATGATATCATATATA)$_2$ (squares), by wild-type *Eco*RV (filled symbols) and the R226V variant (open symbols) under single-turnover conditions. The symbols represent experimental data and the solid lines a best fit to a function with a single exponential with the k_{st} values given in Table 2. The concentrations used were 0.1 μM substrate and 0.2 μM enzyme for wild-type *Eco*RV, and 0.5 μM substrate and 1 μM enzyme for the R226V variant.

selectivity, remains nearly unchanged ({k_{st}[R226V, I]/k_{st}[R226V, II]}/{k_{st}[wild-type, I]/k_{st}[wild-type, II]} = 64; {k_{cat}[R226V, I]/k_{cat}[R226V, II]}/{k_{cat}[wild-type, I]/k_{cat}[wild-type, II]} = 68).

Salt and Temperature Dependence of σ. We measured the dependence of the initial rate, k, for oligodeoxynucleotides I and II on the concentration of sodium chloride or the reaction temperature with wild-type *Eco*RV and the R226V variant. The experiments were performed at a single substrate concentration of about 10 × K_m.

Table 2: First-Order Cleavage Rate Constants (k_{st}) and Apparent Activation Energies (E_a) for Cleavage of Oligodeoxynucleotides I and II by Wild-Type *Eco*RV and the R226V Variant[a]

	mutant			
	wild-type		R226V	
	I	II	I	II
k_{cat} (min^{-1})	63 ± 16	34 ± 7	19 ± 1	0.15 ± 0.04
k_{st} (min^{-1})	124 ± 12	29 ± 2	57 ± 7	0.21 ± 0.12
E_a (kJ/mol)	14 ± 11	34 ± 13	158 ± 16	212 ± 40

[a] For comparision, also the k_{cat} values are given. Standard errors of at least three independent determinations are given.

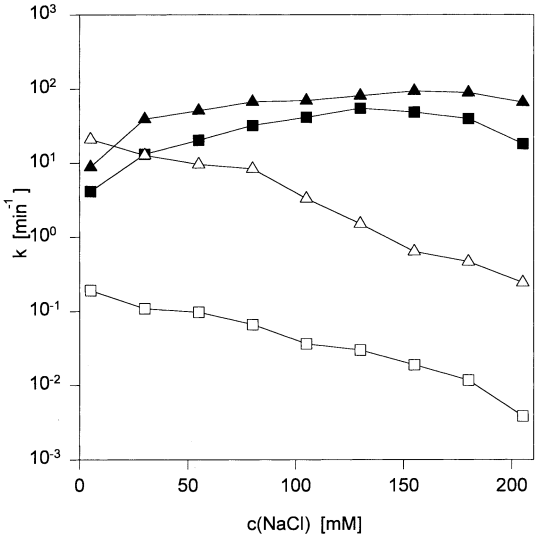

FIGURE 5: Dependence of the initial rates of cleavage of oligodeoxynucleotides I, d(AAAAAAAGATATCTTTTTTT)$_2$ (triangles), and II, d(TATATATGATATCATATATA)$_2$ (squares), by wild-type *Eco*RV (filled symbols) and the R226V variant (open symbols) on different concentrations of NaCl. The symbols represent experimental data; the solid lines are merely drawn to connect the data points.

The wild-type and mutant enzymes respond in a fundamentally different way to variations of the salt concentration in the buffer (Figure 5). Whereas the wild-type enzyme shows with both substrates a broad maximum of activity between 130 and 180 mM NaCl, the initial cleavage rate of the mutant decreases with increasing salt concentration. At 5 mM NaCl, the activity of the mutant with oligodeoxynucleotide I exceeds even that of the wild-type enzyme. Since the activity of the R226V variant toward oligodeoxynucleotide I is slighly more sensitive to increasing salt concentrations than the activity toward oligodeoxynucleotide II (Figure 5), not only the activity but also the ratio of the initial rates for the cleavage of oligodeoxnucleotides I and II by the R226V variant reaches its maximum at low concentrations of sodium chloride.

We measured the temperature dependence for both enzymes with either oligodeoxynucleotide in the range from 15 to 30 °C and obtained linear Arrhenius plots (Figure 6) indicating that the rate-limiting step does not change with temperature in this range; the corresponding apparent energies of activation are shown in Table 2. We could not obtain complete data sets at temperatures ≤ 10 °C because oligodeoxynucleotide II under these conditions was resistant to cleavage by the R226V variant. Similar as observed for the dependence on salt concentration, the temperature

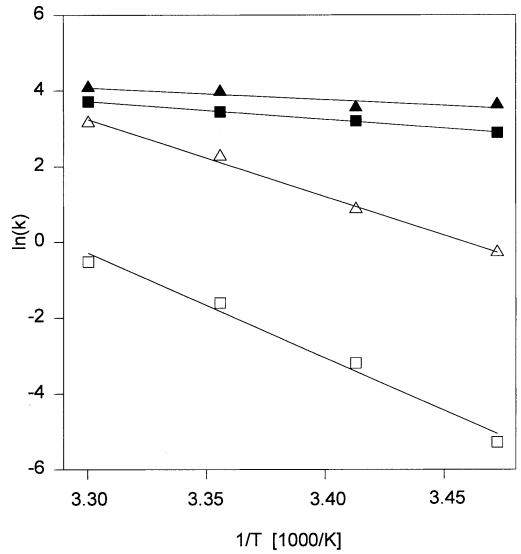

FIGURE 6: Arrhenius plots for the cleavage of oligodeoxynucleotides I, d(AAAAAAAGATATCTTTTTTT)$_2$ (triangles), and II, d(TATATATGATATCATATATA)$_2$ (squares), by wild-type *Eco*RV (filled symbols) and the R226V variant (open symbols). The symbols represent experimental data and the solid lines are a best fit to the Arrhenius equation with the apparent activation energies given in Table 2.

dependence of the cleavage of both substrates is only small for the wild-type enzyme but large for the R226V variant (Figures 5 and 6). The apparent activation energy for the cleavage of oligodeoxynucleotide II by the R226V variant is slightly greater than the corresponding value for oligodeoxynucleotide I (Table 2). This impies that the ratio of the initial rates for the cleavage of oligodeoxynucleotides I and II by the R226V variant increases with decreasing temperature.

Plasmid Substrates. To test whether the high selectivity of the R226V variant toward oligodeoxynucleotides containing *Eco*RV sites in a sequence context of different flexibility is preserved with plasmid substrates, we constructed two pUC8 derivatives as described under Experimental Procedures. These plasmids, pUC8 I and pUC8 II, harbor a single *Eco*RV site with the same flanking sequences as in oligodeoxynucleotides I and II, respectively. We measured the initial rates for the cleavage of both plasmids with wild-type *Eco*RV and the mutant under the same conditions as employed for the steady-state measurements with oligodeoxynucleotides (Figure 7). In accordance with the oligodeoxynucleotide data, the wild-type enzyme cleaves both plasmid substrates with roughly the same rate whereas the mutant hydrolyzes pUC8 I, the substrate with the *Eco*RV site in a nonflexible sequence context, with a 73-fold higher activity than pUC8 II. Furthermore, cleavage of both plasmid substrates by the R226V variant is associated with the accumulation of more nicked circle intermediate than in the case of the wild-type enzyme (Figure 7).

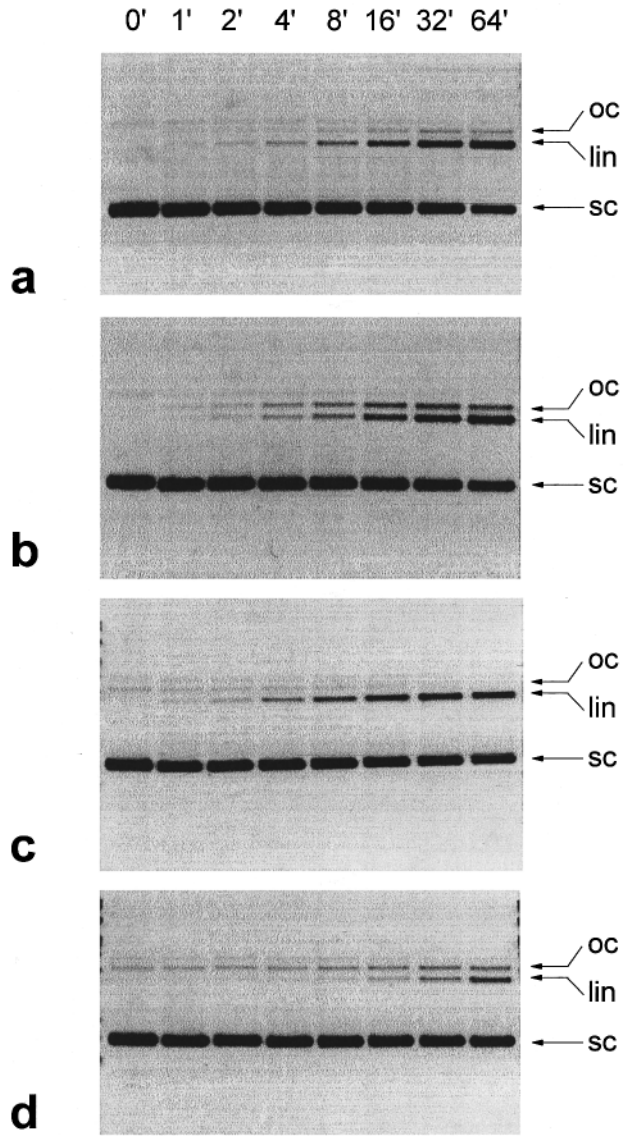

FIGURE 7: Cleavage of 14 nM pUC8 I (harboring a single *Eco*RV site with the flanking sequences of oligodeoxynucleotide I) by 0.1 nM wild-type *Eco*RV (a) and 0.5 nM R226V (b) and of 14 nM pUC8 II (harboring a single *Eco*RV site with the flanking sequences of oligodeoxynucleotide II) by 0.1 nM wild-type *Eco*RV (c) and 14 nM R226V (d) at 23 °C. Positions of supercoiled (sc), open circular (oc), and linear (lin) form of pUC8 I and pUC8 II are indicated. The initial cleavage rates are 3.6 ± 1.0 min^{-1} (a), 0.56 ± 0.27 min^{-1} (b), 4.7 ± 0.9 min^{-1} (c), and 0.0076 ± 0.0065 min^{-1} (d) (standard errors of at least three independent determinations are given).

DISCUSSION

It has long been known that the activity of restriction endonucleases is influenced by the sequences in the vicinity of the recognition site; e.g., some *Eco*RI sites on λ-DNA are hydrolyzed by a factor of 10 faster than others (*19, 20*). In the case of *Eco*RI, similar results were obtained with other substrates (*21*−*24*) and confirmed with many other *Eco*RI variants (T. Oelgeschläger, A. Jeltsch, A. Fritz, and A. Pingoud, unpublished; *25*). Other restriction endonucleases also depend in their activity on sequences flanking the recognition site, e.g., *Nae*I (*26*), *Eco*RV (*27*), and *Eco*RV variants (*16*). The aim of this work was to enhance the potential of variants of the restriction endonuclease *Eco*RV to discriminate between *Eco*RV sites with different flanking sequences and to elucidate the molecular basis for this enhanced selectivity in order to try to further increase the selectivity of this enzyme. To this end, we used two oligodeoxynucleotides, d(AAAAAAAGATATCTTTTTTT)$_2$ and d(TATATATGATATCATATATA)$_2$, as prototypic substrates for an *Eco*RV site in a nonflexible and a flexible sequence context, respectively. Results obtained with various

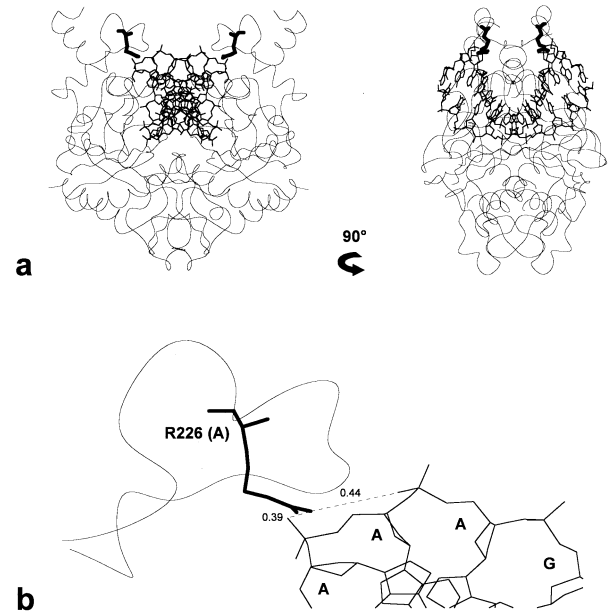

FIGURE 8: Structural model of the *Eco*RV/d(AAAGATATCTT)$_2$ complex (Brookhaven data bank entry 1RVA). (a) Two views of the entire complex with the DNA and residues Arg226 of subunits A and B in boldface; from the rest of the protein only the α carbon trace is shown (in gray). (b) Detail of the structure on the left side of (a) centered on the side chain of residue 226 of subunit A; the distances between the Nη2 atom of Arg266 and the nonbridging oxygen atoms of adjacent phosphate groups of the DNA are marked with dotted lines; distances are given in nanometers.

experimental approaches, such as X-ray crystallography (*28*−*30*), interaction of DNA with nucleosomes (*31, 32*), and sensitivity to unspecific nucleases such as DNase I (*33*), indicate that alternating AT stretches possess a considerably higher degree of flexibility than homo-A/homo-T stretches.

All *Eco*RV variants analyzed in the present study harbor amino acid substitutions that affect contacts of the enzyme to the phosphodiester backbone of the DNA. These contacts contribute to the indirect readout (defined as the contribution of backbone contacts to specific recognition), as opposed to the direct readout (defined as the contribution of base contacts to specific recognition) (review: *34*). An unusual property of the DNA in the crystal structure of the specific *Eco*RV/DNA complex (*9*) is its highly distorted conformation characterized by a sharp central kink of approximately 50° (*9, 35, 36*; Figure 8a). However, this kink originates at the central TA step of the recognition sequence; the rest of the DNA, especially the base pairs flanking the recognition site, is in standard B-conformation (*9*).

Our results implicate an important function of amino acid residue Arg226 in mediating the sensitivity of the enzyme to different flexibilities of sequences flanking the recognition site. The guanidinium group of this residue is in a position suited for the establishment of ionic interactions with DNA phosphates upstream of the recognition sequence (Figure 8). Exchanging Arg226 for various amino acids (L, A, D, Q, H, and V) yields *Eco*RV variants that—in contrast to the wild-type enzyme (and the R226E mutant)—are able to discriminate efficiently between both test substrates; all of them hydrolyze the oligodeoxynucleotide with nonflexible flanking sequences with much higher catalytic efficiency (k_{cat}/K_m) than the one with flexible flanking sequences and therefore show a much higher selectivity than the wild-type enzyme (Table 1). Without the stabilizing effect of an ionic interaction between residue 226 and the DNA phosphates, the enzyme probably is not able to force the substrate with flexible flanking sequences into the correct, catalytically reactive conformation. Rather, the wide range of conformations that this substrate can adopt may allow it to "escape" the force exerted by the enzyme. Only the substrate with the nonflexible flanking sequences is rigid enough to remain without the interaction with Arg226 in a straight, B-like conformation, as is seen for the sequences flanking the recognition site in the structure of the specific complex (Figure 8) and is therefore cleaved much more efficiently.

This enhanced selectivity of the mutants is not only a local effect, because identical oligodeoxynucleotides differing only in the base pairs on both sides of the *Eco*RV recognition site (-AGATATCT- versus -TGATATCA-) are cleaved by the wild-type enzyme and by the R226A variant with the same rate, respectively (data not shown).

One might argue that not flexibility but a sequence-directed bend of the DNA induced by the poly(A) flanks of the oligodeoxynucleotide with the *Eco*RV site in a nonflexible sequence context is responsible for the efficient interaction of this substrate with the mutants in a similar way as was shown for the binding of the TATA-binding protein to a "prebend" promotor sequence (*37*). However, we do not think that such a bend would be advantageous for *Eco*RV variants, because it was shown that poly(A) tracts bend DNA toward the minor groove (*38*), whereas the DNA in the specific *Eco*RV/DNA complex is bent in the opposite direction, toward the major groove (*9*).

The high selectivity of the mutants can be attributed to the k_{cat} (Table 1). For the R226V variant, it was shown in single-turnover experiments that the k_{cat} for oligodeoxynucleotide II is determined by the catalytic step per se (or a step preceding it) whereas the k_{cat} for oligodeoxynucleotide I is determined by the catalytic step (or a step preceding it) and product release (Figure 4, Table 2). However, the ratio of the k_{st} values with substrates I and II for this mutant is even greater than the corresponding ratio of the k_{cat} values (Table 2). Consequently, the restriction afforded by Arg226 on the conformational freedom of the DNA substrate contributes most probably to the stability of the transition state of the reaction. In contrast, binding of the substrate, indicated by the effect on K_m, is favored by flexible flanking sequences, which is observed for all *Eco*RV variants analyzed, with the exception of R226E (Table 1). An explanation may be that a substrate with the *Eco*RV site in a flexible sequence context adapts better to the binding cleft of the enzyme. The flexibility of the sequences flanking the recognition site of *Eco*RV is consequently an example of a feature of the substrate which is favorable in the ground state of the reaction, but unfavorable in the transition state.

The only *Eco*RV variant at position 226 that shows no discrimination between the two substrates is R226E. Rather, this enzyme hydrolyzes both oligodeoxynucleotides very slowly. Presumably, the negatively charged glutamic acid side chain is placed in such an unfavorable position that it interferes with the correct positioning of both substrates because of the repulsive interaction with the negatively charged DNA backbone. In contrast, an aspartic acid at position 226 can be accommodated in the protein/DNA interface without complications associated with the negative

charge, since the R226D variant displays a similar behavior as the mutants with uncharged amino acid substitutions at this position. The differences in selectivity within the group of variants with neutral amino acid substitutions at position 226 are not very pronounced and may be explained by small local rearrangements in the protein/DNA interface.

To examine the possibility of further enhancing the selectivity of *Eco*RV by variation of the reaction conditions, we determined the dependence of the reaction rates for the cleavage of both substrates by wild-type *Eco*RV and the R226V variant on the concentrations of NaCl in the medium and on the reaction temperature (Figures 5 and 6). Whereas the activities of the wild-type enzyme with both substrates had broad maxima around 150 mM NaCl and showed only a slight decrease with decreasing temperatures, corresponding to activation energies typical for enzymatic reations (*39*), the reaction rates of the R226V variant are diminished with increasing salt concentrations or decreasing temperatures. To explain this difference between wild-type *Eco*RV and this variant, one might argue that a variant that is already unable to establish a perfect fit at the protein/DNA interface will be more sensitive than the wild-type enzyme to conditions that globally weaken protein/DNA interactions, as increasing salt concentration, or reduce the total energy of the system. Although the cleavage reactions under nonstandard conditions were done only at a single substrate concentration and therefore do not allow calculation of true selectivities, which are defined as ratios of k_{cat}/K_m values, it seems reasonable to assume that the selectivity of the R226V variant is enhanced by lowering the salt concentration and by performing the reaction at low incubation temperatures, because under these reaction conditions the ratio of the initial rates for the cleavage of oligodeoxynucleotides I and II increases significantly (Figures 5 and 6).

The remarkable sensitivity of the R226V variant for the flexibility of sequences flanking the *Eco*RV site is not a special property confined to oligodeoxynucleotide substrates. In accordance with the oligodeoxynucleotide data, this variant is able to discriminate efficiently between *Eco*RV sites with flexible and nonflexible flanking sequences with plasmid substrates harboring *Eco*RV sites in the sequence context of oligodeoxynucleotides I and II, respectively, while the activity of the wild-type enzyme is not influenced by the flexibility of sequences flanking the *Eco*RV site (Figure 7). The agreement of data obtained with oligodeoxynucleotides and with plasmid substrates implicates that it is reasonable to suppose that the conclusions drawn from experiments with oligodeoxynucleotides in this work are also valid for macromolecular DNAs.

The use of covalently closed supercoiled plasmids as substrates for restriction enzymes allows determination of the individual rates of the two single-strand cuts leading to linear DNA, the reaction product. In the case of concerted cleavage of both strands, the reaction proceeds without accumulation of the open circular DNA intermediate. Since only a faint band of open circular DNA is seen for the cleavage of both plasmid substrates used here by the *Eco*RV wild-type, this enzyme cleaves both strands of the DNA predominantly in a concerted manner (Figure 7). Similar results were reported previously for the wild-type enzyme with other plasmid substrates under optimal reaction conditions (*40*). With the R226V variant, on the other hand, considerably more open circular DNA accumulates during the course of the reaction, indicative for a sequential cleavage of both DNA strands (Figure 7).

In summary, this study shows that it is possible to greatly enhance the selectivity of a restriction endonuclease toward unmodified substrates by exchanging amino acid residues, which contribute to the indirect readout of the enzyme. Similar changes in the selectivity of variants of the restriction enzyme *Eco*RV were reported previously only with modified oligodeoxynucleotides as substrates (*14*−*16*). However, the "price" that the enzyme has to pay for its improved discriminatory power is high, namely, 98% of its catalytic efficiency (Table 1). It remains to be seen if amino acid substitution at other positions or the combination of amino acid substitutions will yield *Eco*RV variants with a similar degree of selectivity but without a considerable loss of specific activity.

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