Probing the Indirect Readout of the Restriction Enzyme EcoRV

MUTATIONAL ANALYSIS OF CONTACTS TO THE DNA BACKBONE*

(Received for publication, October 5, 1995, and in revised form, December 19, 1995)

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According to the crystal structure of the specific EcoRV-DNA complex, not only the functional groups of the nucleobases but also the phosphate groups of the DNA backbone are contacted by the enzyme. To examine the contribution of backbone contacts to substrate recognition and catalysis by EcoRV, we exchanged 12 amino acids residues located close to phosphate groups by site-directed mutagenesis. We purified the resulting EcoRV mutants and characterized them with respect to their DNA binding and cleavage activity. According to our steady state kinetic analysis, there are strong interactions between three basic amino acid residues (Lys-119, Arg-140, and Arg-226) and the phosphate backbone that support specific binding presumably by inducing and maintaining the kinked conformation of the DNA observed in the specific EcoRV-DNA complex. These contacts are important in both the ground state and the transition state. Other, uncharged residues (Thr-93 and Ser-112), which could be involved in hydrogen bonds to the phosphate groups, are needed primarily to stabilize the transition state. An especially important amino acid residue is Thr-37, which seems to couple recognition to catalysis by indirect readout.

The specific interaction of proteins with DNA is of fundamental importance in biology. Much effort, therefore, has been undertaken to understand the molecular basis of the underlying recognition process. It has been suggested that specificity is due to specific hydrogen bonds between the protein and the edges of the bases of the DNA (direct readout) (1). Such contacts are indeed observed in nearly every structure of specific DNA-DNA complexes obtained so far for restriction enzymes, where hydrogen bonds or ionic interactions to the phosphate groups of the bases are needed to stabilize the transition state. Other, uncharged interactions are important in both the ground state and the transition state. Other, uncharged residues (Thr-93 and Ser-112), which could be involved in hydrogen bonds to the phosphate groups, are needed primarily to stabilize the transition state. An especially important amino acid residue is Thr-37, which seems to couple recognition to catalysis by indirect readout.

mediated base contacts as well as DNA-induced tetramerization of the protein on the DNA (3). From comparisons of co-crystal structures of specific and non-specific complexes of DNA binding proteins with DNA, it became evident that specific recognition of DNA by proteins is mediated by direct and indirect readout also in other systems (e.g. glucocorticoid receptor (4); EcoRV (5)); in general, in the specific complexes, much more interactions are observed between the protein and the bases than between the protein and the phosphate backbone of the DNA than in the non-specific complexes.

Steady-state kinetic analysis, there are strong interactions between three basic amino acid residues (Lys-119, Arg-140, and Arg-226) and the phosphate backbone that support specific binding; it was argued, therefore, that in this case, specific recognition of DNA is primarily due to contacts to the phosphate groups of the DNA backbone (indirect readout) (2). This means that during interaction with DNA, the protein recognizes a specific sequence-dependent conformation of the phosphate backbone in addition to functional groups of the bases. This concept was initially met with some reservation, but it has proven to be very fruitful, although it was recently demonstrated that the specific interaction between the trp repressor and its operator is not only due to phosphate contacts but also due to water-mediated base contacts as well as DNA-induced tetramerization of the protein on the DNA (3). From comparisons of co-crystal structures of specific and non-specific complexes of DNA binding proteins with DNA, it became evident that specific recognition of DNA by proteins is mediated by direct and indirect readout also in other systems (e.g. glucocorticoid receptor (4); EcoRV (5)); in general, in the specific complexes, much more interactions are observed between the protein and the bases than between the protein and the phosphate backbone of the DNA than in the non-specific complexes.

It is difficult to assess the relative contributions of both mechanisms of specific recognition because in virtually all complexes between specific DNA binding proteins and their cognate DNA, direct and indirect readout is the result of an interconnected network of hydrogen bonds to functional groups of the bases exposed in the major and minor grooves and hydrogen bonds or ionic interactions to the phosphate groups of the DNA. The crystal structure analyses of restriction enzyme-DNA complexes provide beautiful examples that illustrate the complexity of the network characteristic for the protein-DNA interfaces, as demonstrated for EcoRI (6, 7), EcoRV (5, 8), PvuI (9), and BamHI (10). In the case of EcoRV, there are various reasons to assume that the indirect readout contributes to the recognition process. In contrast with the co-crystal structures of the other restriction enzymes, where direct or water-mediated hydrogen bonds are observed to every base pair of the respective recognition sequences, in the specific EcoRV-DNA co-crystal structure, no hydrogen bonds to the bases of the two inner AT base pairs of the recognition sequence GAT ↓ ATC (the arrow denotes the position of phosphodiester bond cleavage) are seen. Furthermore, among the four co-crystal structures obtained so far for restriction enzymes, the EcoRV-DNA structure is unique in having a highly distorted DNA with a sharp central kink of approximately 50° (5, 11, 12), which renders the inner two base pairs inaccessible to the protein. Recognition of these two base pairs, therefore, cannot be direct. On the other hand, there are a multitude of amino acid residues contacting the phosphate groups of the DNA both within and outside of the recognition sequence (5) (see Fig. 1), which might compensate for the lack of base contacts.

One strategy applied to examine the indirect readout of restriction enzymes has been to use modified oligonucleotides as substrates, e.g. phosphorothioates (EcoRI (13, 14), EcoRV) or S-methylphosphorothioates (TaqI (16)). Here we present a complementary approach focussing on the contribution of the protein to the indirect readout by a systematic mutational analysis of amino acid residues, which in the crystal structure are located sufficiently close to the phosphodiester backbone to...
be considered candidates for indirect readout. We will present evidence that the catalytic efficiency of the restriction enzyme EcoRV depends on both modes of specific recognition.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis and Purification of EcoRV Variants—Site-directed mutagenesis of the EcoRV gene was carried out following the method described by Ito et al. (17). This technique is based on the incorporation of a desired mutation with a PCR™ primer by two successive PCR™ reactions. The presence of the mutations, as well as the absence of unwanted mutations elsewhere in the gene were confirmed by sequencing both strands of the entire gene of each mutant. All mutant proteins had an affinity tag of six His residues on their N terminus to facilitate purification (18). Protein expression and purification was carried out essentially as described by Wenz et al. (18).

Cleave Assays with Plasmid DNA—For plasmid cleavage assays, 21 nM pATRV (a derivative of pAT153 containing an additional EcoRV cleavage site) linearized with Asel (U. S. Biochemical Corp.) was incubated at 37 °C in cleavage buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, or star buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MnCl₂) with varying amounts of wild-type or mutant EcoRV (diluted in 10 mM Tris-HCl, pH 7.5). After defined time intervals, aliquots were withdrawn, and the reaction was stopped by adding one-fifth volume 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). Cleave activity is given in relative units defined by the amount of enzyme needed to completely digest the DNA under standard conditions.

Oligodeoxynucleotide Substrate—The oligodeoxynucleotide employed in the cleavage assay was synthesized on solid support with a Milligen Cyclone DNA synthesizer and purified by denaturing polyacrylamide gel electrophoresis. It was labeled at the 5'-end with γ-[^32]P-ATP (Amersham Corp.) and T4 polynucleotide kinase (MBI Fermentas) according to the protocol of the suppliers. Throughout the text, oligodeoxynucleotides that carry a 5'-phosphate group are abbreviated by d(…). Indirect Readout of EcoRV—The Indirect Readout of EcoRV was investigated as a function of oligodeoxynucleotide and mutant proteins had an affinity tag of six His residues on their N-terminus to facilitate purification (18). Protein expression and purification was carried out essentially as described by Wenz et al. (18).

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**RESULTS**

Selection, Generation, and Purification of EcoRV Mutants—According to the available EcoRV-DNA co-crystal structures (Brookhaven databank entries 2RVE, 4RVE, 1RVA, 1RVB, 1RVC) (5, 8), there are 12 amino acid side chains with hydrogen bond donors in close vicinity to the nonbridging oxygen atoms of the phosphate groups of the DNA substrate (heteroatom distance < 0.4 nm, Fig. 1, Table I). Residues of the catalytic center, the R- and the Q-loops, were the target of previous mutational studies (21, 22) not included in the work described here. Amino acid residues, which due to their proximity to the DNA backbone could be responsible for the indirect readout of EcoRV, were exchanged by PCR mutagenesis to Ala (in the case of Arg, Lys, Ser, Thr) or to Phe (Tyr). Wild-type EcoRV and the 12 mutants were produced as N-terminally His₆-tagged proteins and purified to near homogeneity (better than 95%) by chromatography on nickel chelate columns. The specific activity of the wild-type EcoRV preparation determined with λ-cleavage assays was, within the limits of error, the same as measured previously in our experiments (18) or in other groups (23, 24).

Cleave of a Plasmid Substrate by EcoRV Mutants—The mutant EcoRV proteins were characterized first by cleavage assays with pATRV. This plasmid harbors two EcoRV sites with very different flanking sequences (... GCC-GATATCGTCC... versus ... GAAAGATATC TTTT ...). To allow for the identification of the products of cleavage of pATRV, the plasmid was linearized with Asel prior to cleavage with EcoRV. All mutants display specific EcoRV activity in normal cleavage buffer (containing MgCl₂) and star buffer (containing MnCl₂); the mutants T111A, S112A, and, to a smaller extent, S41A, show an enhanced star activity compared with the wild-type enzyme, i.e. cleavage at sites that differ in one base pair from the canonical site (Fig. 2, 25). The substrate concentration used in this assay (21 nM), most of the mutants display moderately altered relative activities in cleavage buffer, ranging from a slightly enhanced to by a factor of 50 decreased activity in comparison with the wild-type enzyme (Table II). Only one EcoRV variant, T37A, shows a 1000-fold diminished relative activity. This is comparable in magnitude with the activity of various mutants with substitutions in the recognition loop, for example N185A, N188A, and S183A, which have residual activities of 1/5000, 1/600–1/5000, and 1/1000, respectively (26). These data indicate that Thr-37 is as important for the catalytic efficiency of EcoRV as amino acid residues of the recognition loop, which are engaged in specific hydrogen bonds to the bases of the recognition sequence.

It is known that the DNA cleavage rate of EcoRV is influenced by sequences flanking the recognition sequence (27). The use of a plasmid substrate with two EcoRV sites embedded in very different sequence surroundings offers the possibility to screen the mutants for an enhanced sensitivity toward flanking sequences. Whereas in cleavage buffer the wild-type enzyme cleaves both sites with similar rates, three EcoRV variants, namely R226A, R140A, and T93A, show a preference for one site (Table II, Fig. 3), all three mutants prefer the EcoRV site...
Fig. 1. Amino acid residues contacting the phosphate backbone of the DNA. In a, a schematic representation of backbone contacts between EcoRV and one strand of the DNA are given. Only residues that harbor a hydrogen bond donor in a distance <0.4 nm from one of the nonbridging oxygen atoms of the phosphate groups in at least one of the EcoRV-DNA co-crystal structures are shown. These residues and the contacted DNA strand are highlighted in the structural model of the specific undecamer complex (Brookhaven data bank entry 1RVA) shown in b. O₂ refers to side chain oxygen of Ser and Thr residues, O₁ refers to the side chain oxygen of a Tyr residue, and N₁ and N₂ refer to the terminal nitrogen atoms of Arg and Lys residues, respectively.

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with AT rich flanking sequences.

Cleavage of Oligodeoxynucleotide Substrates by EcoRV Mutants—As the crystal structure analysis of the EcoRV × d(AAA-GATATCTT)₂ complex has produced evidence for an interaction between EcoRV and position −5 of the substrate DNA (cf. Fig. 1a), a 20-mer oligonucleotide substrate was chosen for a detailed kinetic analysis of the mutant proteins under steady-state conditions. This substrate can be expected to mimic a high molecular weight DNA as it is longer than the entire binding pocket of EcoRV, which covers 14–16 bp (5, 20). By variation of the substrate concentration over 5 orders of magnitude, it was possible to determine the kinetic parameters \( K_m \) and \( k_{cat} \) for all mutants except the T37A mutant (Table III).

Fig. 4 shows as an example the Michaelis-Menten diagrams for the wild-type EcoRV and the mutant R226A. The steady-state kinetic parameters determined for the cleavage of the 20 bp substrate by the wild-type enzyme are in the same order of magnitude as corresponding values reported previously for plasmid substrates, e.g. for pAT153, the reported \( K_m \), \( k_{cat} \), and \( k_{cat}K_m \) values are 0.5 mM, 0.9 min⁻¹, and \( 3.0 \times 10^7 \) M⁻¹ s⁻¹, respectively (25).

Depending on their \( K_m \) and \( k_{cat} \) values, it is possible to divide the EcoRV mutants into three groups (Table III).

Group I consists of mutants with a similar \( k_{cat} \)/\( K_m \) value as the wild-type enzyme: S41A, T94A, Y95F, T111A, R221A, S223A.

Group II consists of mutants with lower \( k_{cat} \)/\( K_m \) values due to an increase in \( K_m \): K119A, R140A, R226A. The \( k_{cat} \)/\( K_m \) values of these mutants are reduced by 2–3 orders of magnitude compared with the wild-type enzyme, and this group, therefore, comprises the EcoRV variants with the lowest activities.

Group III consists of mutants with lower \( k_{cat} \)/\( K_m \) values due to a decrease in \( k_{cat} \): T93A and S112A. The \( k_{cat} \)/\( K_m \) values of these mutants are by more than 1 order of magnitude lower than that of the wild-type enzyme.

The only EcoRV variant that could not be classified according to the above scheme is the T37A mutant. Due to its low activity, DNA cleavage by T37A is only measurable at equimolar concentrations of enzyme and substrate, i.e. under single turnover conditions. In good agreement with the results of the plasmid DNA cleavage experiments (Table II), the single turnover cleavage rate constant \( k \) for oligodeoxynucleotide DNA cleavage by this mutant is 700 times lower than the \( k_{cat} \) of the wild-type enzyme (Table III). As the \( K_d \) value measured for T37A (see below) is well below the substrate concentration that was employed in the single turnover experiments (0.5 μM), the enzyme is likely to be saturated with substrate. Hence, it is reasonable to compare the \( k \) value of the mutant with the \( k_{cat} \) value of the wild-type enzyme. Thr-37 is the only amino acid residue involved in a phosphate contact that has an impact on both specific binding (\( K_d \)) and catalysis (\( k \)), although the effect on catalysis is much more pronounced.

DNA Binding Experiments with EcoRV Mutants—In addition to the kinetic analyses, equilibrium binding studies with a 381-bp DNA fragment harboring a single EcoRV site were performed and analyzed by gel electrophoretic mobility shift assays. These experiments were carried out in the presence of 10 mM CaCl₂ (instead of MgCl₂), which inhibits cleavage but supports specific DNA binding (12). The equilibrium DNA binding constant measured for binding of the wild-type enzyme to the 381-bp DNA fragment is the same as that determined previously under similar reaction conditions (12). By variation of the protein concentration over 4 orders of magnitude, it was possible to detect a specific band shift for all mutants (Fig. 5). It was not possible, however, to monitor complete binding curves for the mutants K119A and R226A because of their low affinity to DNA. The retardation of the specific complex in comparison with the free DNA was identical for all EcoRV variants, implying that the degree of DNA binding is within ±5° (the estimated accuracy of the determination), the same for the wild-type enzyme and all EcoRV mutants.
For the wild type enzyme and most of the mutants, the $K_d$ values measured in the presence of Ca$^{2+}$ (which permits cleavage, but supports binding) are 1 order of magnitude lower than the $K_m$ values measured in the presence of Mg$^{2+}$. In a double logarithmic plot of $K_d$ versus $K_m$, most mutants are well represented by the regression line (Fig. 6). Exceptions are the mutants T93A and S112A; their $K_d$ values are more than 1 order of magnitude higher than the corresponding $K_m$ values. As in the two sets of experiments (binding versus cleavage) only the divalent cation used was different; this finding may suggest that in the presence of specific DNA, these two mutants have a lower affinity for Ca$^{2+}$ than for Mg$^{2+}$ compared with the wild-type enzyme and the other mutants.

**DISCUSSION**

Restriction endonucleases recognize their cleavage sites on double-stranded DNA with remarkable high specificity by forming contacts both to the bases and to the backbone of the DNA (28, 29). As the essential role of the base contacts had been established before (21, 18), it has been the aim of the work presented here to define the importance of contacts between amino acid residues and phosphate groups for the mechanism of DNA recognition by the restriction endonuclease EcoRV. For this purpose, we have exchanged all amino acid residues that present here to define the importance of contacts between amino acid residues and phosphate groups for the mechanism of DNA recognition by the restriction endonuclease EcoRV. For this purpose, we have exchanged all amino acid residues that
is in agreement with the results of a complementary analysis in which phosphate contacts of EcoRV were probed by phosphoro-thioate containing oligonucleotides; it was shown that all phosphates demonstrated by our study to be indirect readout show effects upon modification (the position +5 was not subject of this study).  

The various mutants can be classified according to whether they are impaired in $K_m$ and/or $k_{cat}$. In the subsequent discussion this classification will be used.

Mutants with Similar Catalytic Efficiency as the Wild-type Enzyme (group I in Table III)—Six out of 11 EcoRV mutants (S41A, T94A, Y95F, T111A, R221A, S223A) display a similar catalytic efficiency $(k_{cat}/K_m)$ as the wild-type enzyme when analyzed in steady state cleavage experiments. The energy penalty $\Delta G_{app}$ associated with the deletion of the respective functional group in one enzyme monomer is less than 1 kJ/mol or 0.24 kcal/mol $(\Delta G_{app} = -0.5 \cdot RT \ln ([K_{cat}/K_m]_{mutant}/(K_{cat}/K_m)_{wild\ type})$, Table III), which is well below the value of 5.5 kJ/mol or 1.3 kcal/mol estimated for an average protein phosphate contact (30, 31). Consequently, during the enzymatic turnover, the amino acid residues Ser-41, Thr-94, Tyr-95, Thr-111, Arg-221, and Ser-223 most likely interact only weakly with the phosphates of the DNA substrate and are of minor importance for substrate binding and cleavage.

Interestingly, some of the mutants (T94A, Y95F, and R221A), which have a similar $k_{cat}/K_m$ value as the wild-type enzyme, have a 2-fold higher $k_{cat}$ and $K_m$ value (group I* in Table III). Moreover, their $K_d$ is also higher than that of the wild-type enzyme. These results may be interpreted in terms of small differential effects on substrate binding and transition state stabilization; it appears that these residues are involved in contacts that stabilize the ground state of the enzyme-substrate complex, not, however, the transition state (Fig. 7).

Mutants with a Reduced DNA Affinity (Group II in Table III)—During the recognition process, EcoRV distorts the cognate sequence, which leads to the unstacking of the two central base pairs and a bending of approximately 50° as seen in the crystal structure of the specific EcoRV-DNA complexes (5, 8) and confirmed by gel shift experiments with catalytically inactive mutants (11) or with Ca$^{2+}$ as a substitute for Mg$^{2+}$ (12). If the DNA distortion were of similar importance for the transition state as it is for the ground state, one would expect that mutants defective in contacts responsible for bending will have a higher $K_m$ or $K_d$ and a more or less unaltered $k_{cat}$, as the energy difference between ground and transition state is the same (Fig. 7). There are three amino acid residues contacting the phosphate backbone which fit into this scheme, namely the basic amino acid residues Arg-226, Lys-119, and Arg-140; the corresponding alanine mutants show a drastic increase in $K_m$ and $K_d$, but an only slightly changed $k_{cat}$ (Table III). Consequently, the results of both steady state cleavage and equilibrium binding experiments demonstrate that basic residues that are not in direct contact with functional groups of the bases of the recognition sequence but close to the phosphate groups of the DNA backbone, are important for specific binding. The only exception to this rule is the amino acid Arg-221, as the R221A mutant shows only moderately enhanced $K_m$ and $K_d$ values.

We suggest that the main function of the residues Arg-226, Lys-119, and Arg-140 is to stabilize the distorted conformation of the DNA in the specific EcoRV-DNA complex. The results obtained for the K119A and R140A mutants can be rationalized by a comparison of the co-crystal structures of EcoRV with specific and nonspecific DNA. Upon formation of the specific complex, there is a significant movement of the residues Lys-119 and Arg-140 with respect to the DNA that shortens the...
distance between the interacting partners (Fig. 8, Table I). Thus, weak ionic interactions with nonspecific DNA become considerably stronger in the complex with the specific substrate leading to a gain in binding energy at the transition from the nonspecific to the specific binding mode. The distances between Lys-119, Arg-140, and the phosphate groups (Table I) are not the only ones that are altered in the co-crystal structures with nonspecific and specific DNA, but we suppose that the changes in positions of Lys-119 and Arg-140 are the most important ones because the interacting partners are charged.

Whereas the decrease in apparent binding energy that accompanies the substitution of Lys-119 (6.7 kJ/mol or 1.6 kcal/mol) and Arg-140 to alanine (4.0 kJ/mol or 0.95 kcal/mol), respectively, are roughly compatible with the deletion of a single phosphate contact, the energy penalty associated with the exchange at position 226 (8.2 kJ/mol or 2.0 kcal/mol) is significantly higher. The large effect observed with the R226A mutant is particularly noteworthy as Arg-226, which is in a disordered region in the co-crystal structure of the unspecific EcoRVz DNA complex, contacts the DNA backbone two residues outside of the recognition sequence. This implies that Arg-226 must have an additional role. It has been suggested that this amino acid residue apart from promoting specific binding is responsible for the stabilization of a loop between α helices D and E encompassing residues 221–228 (8) (cf. Fig. 1b). The importance of the C terminus of EcoRV for catalysis was dem-

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**Table III**

| Mutant  | Group | $K_m$ [nM] | $k_{cat}$ [s$^{-1}$] | $k_{cat}/K_m$ [s$^{-1}$] | $\Delta G_{exp}$ [kJ/mol] | $\Delta G_{app}$ [kJ/mol] | $K_d$ [nM] |
|---------|-------|-----------|---------------------|-------------------------|--------------------------|------------------------|-----------|
| Wild-type | II    | 2.1 ± 0.6 | 2.9 ± 0.2           | 23 ± 5.4                | 100                      | 0.20                   |           |
| T37A    | I     | 3.2 ± 0.8 | 6.7 ± 0.5           | 37 ± 7.1                | 161                      | 0.59                   | 0.14      |
| T93A    | II    | 4.0 ± 0.9 | 0.33 ± 0.02         | 1.6 ± 0.33              | 7.0                      | 3.3                    | 0.79      |
| T94A    | I*    | 5.1 ± 0.9 | 0.13 ± 0.01         | 13 ± 1.4                | 57                       | 0.71                   | 0.17      |
| T111A   | I     | 0.83 ± 0.14| 0.06 ± 0.03        | 1.8 ± 0.52              | 7.8                      | 3.2                    | 0.75      |
| K119A   | II    | 0.64 ± 0.3| 4.0 ± 0.5           | 0.10 ± 0.025            | 0.43                     | 6.7                    | 1.6       |
| R140A   | I     | 120 ± 33  | 6.0 ± 0.5           | 0.92 ± 0.20             | 4.0                      | 0.95                   | 14        |
| R221A   | I*    | 4.8 ± 1.7 | 4.3 ± 0.4           | 15 ± 4.5                | 65                       | 0.53                   | 0.13      |
| S223A   | II    | 0.86 ± 0.17| 1.1 ± 0.04         | 21 ± 3.6               | 1.1                      | 0.11                   | 0.027     |
| R226A   | II    | 1300 ± 310| 2.4 ± 0.2           | 0.031 ± 0.0051          | 0.13                     | 8.2                    | 2.0       |

* Determined in single turnover cleavage experiments.

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**Fig. 4.** Michaelis-Menten diagrams for the cleavage of d(p-GATCGACGATATCGTCGAC) with wild-type EcoRV (a) and the mutant R226A (b). The values for the initial rates are averages from three independent experiments; they are accurate within ±30%.

**Fig. 5.** Binding of wild-type EcoRV (a) and the mutant R226A (b) to a 382-bp DNA fragment in the presence of CaCl$_2$. The binding was studied with 20 pM (a) and 1 nM (b) 32P-labeled DNA and EcoRV at the concentrations indicated. The bands corresponding to the specific complex and the free DNA are indicated.
onstrated previously with a mutant whose C-terminal end, starting from position 216, had been deleted. This mutant showed no activity in cleavage assays with \( \lambda \)-DNA (21). Furthermore, the W219C mutant was shown to be very defective in DNA binding in the presence of Mg\(^{2+} \) (20).

In comparison with the wild-type enzyme, two mutants of group II (R140A, R226A) show an enhanced selectivity toward different flanking sequences (Table II, Fig. 3). Flanking sequences are likely to influence the structure and dynamics of the DNA, both of which are important in the recognition process, especially for the propensity of the recognition sequence to be bent in a unique way. The EcoRV restriction endonuclease distorts the recognition sequence GATATC during the recognition process by introducing a sharp central kink (5). It appears, as if reducing the ability of EcoRV to deform the DNA, for example through mutation of an amino acid residue that stabilizes the kinked conformation, leads to an increased sensitivity of EcoRV to flanking sequence effects. This consideration may explain the preferences of some phosphate contact mutants for EcoRV sites with AT-rich flanking sequences because these sequences may facilitate the distortion of the cognate DNA during the recognition process.

Mutants That Selectively Affect the \( k_{cat} \) of the Reaction (Group III in Table III)—Compared with the basic amino acid residues discussed above, Thr-93 and Ser-112 interact with the phosphate backbone in a fundamentally different way. They are responsible for backbone contacts required for the catalytic efficiency of the enzyme. Exchanging these residues leads to EcoRV variants with a reduced \( k_{cat} \) value, whereas their ability to bind the substrate is not or only marginally decreased (Table III), which means that the energy of the ground state complex is unaltered but that the energy of the transition state complex is higher than for the wild-type enzyme (Fig. 7). The way in which the residues Thr-93 and Ser-112 influence the catalytic machinery of the enzyme is obvious, because they contact the phosphate groups \( 1 \) and \( 2 \) (Fig. 1a), respectively, which are in close vicinity to the catalytic center and participate in the hydrolysis of the phosphodiester bond (32, 33). Thus, hydrogen bonds established by these residues may help directly to stabilize the precise assembly of the catalytic center.

A remarkable property of these mutants is a pronounced metal ion effect on the binding of DNA. In the presence of Ca\(^{2+} \), T93A and S112A bind to the DNA by more than 1 order of magnitude more weakly than in the presence of Mg\(^{2+} \), whereas the wild-type enzyme and all other mutants bind to DNA by 1 order of magnitude more strongly in the presence Ca\(^{2+} \) than in Mg\(^{2+} \).
with a relaxed specificity in the presence of Mn$^{2+}$Ser-112 (and of Thr-111) by alanine lead to an proximity to the catalytic center and hence may affect this center of the enzyme (5, 8) and at a site remote from the presence of Mg$^{2+}$

between Thr37 and other functional important regions of depicted. The thin line represents helix B, distances (in nm) are marked with dashed lines. The capital letters in parenthesis refer to the different EcoRV subunits A and B, respectively.

Thr-37 is located at the N-terminal end of the long connecting Thr-37 to the hydrophobic core of the protein. Thr-37 is not only in hydrogen bonding distance to the phosphate backbone of the DNA is to stabilize the kinked DNA conformation in the specific EcoRV-DNA complex. Other, uncharged residues help to assemble the catalytic center in the transition state. An especially important residue, Thr-37, is most probably responsible for the coupling of specific recognition and catalysis.

The effects seen with mutants of amino acid residues in close vicinity to the phosphate backbone are in general not as severe as observed with mutants of the recognition loop of EcoRV (21) but together may contribute to a considerable extent to the specificity of the recognition process and to the catalytic efficiency.

Acknowledgments—We thank Dr. J. Alves, Dr. B. A. Connolly, Dr. S. E. Halford, and Dr. F. K. Winkler for communication of results prior to publication as well as many valuable discussions.

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