Intra- vs Intersubunit Communication in the Homodimeric Restriction Enzyme *Eco*RV: Thr 37 and Lys 38 Involved in Indirect Readout Are Only Important for the Catalytic Activity of Their Own Subunit†

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ABSTRACT: *Eco*RV is a dimer of two identical subunits which together form one binding site for the double-stranded DNA substrate. Concerted cleavage of both strands of the duplex requires intersubunit communication to synchronize the two catalytic centers of *Eco*RV. Here we address the question of how contacts to the DNA backbone trigger conformational changes which lead to the activation of both catalytic centers. The structure of the specific *Eco*RV−DNA complex shows that a region including amino acids Thr 37 and Lys 38 is involved in interactions with the DNA backbone and is a candidate for intersubunit communication. Homodimeric *Eco*RV T37A and K38A variants have a 1000-fold reduced catalytic activity. To examine whether Thr 37 and Lys 38 of one subunit affect the catalytic center in the same subunit and/or in the other subunit, we have produced heterodimeric variants containing a Thr 37 → Ala or Lys 38 → Ala substitution in one subunit combined with a wild type (wt) subunit (wt/T37A and wt/K38A) or with a subunit which contains an amino acid substitution (Asp 90 → Ala) in the active site (D90A/T37A and D90A/K38A). Cleavage experiments with supercoiled pAT153 show that wt/T37A and wt/K38A preferentially nick the DNA. A steady-state kinetic analysis of the cleavage of an oligodeoxynucleotide substrate shows that the activity of wt/T37A and wt/K38A is half of that of wild type *Eco*RV, whereas D90A/T37A and D90A/K38A are almost inactive. These results demonstrate that Thr 37 and Lys 38 affect primarily the catalytic center in their own subunit and that both subunits of *Eco*RV can be activated independently of each other. We suggest that Thr 37 and Lys 38 control the catalytic activity of the active site in their own subunit by positioning α-helix B.

One of the most remarkable characteristics of type II restriction endonucleases is their high specificity (reviews in refs *1* and *2*). These enzymes recognize short DNA sequences comprising 4−8 bp and cleave DNA at these recognition sites by several orders of magnitude more readily than all other even very related sequences. The discriminating power of these enzymes is based on many contacts between enzyme and substrate. Although binding of restriction endonucleases to the specific site is thermodynamically strongly favored as opposed to binding to nonspecific sites [some restriction enzymes require the presence of divalent metal ions for this discrimination (*3*−*12*)], differences in affinity alone cannot explain the high specificity of these enzymes. A considerable contribution must come from the kinetics of cleavage, i.e., from the approach to the transition state. This implies that in order to understand the specificity of restriction enzymes one has to analyze in molecular terms how the formation of specific contacts is signaled to the catalytic center.

*Eco*RV is one of the best characterized type II restriction enzymes. It is a dimer of two identical subunits (*13*) which together form one binding site for the double-stranded DNA substrate. *Eco*RV recognizes the palindromic DNA sequence GAT/ATC (*14*) and in the presence of Mg^{2+} cleaves both DNA strands in a concerted manner (*15*) at the position indicated (*16*). Each subunit has its own catalytic center consisting of amino acid residues Asp 74, Asp 90, and Lys 92 (*17*, *18*) which are part of the [PD...(D/E)XK] motif (*5*, *19*, *20*), also present and identified as essential for catalytic activity in other restriction endonucleases (*18*, *21*−*27*). Both catalytic centers are normally activated together, but in principle can work independently of each other (*28*, *29*). Like other restriction enzymes, *Eco*RV forms a multitude of contacts to its recognition sequence both to the bases and to the phosphodiester backbone. In the specific complex, the DNA is bent and unwound (*10*, *30*−*34*). If all contacts are properly formed, a conformational change must occur that activates the catalytic centers in both subunits. The findings that each subunit does not contact all bases of the recognition sequence (*30*, *32*, *34*) and that a concerted cleavage of both strands of the DNA occurs (*10*) demonstrate that each subunit via its DNA recognition interface must control the active sites of both subunits. Intersubunit communication can be studied by a heterodimer approach developed recently by us (*28*, *29*) that allows us to introduce amino acid substitu-

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tions in only one subunit of a homodimeric protein and thereby to analyze whether the effect of a mutation is localized to one subunit and/or transmitted to the other subunit. Indeed, we have shown that mutations within the DNA recognition loop (R-loop) of one subunit strongly reduce the catalytic activity of both active sites (*28*). Asn 188 presumably couples DNA recognition by base specific contacts (direct readout) to cleavage via a "signal transduction" between the R-loop and the catalytic center (*28*, *30*, *32*). As proposed by Wenz et al. (*35*), Thr 37 could fulfill a similar role for the contacts to the phosphodiester backbone (indirect readout); Thr 37 may trigger activation of the catalytic center in the same subunit by positioning α-helix B comprising amino acid residues 36−58 which in turn affects the catalytic center of the same subunit via Glu 45. Alternatively, it was suggested that Thr 37 activates the catalytic center in the opposite subunit via a main chain contact to Gln 69 of the other subunit. In the vicinity of Thr 37 is another interesting candidate for coupling indirect readout to catalysis and for intersubunit crosstalk; Lys 38 forms a contact to the deoxyribose of the GAT**A**TC adenosine residue of the DNA strand cut by the other subunit (*30*, *32*). In addition, Kostrewa and Winkler (*32*) observed that in the *Eco*RV product complex the 5′-phosphate group of the same adenosine residue interacts with this lysine. These crossover interactions suggest intersubunit communication (Figure 1).

In this study, we investigate whether amino acid residues Thr 37 and Lys 38 affect the catalytic center in the same, in the opposite, or in both subunits of *Eco*RV. To this end, we have produced the *Eco*RV heterodimers wt/T37A,[1] D90A/T37A, wt/K38A, and D90A/K38A and characterized these artificial heterodimers in terms of their cleavage activity toward oligodeoxynucleotide and plasmid substrates. Our results demonstrate that both amino acid residues primarily affect the catalytic center in their own subunit and, therefore, are not involved in intersubunit crosstalk.

MATERIALS AND METHODS

Generation, Expression, and Purification of the Homodimeric K38A Variant (K38A/K38A). Site-directed mutagenesis of the *eco*RV gene was performed in a manner similar to that described by Ito et al. (*36*). Protein expression and purification of the His_6-tagged *Eco*RV variant was carried out as described (*37*).

Expression and Purification of the EcoRV Heterodimers. Heterodimers were produced as described by Wende et al. (*29*). Briefly, this has been achieved by transforming *Escherichia coli* cells with two plasmids carrying the *eco*RV gene fused to the coding region for the glutathione *S*-transferase or a His_6 tag, respectively. Upon induction of protein expression, *Eco*RV homo- and heterodimers are statistically generated *in vivo*. Two affinity chromatography steps carried out in series (Ni−NTA and GSH columns) allow for a separation and purification of the homo- and heterodimers. The absence of homodimeric enzymes in the heterodimer preparations was shown by gel shift experiments as described (*28*, *29*).

Gel Electrophoretic Mobility Shift Experiments. A ^{32}P-labeled 382 bp DNA fragment (100 pM) with a single *Eco*RV site (*38*) was incubated with 0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 nM *Eco*RV heterodimers in binding buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM 2-mercaptoethanol, 2 mM spermidine, 0.1 mg/mL bovine serum albumin, and 10 mM $CaCl_2$] for at least 30 min at room temperature. To 10 μL of this binding mixture was added

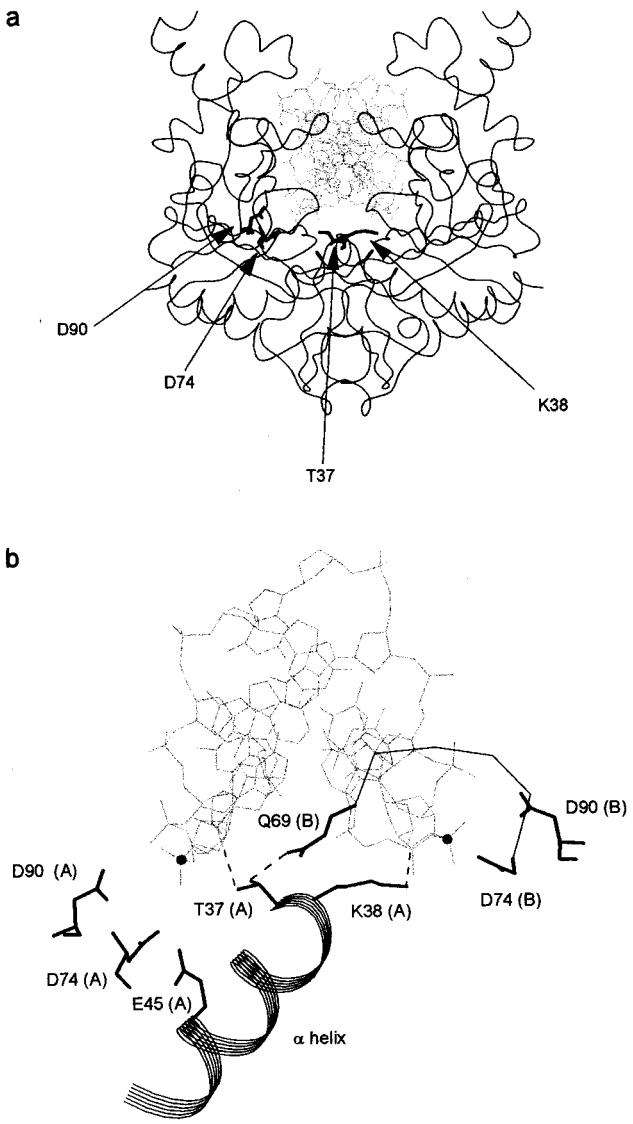

FIGURE 1: (a) Structure of the *Eco*RV−DNA complex with specific DNA (Brookhaven Protein Data Bank entry 1RVB). The enzyme is a dimer of two identical subunits. The side chains of amino acid residues Thr 37 and Lys 38, involved in indirect readout, and the side chains of amino acid residues Asp 74 and Asp 90, involved in catalysis, are indicated only in one subunit. (b**)** Detail of the *Eco*RV−DNA cocrystal structure. The connections of Thr 37 and Lys 38 to the catalytic centers of both subunits represented by Asp 74 and Asp 90 are displayed. The helix represents part of α-helix B. The capital letters in parentheses refer to *Eco*RV subunits A and B, respectively. DNA strand C which is cleaved by subunit A and DNA strand D which is cleaved by subunit B are displayed for the recognition sequence GATATC. The scissile phosphate is indicated by a dot in both strands. Contacts between Thr 37 (subunit A) and GATAT**p**C (strand C) as well as between Lys 38 (subunit A) and the deoxyribose of GAT**A**TC (strand D) are shown by dashed lines.

[1] wt/T37A is an artificial heterodimer which contains one wild type and one mutant subunit with a Thr 37 → Ala substitution. Similarly, D90A/T37A denotes a heterodimer consisting of two mutant subunits, one of them carrying a Asp 90 → Ala and the other one a Thr 37 → Ala substitution.

3 μL of gel loading buffer [50% (v/v) glycerol, 0.25% (w/v) xylene cyanol, and 0.15% (w/v) azorubin in binding buffer]. Electrophoresis was carried out on 10 × 10 cm 6% polyacrylamide gels at room temperature in 50 mM Tris-taurine (pH 8.0) and 10 mM $CaCl_2$. Radioactive bands were detected and quantitated using an Instant Imager (Canberra Packard). Binding constants were determined as described (*35*).

Cleavage Experiments with Plasmid DNA Fragments. All cleavage experiments were performed at 37 °C in reaction buffer [20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 10 mM $MgCl_2$] with the 382 bp DNA fragment mentioned above as substrates containing a unique site for *Eco*RV. Cleavage experiments were performed with 50 nM heterodimers and 100 nM 382 bp fragment in reaction buffer. After defined time intervals, aliquots were withdrawn and immediately mixed with stop mix [0.25 M EDTA, 25% sucrose (w/v), 1.2% (w/v) SDS, 0.1% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanol (pH 8.0)]. Substrates and products were separated by electrophoresis in 8% polyacrylamide gels under denaturing conditions (7 M urea) in TTE buffer [80 mM Tris-taurine (pH 9.0) and 2 mM EDTA] and quantitated using an Instant Imager.

Cleavage Experiments with Plasmid DNA. Cleavage experiments with homodimeric *Eco*RV variants were carried out as described recently (*37*). Cleavage experiments with heterodimeric *Eco*RV variants were performed with the supercoiled plasmid pAT153 containing a single site for *Eco*RV, at 37 °C in reaction buffer. Reaction mixtures contained 1 nM wt *Eco*RV or 30 nM heterodimers and 100 nM pAT153 in reaction buffer. After defined time intervals, aliquots were withdrawn and immediately mixed with stop mix. Substrates and products were separated by electrophoresis in 1% agarose gels under native conditions in TPE buffer [80 mM Tris-phosphate (pH 8.0) and 2 mM EDTA], stained with ethidium bromide, and quantitatively analyzed.

Cleavage Experiments with Oligodeoxynucleotides. The self-complementary 20mer d(GATCGAC**GATATC**GTCGATC) (with the *Eco*RV site in bold type) was synthesized with a Milligen Cyclone DNA synthesizer. The eicosadeoxynucleotide was labeled at its 5′-end using T4 polynucleotide kinase (U.S. Biochemicals) and [γ-^{32}P]ATP (Amersham). Steady-state cleavage experiments were performed using 0.1−1 μM oligodeoxynucleotide and 10 nM to 0.5 μM enzyme in reaction buffer. For wt *Eco*RV, the enzyme concentration was 10 times lower than the lowest substrate concentration; for the heterodimers, the enzyme concentration was 2−10 times lower than the substrate concentration. Single-turnover experiments with heterodimers of very low activity were carried out with an excess of enzyme over substrate. After defined time intervals, aliquots were withdrawn and spotted onto DEAE-cellulose thin layer plates (Machery and Nagel, Düren, Germany) which were then subjected to homochromatography. The separated substrate and product spots were detected and quantitatively analyzed using an Instant Imager. For K_m and k_{cat} determinations, at least four reaction progress curves at four different substrate concentrations were measured for each protein. The initial velocities were used to determine K_m and k_{cat} values by a least-squares fit to the Michaelis−Menten equation.

RESULTS

On the basis of the crystal structure of the specific *Eco*RV−DNA complex (*30*, *32*, *34*), Thr 37 and Lys 38, located close to the subunit−subunit interface, make contacts to the phosphodiester backbone of the DNA substrate (Figure 1). It was shown by Wenz et al. (*35*) that Thr 37 is a very important residue for the indirect readout because the homodimeric T37A variant has a cleavage activity toward plasmid DNA cleavage 1000-fold lower than that of the wt enzyme (*35*). We have now produced the homodimeric K38A variant. It also shows a cleavage activity 1000-fold lower than that of the wild type enzyme, and therefore, Lys 38 appears to be as important as Thr 37 for the function of the enzyme. To study the possible involvement of Thr 37 and Lys 38 in intersubunit communication in *Eco*RV, we have produced and purified to homogeneity the heterodimeric variants wt/T37A, D90A/T37A, wt/K38A, and D90A/K38A. Like the heterodimers produced and analyzed previously (*20*, *28*, *29*), the heterodimers studied here are stable. Their purified preparations do not contain detectable amounts of homodimeric proteins, as analyzed by gel shift experiments (data not shown).

Cleavage experiments with plasmid DNA show that wild type *Eco*RV cleaves the supercoiled plasmid in a concerted manner (Figure 2). This means that the supercoiled plasmid is converted directly to the linear form without detectable amounts of the open circle DNA occurring as an intermediate of the cleavage reaction. In contrast, the heterodimers wt/T37A and wt/K38A cleave supercoiled pAT153 DNA with a pronounced accumulation of the nicked intermediate (Figure 2). The same mode of cleavage has been observed with the heterodimer wt/D90A which contains one active and one inactive subunit (Figure 2) (*28*). This finding is most easily explained by assuming that the heterodimers wt/T37A and wt/K38A, like wt/D90A, consist of one active and one inactive catalytic center. This would mean that in the heterodimers wt/T37A and wt/K38A one catalytic center is affected and the other catalytic center unaffected by the mutation in one subunit. To determine the catalytic activities of each subunit, heterodimers consisting of a D90A subunit and a T37A or K38A subunit were produced. Residual catalytic activity if observed with these variants could only be due to the T37A or K38A subunit, because the D90A subunit is completely inactive. As cleavage experiments with these heterodimers show that heterodimers D90A/T37A and D90A/K38A are inactive (Figure 2), we conclude that the mutations Thr 37 → Ala and Lys 38 → Ala only affect the catalytic center of their own subunit.

The asymmetry introduced into the *Eco*RV dimer by amino acid substitutions in one subunit leads not only to an asynchrony in cleavage of the two strands but also to preferential cleavage of one strand of a 382 bp DNA fragment by the D90A/T37A and D90A/K38A mutants (Figure 3). D90A/T37A and D90A/K38A cleave the AAGT**GATATC**GGAT strand 5 times faster than the ATCC**GATATC**ACTT strand. The most straightforward explanation for this result is that the two strands of the DNA duplex have different flanking sequences; these may interact differently with the two different *Eco*RV subunits, leading to different rates of cleavage of the two strands of the DNA duplex by D90A/T37A and D90A/K38A, as observed

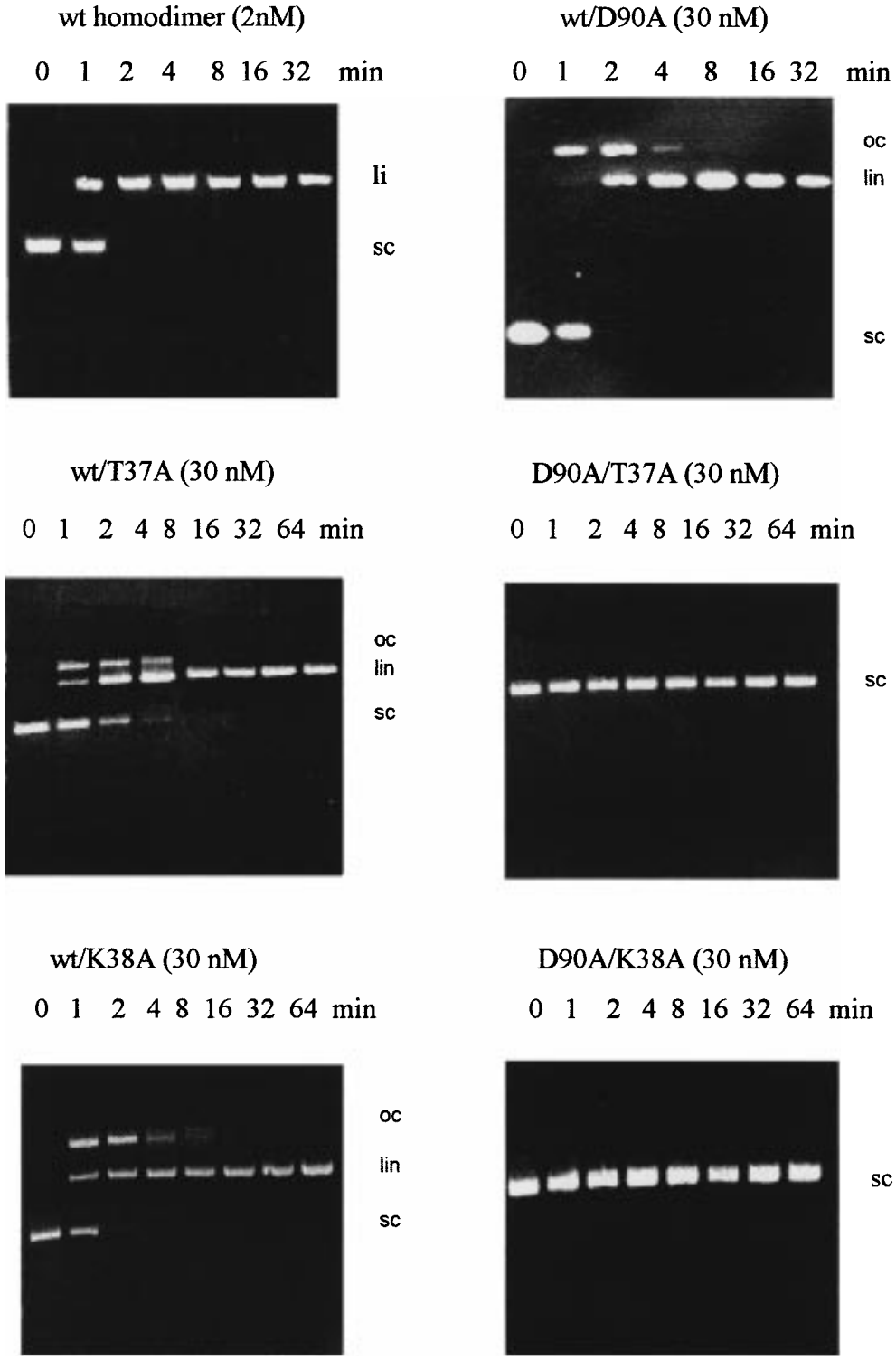

FIGURE 2: DNA cleavage by wild type *Eco*RV and heterodimers. The cleavage reactions were carried out with 100 nM pAT153 DNA (which has a single *Eco*RV site), 2 nM wild type *Eco*RV, and 30 nM heterodimers. The wild type enzyme cleaves both strands in a concerted reaction; the heterodimers wt/D90A, wt/T37A, and wt/K38A cleave one strand after the other, which leads to the accumulation of the nicked circle intermediate. The heterodimers D90A/T37A and D90A/K38A are nearly inactive. The positions of the bands of supercoiled substrate (sc), open circle intermediate (oc), and linear product (lin) are indicated.

previously with the D90A/N188Q heterodimer (*28*). In contrast, preferential cleavage of one strand over the other is not observed with the wild type enzyme as determined in pre-steady-state experiments (F. Stahl, unpublished).

While the plasmid cleavage experiments were only evaluated in a qualitative manner to demonstrate whether the cleavage of the two strands of the duplex occurs in a concerted or in a sequential manner, oligodeoxynucleotide cleavage experiments were carried out to obtain k_{cat} and K_m values, independent of processes such as nonspecific binding and target site location. Steady-state kinetic parameters were obtained for the cleavage of a 20mer oligodeoxynucleotide by the various heterodimers. wt/T37A and wt/K38A show a 50% reduced k_{cat}/K_m as compared to wild type *Eco*RV

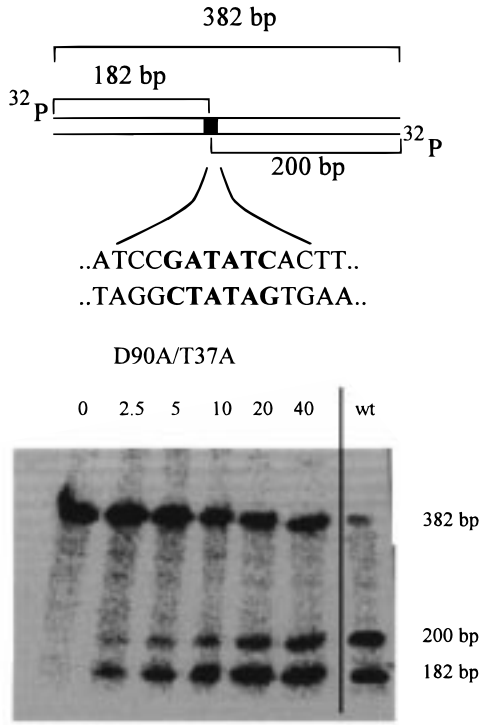

FIGURE 3: DNA cleavage of a 382 bp DNA fragment (5′-end labeled) by the D90A/T37A and heterodimer. The reaction was carried out with 100 nM DNA and 50 nM enzyme. The electrophoretic analysis of the reaction products (the relationship between substrate and products is depicted in a schematic form on the top of the figure) was carried out on denaturing gels. The quantitative analysis shows that cleavage is 5-fold faster within the AAGT-**GATATC**GGAT strand than within the ATCC**GATATC**ACTT strand. To demonstrate equal specific activity of the radioactive label in two strands, in the lane designated with wt, the result of an almost complete digestion of the 382 bp DNA fragment with wt *Eco*RV is shown.

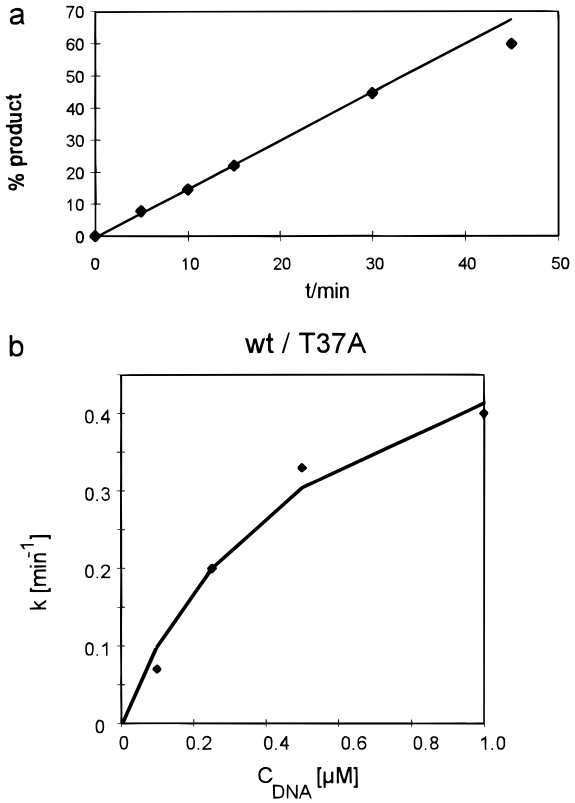

FIGURE 4: (a) Example of an oligodeoxynucleotide cleavage experiment. d(pGATCGACGATATCGTCGATC) (1 μM) was cleaved using 100 nM wt/T37A in reaction buffer. Substrate and reaction products were separated by homochromatography and quantified using an instant imager. **(**b**)** Steady-state kinetic analysis of the cleavage of d(pGATCGACGATATCGTCGATC) by the wt/T37A heterodimer. Data points were derived from at least four independent measurements. The line represents a theoretical curve for $K_m = 0.55$ μM and $k_{cat} = 0.64$ min^{-1}.

(Figure 4 and Table 1). This is exactly what would be expected if one active and one inactive subunit were present, as demonstrated for the wt/D90A combination which contains one inactive subunit and behaves in a manner very similar to that of wt/T37A and wt/K38A (*28*, *29*). The results of the cleavage experiments with plasmid DNA make it very unlikely that the catalytic activities of both subunits of wt/T37A and wt/K38A are reduced by a factor of 2. Hence, the straightforward interpretation of all data is that the mutations T37A and K38A in one subunit primarily affect the catalytic center only of their own subunit and leave the other subunit unimpaired.

Interestingly, the Michaelis−Menten analysis of oligodeoxynucleotide cleavage by these mutants shows that the reduced catalytic activity of these mutants is mainly due to a reduced k_{cat} value, suggesting that DNA binding of these heterodimers is not much reduced by the T37A and K38A mutations in one subunit. Gel shift experiments carried out with a 382 bp DNA fragment in the presence of Ca^{2+} demonstrate that indeed the heterodimers wt/T37A and wt/K38A have only a 2-fold lower affinity for DNA compared to the wild type enzyme (data not shown). Therefore, these mutations disturb coupling of indirect readout and catalysis in the same subunit but hardly binding of the substrate.

Due to their low activities, oligodeoxynucleotide cleavage by the heterodimers D90A/T37A and D90A/K38A is readily detected only under single-turnover conditions. The $k_{single\ turnover}$ values determined for the D90A/T37A and D90A/K38A heterodimers are 0.002 and 0.004 min^{-1}, respectively (Table 2). This is approximately half of the single-turnover rate constants determined for T37A (0.0043 min^{-1}) and the K38A (0.01 min^{-1}) homodimers, again a result that is most

Table 1: Steady-State Kinetic Parameters for the Cleavage of d(GATCGAC**GATATC**GTCGATC) by *Eco*RV Homo- and Heterodimers

enzyme	K_m (μM)[a]	k_{cat} (min^{-1})[a]	k_{cat}/K_m (min^{-1} μM^{-1})
wt/wt[c]	0.82	1.85	2.26 (1.0)[b]
wt/D90A[c]	0.54	0.57	1.05 (0.47)
wt/T37A	0.55	0.64	1.16 (0.51)
wt/K38A	0.61	0.74	1.21 (0.54)

[a] K_m and k_{cat} values are derived from at least four different experiments; they are accurate within ±30%. [b] Relative values with k_{cat}/K_m set to 1 for the wild type enzyme. [c] Taken from Stahl et al. (*28*).

Table 2: Single-Turnover Kinetic Parameters for the Cleavage of d(GATCGAC**GATATC**GTCGATC) by Mutant *Eco*RV Homo- and Heterodimers

enzyme	$k_{single-turnover}$
D90A/T37A	0.002
D90A/K38A	0.004
T37A/T37A	0.0043[a]
K38A/K38A	0.01

[a] Taken fromWenz et al. (*35*).

easily explained by assuming that the substitution in one subunit only affects this subunit. Taken together, our results demonstrate that mutations T37A and K38A in one subunit almost completely inactivate their own but do not influence the other subunit.

DISCUSSION

Type II restriction enzymes are dimers of identical subunits, each of which harbors a catalytic center. As only one substrate molecule is bound by the homodimeric enzyme, the two subunits must cooperate in the recognition process which includes formation of a number of specific contacts to the DNA and activation of the catalytic centers. To inactivate an invading phage, the two cleavage events required to cut the double-stranded DNA substrate should be carried out in a concerted way to prevent repair of a nicked intermediate by DNA ligase. This could be achieved by coupling the two cleavage events in an obligatory manner by a cooperative conformational change triggered by the formation of specific contacts between enzyme and DNA. Hence, both the recognition process and DNA cleavage depend on communication between the two subunits. In *Eco*RV, the two catalytic centers in principle are able to operate independently of each other, but normally are activated together, which means that signals originating from specific base contacts (direct readout) of one subunit are transmitted to both catalytic centers (*28*, *29*), thereby synchronizing the two cleavage reactions. To investigate whether contacts to the phosphodiester backbone (indirect readout) in one strand of the duplex also serve to activate the catalytic centers of both subunits in *Eco*RV, we have produced artificial heterodimers carrying substitutions of two amino acid residues especially important for indirect readout of *Eco*RV [Thr 37 (*35*) and Lys 38 (this paper)], namely, wt/T37A, wt/K38A, D90A/T37A, and D90A/K38A.

Thr 37 is at a key position of a complex network of interactions as shown in the structure of the specific *Eco*RV−DNA complex (*30*, *32*, *34*) and makes a contact to the phosphodiester backbone to the last phosphate of the recognition sequence (GATAT**p**C) (Figure 1). In this position, one could assume a connection to the catalytic centers of the same as well as of the opposite subunit. On one hand, Thr 37 is located at the N-terminal end of α-helix B and may be important for the positioning of this secondary structural element, which harbors residue Glu 45 that is near the catalytic center and therefore in an ideal position for affecting the juxtaposition of catalytic residues Asp 74, Asp 90, and Lys 92 by electrostatic effects. On the other hand, Thr 37 contacts the catalytic center of the opposite subunit via Gln 69 of the other subunit. Gln 69 is located in the Q-loop which is in close proximity to Asp 74, a catalytically essential residue. The results presented here, however, argue against any influence of Thr 37 on the active site of the other subunit such that the connections

Thr 37 (subunit A) ↔ Glu 69 (subunit B) ↔ active site (subunit B)

do not seem to be important. Our results rather demonstrate that Thr 37 affects only the catalytic center in the same subunit. We suggest that Thr 37 is responsible for the correct positioning of α-helix B and thereby of Glu 45 which is near the active site of the same subunit:

Thr 37 (subunit A) ↔ α-helix B (subunit A) ↔ active site (subunit A)

According to the crystal structure of the specific *Eco*RV−DNA complex (*30*, *32*, *34*), Lys 38 is involved in indirect readout. We have shown here that it is as important for DNA cleavage by *Eco*RV as Thr 37. Lys 38 is close to the deoxyribose of the adenosine (GAT**A**TC) residue at the scissile phosphodiester bond of the DNA strand that is cleaved by the catalytic center of the opposite subunit (Figure 1). It seems to be possible that by altering the DNA conformation Lys 38 of one subunit could affect the catalytic center of the other subunit. On the other hand, Lys 38 like Thr 37 is located at the N-terminal end of α-helix B and may be important for the positioning of this secondary structural element. Our data present clear evidence that the Lys 38 → Ala substitution affects the catalytic center in the subunit carrying the substitution. Thus, as observed with Thr 37, positioning of α-helix B by Lys 38 of the same subunit appears to be extraordinarily important for the catalytic activity of *Eco*RV.

The contacts of Thr 37 and Lys 38 to the backbone of the DNA substrate are almost as important for the overall reaction of *Eco*RV as the base specific contacts (*35*). This can hardly be explained if the sole function of these residues were participation in binding of the substrate by formation of a single hydrogen bond each. Indeed, the T37A mutant is mainly affected in its k_{cat} (*35*) which means that Thr 37 is involved in catalysis. On the basis of the results presented here, we propose that its function is to activate the catalytic center in its own subunit. Our data suggest that the same is true for Lys 38. Thr 37 and Lys 38 are located at the end of α-helix B at the bottom of the DNA binding cleft which carries Glu 45 that is close to the active site. We propose that the Thr 37 → Ala and Lys 38 → Ala substitutions displace α-helix B from its position in wild type *Eco*RV. This conformational change could be transmitted to the active site via Glu 45. However, a Glu 45 → Ala substitution does not reduce the catalytic activity of *Eco*RV as much (*39*) as the Thr 37 → Ala or Lys 38 → Ala substitutions. These findings and our conclusion that Glu 45 transmits conformational changes of α-helix B to the active site can be reconciled by assuming that the catalytic center is under negative control of Glu 45. If Glu 45 is not properly positioned, it interferes with the formation of a catalytically competent conformation of the catalytic center. When Glu 45 is substituted by alanine, the transmission of conformational changes at the protein−DNA interface is relaxed. While this detail is speculative, the role of α-helix B in coupling indirect readout and activation of the catalytic center is evident from our results. With a similar argument, Perona and Martin (*34*) have rationalized the reduced catalytic activity of the T93A mutant (*10*, *35*). In the structure of the T93A variant, the linker region (which contains Thr 37) between the cleavage domain and the dimerization domain of each subunit changes its conformation in response to the amino acid substitution at position 93, leading to new positions of the cleavage domain relative to the dimerization domain. It is argued that the catalytic deficiency of T93A may be due in part to an indirect effect on the positioning of Glu 45.

The indirect readout contacts investigated here do not influence both active sites. This result demonstrates once more (*35*) that both catalytic sites in principle can be activated independently of each other, suggesting that both subunits can have different conformations. This result differs from that obtained with mutants that form specific contacts to the bases of the DNA (*28*). In these cases, we observed that a mutation in one subunit reduces the catalytic activity of both active sites, presumably because the DNA recognition loops of both subunits are in close contact with each other (*28*). Thus, direct readout through base recognition is cooperative, because an altered conformation of one recognition loop can prevent the formation of many specific contacts in both loops. Thereby, it is guaranteed that both catalytic centers are only activated if all specific contacts by both subunits are formed. This seems to be sufficient for synchronizing the two cleavage events. Indirect readout by one subunit at the two most important backbone contacts, in contrast, is not transmitted to the other subunit.

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