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Crosslinking the *Eco*RV restriction endonuclease across the DNA-binding site reveals transient intermediates and conformational changes of the enzyme during DNA binding and catalytic turnover

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***Eco*RV completely encircles bound DNA with two loops, forming the entry and exit gate for the DNA substrate. These loops were crosslinked generating CL-*Eco*RV which binds and releases linear DNA only slowly, because threading linear DNA into and out of the DNA-binding 'tunnel' of CL-*Eco*RV is not very effective. If the crosslinking reaction is carried out with a circular bound DNA, CL-*Eco*RV is hyperactive towards the trapped substrate which is cleaved very quickly but not very accurately. CL-*Eco*RV also binds to, but does not cleave, circular DNA when added from the outside, because it cannot enter the active site. Based on these results a two-step binding model is proposed for *Eco*RV: initial DNA binding occurs at the outer side of the loops before the gate opens and then the DNA is transferred to the catalytic center.**

Keywords: protein–DNA association/protein–DNA interaction/restriction endonuclease/restriction–modification system/specificity

Introduction

Type II restriction enzymes cleave DNA molecules specifically at recognition sites comprising 4–8 base pairs. These enzymes have two outstanding properties: (i) they associate to their recognition sites at a diffusion-controlled rate, in spite of the fact that there is a large excess of non-target DNA; and (ii) they display a very high degree of sequence specificity (for review see Roberts and Halford, 1993; Pingoud and Jeltsch, 1997). Typically, sequences differing by one base pair from their recognition sites are cleaved three to five orders of magnitude more slowly than the canonical site. On the basis of detailed structural and biochemical work, the mechanistic basis of both of these properties is well understood for *Eco*RV (recognition site: GATATC), which is one of the best studied restriction endonucleases. *Eco*RV is a homodimeric enzyme (2×29 kDa) whose DNA-binding site is located at the bottom of a deep cleft and flanked by two arms of the enzyme (Figure 1A) (Winkler *et al*., 1993). The reaction mechanism of *Eco*RV comprises several steps: first, the enzyme associates non-specifically to DNA. This occurs in a diffusion-controlled reaction (Erskine *et al*., 1997). In apparent contrast to the very high association rate constant of *Eco*RV to DNA is the finding that the free enzyme is in a closed conformation in the crystal in which the arms approach each other thereby preventing direct entry of the DNA to the binding cleft (Figure 1A). Consequently, during the association reaction conformational changes must take place that open up the DNA-binding site of the enzyme. In the crystal structure, the non-specific *Eco*RV–DNA complex has a more open conformation than either free *Eco*RV or the specific *Eco*RV–DNA complex (Winkler *et al*., 1993). In the non-specific complex, significant parts of the arms appear not to be ordered (Figure 1A). This may be important for the mechanism of target site location, because *Eco*RV while being bound non-specifically to DNA, scans several hundreds of base pairs by linear diffusion searching for its recognition site (Taylor *et al*., 1991; Jeltsch *et al*., 1996; Jeltsch and Pingoud, 1998). Upon encountering a GATATC site numerous contacts of the enzyme to the edges of the bases and the DNA backbone are formed which together trigger formation of a specific complex (Thielking *et al*, 1991; Winkler *et al*., 1993; Stahl *et al*., 1996). In the specific complex formed, the arms of *Eco*RV are ordered and completely encircle the DNA to form a tightly closed complex (Figure 1A) (Winkler *et al*., 1993; Kostrewa and Winkler, 1995; Perona and Martin, 1997). Upon transition of the non-specific to the specific complex the catalytic center becomes activated leading to DNA cleavage. Finally, the cleavage products dissociate from the enzyme.

In the present study we have investigated how conformational changes of the arms of *Eco*RV are involved in the mechanism of action of this enzyme. To this end, both subunits of *Eco*RV were crosslinked across the DNA-binding site at the tips of the arms using a series of divalent chemical crosslinkers. Thereby, a covalently closed 'ring-shaped' enzyme is generated, whose DNA-binding site is located in an artificially created 'tunnel' in the center of the protein (Figure 1B). These crosslinked proteins were purified and analyzed with respect to the thermodynamics and kinetics of DNA binding and cleavage. Our results allow us to draw important conclusions regarding the mechanism by which *Eco*RV acts, the most important of which is that DNA binding occurs in at least two steps. At first, DNA is bound to the outside of the homodimeric protein. Then, the arms open up to allow the DNA to bind at the catalytic site of the enzyme.

Results

Preparation of intramolecularly crosslinked EcoRV

To crosslink the two subunits of an *Eco*RV dimer specifically at the arms of the molecule, we inspected the structure for suitably located amino acid residues. Thr222 is located at the inner side of the arms of *Eco*RV. The distance

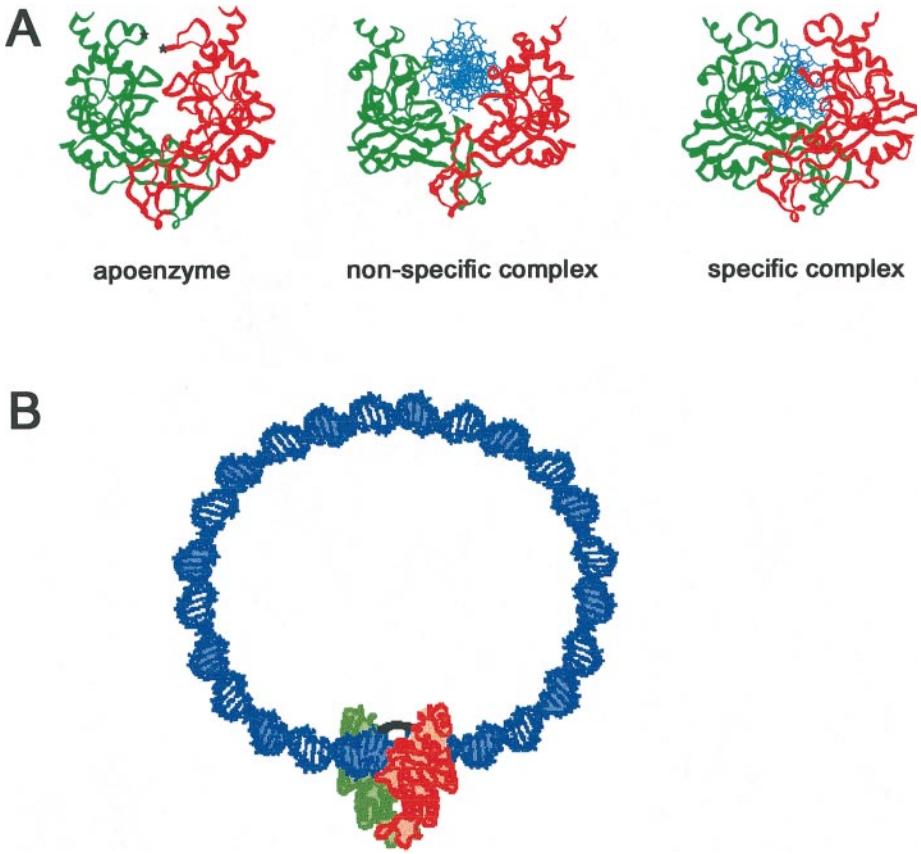

Fig. 1. (**A**) Structure of the *Eco*RV apoenzyme, and the *Eco*RV in complex with non-specific and specific DNA (Brookhaven databank entries: 1RVE for the free enzyme, 2RVE for the complex with non-cognate DNA and 4RVE for the complex with cognate DNA). The position of Thr222 is labeled in the apoenzyme by *. (**B**) Schematic drawing of CL-*Eco*RV catenated to a circular DNA molecule. The DNA is drawn to scale, but comprises only ~130 bp.

between the Cα atoms of the two symmetry-related Thr222 residues of the two subunits is 18 Å in the specific *Eco*RV DNA complex 5RVE (Kostrewa and Winkler, 1995). Thr222 was exchanged for cysteine and the only other cysteine in *Eco*RV (Cys21) was substituted by serine. This C21S/T222C mutant behaves like wild-type *Eco*RV with respect to kinetic properties and DNA binding (data not shown). All control experiments reported in this work were carried out with *Eco*RV C21S/T222C. Throughout this paper 'uncrosslinked *Eco*RV' refers to the C21S/T222C variant and 'CL-*Eco*RV' refers to the purified *Eco*RV C21S/T22C variant carrying an intramolecular crosslink. Crosslinking was carried out using a set of bis(maleimido)alkanes as bifunctional SH-specific reagents. In these reagents two reactive maleimide groups are linked by three (CL3), five (CL5), six (CL6) or seven (CL7) methylene groups (Franke and Pingoud, 1998). In spite of the different length of the bis(maleimido)alkanes crosslinking yields obtained with the C21S/T222C mutant were similar with all four reagents (Figure 2A). Crosslinking was carried out at different temperatures with the free enzyme as well as with the enzyme in complex with DNA either in the presence of EDTA to give a non-specific complex (Taylor *et al*., 1991) or in the presence of Ca^{2+} to give a specific but catalytically incompetent complex (Vipond and Halford, 1995). The yields of crosslinking increased significantly with increasing incubation temperature (Figure 2A). At low temperatures high yields were only observed with the non-specific *Eco*RV–DNA complex.

If crosslinking occurs while a circular plasmid molecule is bound by *Eco*RV, enzyme and DNA become catenated, i.e. topologically interconnected such that dissociation of the enzyme from the DNA is impossible unless the circular DNA is cleaved or the enzyme denatured (Figure 1B). To purify CL-*Eco*RV, we took advantage of the irreversible nature of CL-*Eco*RV–DNA complexes. For preparative isolation of CL-*Eco*RV, crosslinking was carried out in the presence of Ca^{2+} using the CL6 crosslinker and *Eco*RV in complex with pAT153 plasmid DNA which contains one *Eco*RV recognition site. Under the experimental conditions used, ~20 molecules of *Eco*RV were trapped on each plasmid DNA by the crosslinking reaction (data not shown). CL-*Eco*RV catenated with the circular plasmid was purified to homogeneity (Figure 2B). For experiments with CL-*Eco*RV without an internally bound plasmid, the CL-*Eco*RV preparation was incubated with the non-specific nuclease from *Serratia marcescens* and the free CL-*Eco*RV separated from the digestion products (Figure 2B). We have proven by three experiments that the crosslink of the *Eco*RV subunits occurred intramolecularly as desired and not intermolecularly. (i) Plasmids bound by CL-*Eco*RV are not released even under high salt conditions (1 M NaCl) under which uncrosslinked *Eco*RV no longer binds to DNA. (ii) Gel-shift experiments carried out with CL-*Eco*RV (the protein still binds to

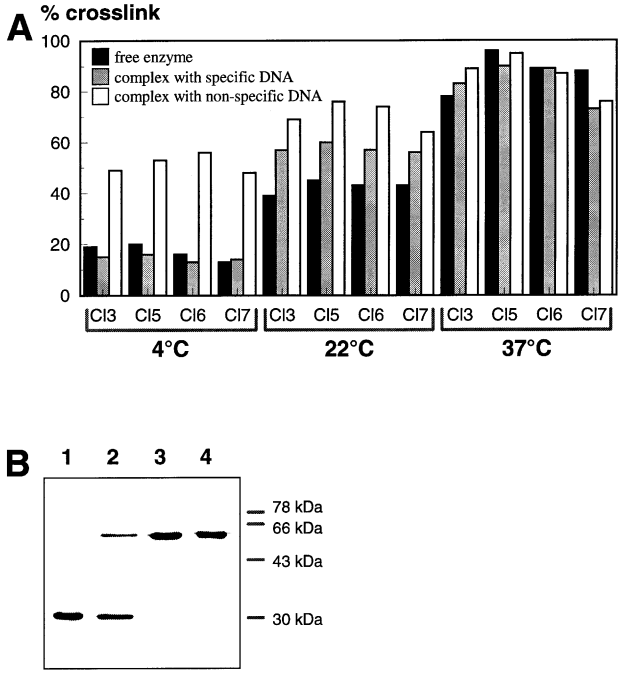

Fig. 2. (**A**) Compilation of the yields of the crosslinking reactions with four different crosslinkers (CL3, CL5, CL6 and CL7, having 3, 5, 6 and 7 methylene groups separating the maleimide groups, respectively). Reactions were performed at three different temperatures using free *Eco*RV as well as *Eco*RV bound specifically or non-specifically to DNA as described in Materials and methods. All experiments were performed at least in triplicate. (**B**) Purification of CL-*Eco*RV catenated to DNA and in a DNA-free form. Coomassie-stained SDS–PAGE gel of the *Eco*RV preparations obtained [lane 1, uncrosslinked *Eco*RV; lane 2, unpurified CL-*Eco*RV; lane 3, purified CL-*Eco*RV catenated to DNA (the DNA is released from the catenated complex upon incubation with SDS); lane 4, purified CL-*Eco*RV free of DNA (after treatment with *S.marcescens* endonuclease)].

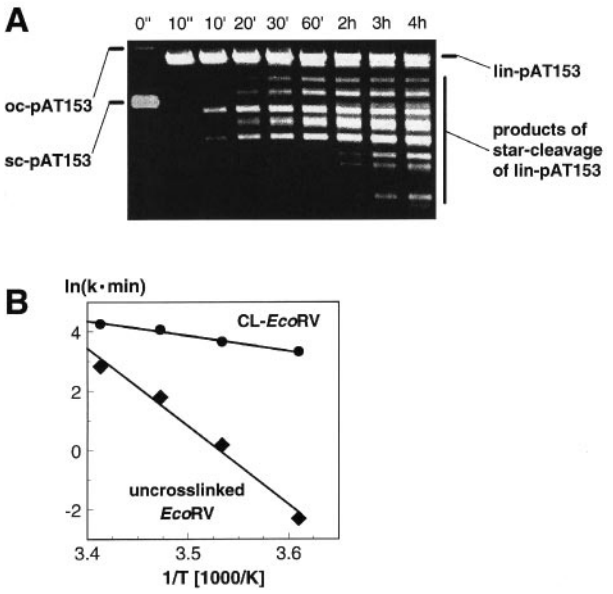

Fig. 3. Cleavage of pAT153 plasmid catenated to CL-*Eco*RV. (**A**) Agarose gel electrophoretic analysis showing the cleavage of pAT153 catenated to CL-*Eco*RV. Note that the plasmid which contains one *Eco*RV site is converted from the superhelical to the linear form within 10 s. After 10 min additional bands appear indicating DNA cleavage at non-canonical sites. (**B**) Temperature dependence of the rate of DNA cleavage of CL-*Eco*RV and uncrosslinked *Eco*RV determined using a quenched-flow device. In these experiments the temperature was varied between 4 and 20°C.

linear DNA, see below) using a DNA fragment that contains one *Eco*RV site show only one band shift under conditions of specific DNA binding. No band shifts of low electrophoretic mobility are observed that would correspond to intermolecularly crosslinked *Eco*RV species bound to the DNA, which would contain four subunits of *Eco*RV under native conditions. (iii) The S-values of CL-*Eco*RV and uncrosslinked *Eco*RV determined in sedimentation velocity runs in the analytical ultracentrifuge are identical within experimental error (CL-*Eco*RV, 4.3S; uncrosslinked *Eco*RV, 4.2S) again demonstrating the absence of tetrameric enzymes, which would be the result of an intermolecular crosslink.

Kinetic properties of CL-EcoRV

In order to determine if CL-*Eco*RV retains catalytic activity, it was purified in complex with circular pAT153 plasmid, catenated to the enzyme. Upon incubation with Mg^{2+}, complete cleavage of the bound plasmid was observed within 10 s (Figure 3A). To prove that the cleavage of the internal plasmid is carried out by CL-*Eco*RV and not by a small contamination of uncrosslinked *Eco*RV, a second plasmid of different length which contains an *Eco*RV site was added to the CL-*Eco*RV preparation prior to the cleavage reaction. The rationale of this control experiment is that an external plasmid cannot be cleaved by CL-*Eco*RV but only by a contamination of uncrosslinked *Eco*RV. Our data show that after 60 min incubation only 15% of externally added plasmids were cleaved, whereas after 10 s all internal plasmids were cleaved, demonstrating that the activity due to a contamination with uncrosslinked *Eco*RV is $<1/1000$ of the activity displayed towards the internal plasmid. Thus, the cleavage of the internal plasmid must have been carried out by the CL-*Eco*RV molecules bound to this substrate. The rate of DNA cleavage at ambient temperature by CL-*Eco*RV at GATATC sites as determined by quenched-flow experiments (71 min^{-1}) is roughly four times faster than that measured for uncrosslinked *Eco*RV (17 min^{-1}) under identical conditions (Figure 3B). These results demonstrate that CL-*Eco*RV is a very efficient endonuclease towards the internal plasmid DNA. However, we observed three dramatic differences in the catalytic properties of CL-*Eco*RV and uncrosslinked *Eco*RV, as follows:

(i) CL-*Eco*RV cleaves DNA at 2 M NaCl with high rate ($\geqslant$6 min^{-1}), whereas uncrosslinked *Eco*RV is catalytically inactive at $c_{NaCl} \geqslant$500 mM (data not shown). This result was expected, because uncrosslinked *Eco*RV is unable to bind the substrate in such high salt buffer, whereas CL-*Eco*RV carries its substrate irreversibly bound and does not depend on binding to DNA added from the outside.

(ii) CL-*Eco*RV shows strong cleavage activity at non-canonical sites as indicated by the rapid occurrence of additional bands in the DNA-cleavage reaction of the internal plasmid (Figure 3A). Analysis of the resulting cleavage pattern reveals that *Eco*RV 'star' cleavage occurs, i.e. cleavage at sites that differ in one base pair from the GATATC sequence. Such relaxation of specificity has already been observed with *Eco*RV under certain buffer conditions, for example in the presence of Mn^{2+}, under

low ionic strength or in the presence of organic solvents (Halford *et al*., 1986). However, with CL-*Eco*RV star cleavage is observed under normal buffer conditions (20 mM Tris–HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, hereafter termed 'cleavage buffer'). Under these conditions star cleavage is not observed with uncrosslinked *Eco*RV, not even with 20-fold higher enzyme concentrations and after 10-fold longer incubation times (data not shown). This means that star activity of CL-*Eco*RV must be at least 2000-fold higher than that of uncrosslinked *Eco*RV, a very interesting result suggesting that the crosslink has frozen *Eco*RV in a hyperactive conformation (see below).

(iii) As already mentioned, the rate constant of DNA cleavage by CL-*Eco*RV at GATATC sites is ~4-fold higher than that measured for uncrosslinked *Eco*RV at ambient temperature. However, if the temperature is decreased this difference increases dramatically, because CL-*Eco*RV is much less sensitive towards a decrease in reaction temperature than uncrosslinked *Eco*RV (Figure 3B). At 4°C, CL-*Eco*RV cleaves the internal DNA ~300-fold more efficiently than uncrosslinked *Eco*RV cleaves external DNA. The temperature dependence of the DNA-cleavage rate [k(T)] was analyzed according to the Arrhenius model to determine the apparent activation energy of DNA cleavage (E_A):

$$k(T) = k_0 \exp(-E_A/RT)$$

An ln(k) versus 1/T fit of data allowed to calculate an activation energy of 40 kJ/mol for CL-*Eco*RV but of 220 kJ/mol for uncrosslinked *Eco*RV, supporting the conclusion that CL-*Eco*RV is frozen by the crosslink in an activated conformation that is on the way to the transition state of DNA cleavage.

Interaction of CL-EcoRV with linear DNA

CL-*Eco*RV cannot cleave circular DNA when added as an external substrate, because the two subunits form a stable dimer (Stahl *et al*., 1996; Wende *et al*, 1996) and, therefore allow access of substrates to the catalytic center only after opening the arms. However, the question arises of whether CL-*Eco*RV might be able to bind to and cleave linear DNA molecules which in principle could also enter the binding site by threading from one of the two ends. A 20mer oligonucleotide substrate is indeed hydrolyzed by CL-*Eco*RV when added as an external substrate (Figure 4A). We determined a rate constant for DNA cleavage of 0.04 min^{-1} per enzyme molecule (Figure 4B) which is much smaller than that observed for uncrosslinked *Eco*RV (k_{cat} = 2.9 min^{-1}). This finding implies that CL-*Eco*RV can bind to linear DNA substrates, a conclusion that was confirmed by binding studies with CL-*Eco*RV using linear DNA fragments in the presence of Ca^{2+} which supports specific DNA binding but not cleavage (Vipond and Halford, 1995). The specific binding constant of CL-*Eco*RV measured to a 382mer substrate that contains one *Eco*RV site ($K_{Ass} = 5.0\times10^9$ M^{-1} ± 20%) within the limits of error is identical to that of uncrosslinked *Eco*RV ($K_{Ass} = 3.3\times10^9$ M^{-1} ± 20%) (data not shown). Similarly, the binding constants of CL-*Eco*RV and uncrosslinked *Eco*RV to a non-specific 20mer determined under cleavage conditions (10 mM $MgCl_2$, 50 mM NaCl) are identical (CL-*Eco*RV, $K_{Ass} = 2.6\times10^6$ M^{-1} base pair; uncrosslinked

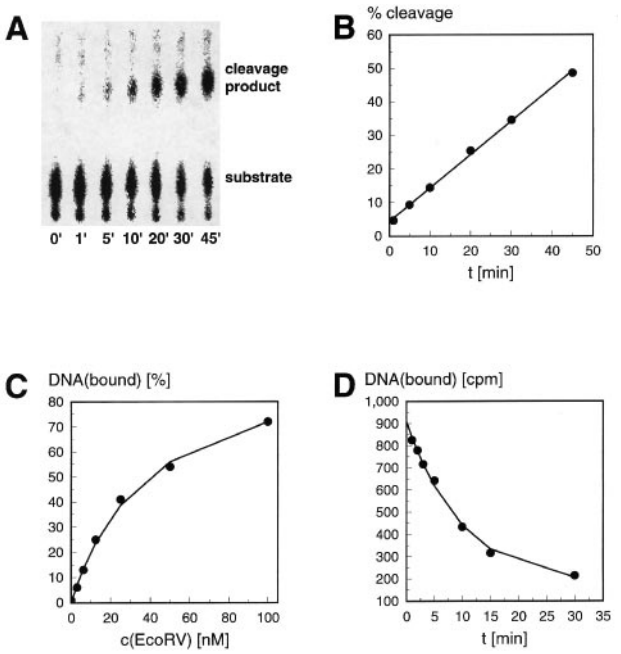

Fig. 4. Interaction of CL-*Eco*RV with linear DNA. (**A**) Cleavage of the specific 20mer oligonucleotide by CL-*Eco*RV as analyzed by homochromatography. 20mer (0.5 μM) was incubated with 100 nM CL-*Eco*RV in cleavage buffer. (**B**) Quantitative analysis of the data shown in (A). (**C**) Equilibrium-binding analysis of CL-*Eco*RV to the non-specific 20mer oligonucleotide. Experiments were carried out in cleavage buffer with 100 μg/ml BSA using 2 nM oligonucleotide. (**D**) Dissociation kinetics of CL-*Eco*RV from non-specific DNA. The experiment was carried out in cleavage buffer as described in Materials and methods.

*Eco*RV, $K_{Ass} = 1.4\times10^6$ M^{-1} base pair) (Figure 4C). The result that the equilibrium constant for binding of *Eco*RV to DNA are almost unaffected by the crosslink implies that the crosslink does not influence the ground state of the *Eco*RV–DNA complex in contrast to the significant stabilization of the transition state as shown in the previous section.

We have also determined the kinetics of dissociation of CL-*Eco*RV from linear DNA by nitrocellulose-filter binding experiments using the 382mer substrate. Experiments were carried out in the presence of 10 mM $MgCl_2$. Under these conditions CL-*Eco*RV binds to the DNA, cleaves it and subsequently binds non-specifically to the cleavage products. Thus, dissociation from non-specific DNA is observed in our experiments. The dissociation rate constant for CL-*Eco*RV and linear DNA (k_{off} = 0.1 min^{-1}; Figure 4D) is much smaller than that measured for uncrosslinked *Eco*RV (k_{off} = 1.2 s^{-1}; Erskine *et al*., 1997). The comparison of the thermodynamic data (no difference between *Eco*RV and CL-*Eco*RV) and the kinetic data (~1000-fold higher dissociation rate for *Eco*RV compared with CL-*Eco*RV) suggest that the association rate must be affected similarly as the dissociation rate (much smaller association rate for CL-*Eco*RV compared with *Eco*RV).

Interaction of CL-EcoRV with circular DNA substrates

We have shown so far, that CL-*Eco*RV binds to linear DNA via threading using the ends of the DNA. For theoretical reasons (see Discussion), we speculated

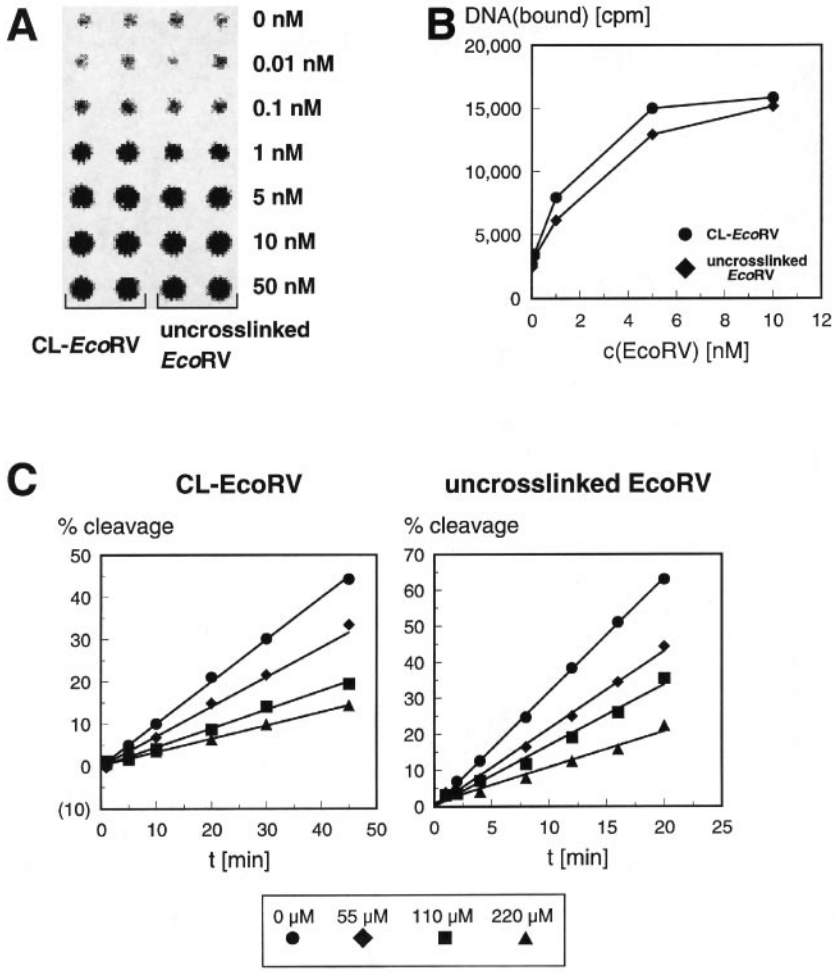

Fig. 5. Interaction of CL-*Eco*RV with circular DNA. (**A**) Binding of CL-*Eco*RV and uncrosslinked *Eco*RV to circular DNA. Experiments were performed in washing buffer containing 0.7 pM radioactively labeled pAT153. (**B**) Quantitative analysis of the data shown in (A). (**C**) Reduction of the rate of cleavage of the specific 20mer by CL-*Eco*RV and uncrosslinked *Eco*RV by addition of circular plasmid as competitor. Cleavage experiments were performed in cleavage buffer. The concentrations of pUC8 present as competitor are given in mole base pairs.

whether CL-*Eco*RV might also be able to bind to circular DNA. Therefore, radioactively labelled circular DNA was incubated with CL-*Eco*RV and DNA binding was measured by nitrocellulose-filter binding experiments. As shown in Figure 5A, significant binding is observed. We had confirmed that the substrate preparation did not contain a contamination by linear DNA and that it remained circular during the binding experiment (data not shown). These data could not be analyzed in terms of microscopic binding constants, because many enzyme molecules can bind to each substrate molecule. However, binding isotherms measured with CL-*Eco*RV and uncrosslinked *Eco*RV are virtually identical, with half saturation of binding observed in both cases at c_{EcoRV} = 2–4 nM. These results demonstrate that both enzyme species have a similar affinity for circular DNA (Figure 5B). This conclusion was corroborated by competition experiments in which it was observed that cleavage of the 20mer substrate by CL-*Eco*RV is strongly inhibited by addition of circular pUC8 plasmid DNA which does not contain an *Eco*RV site (Figure 5C)—a result which can only be rationalized if CL-*Eco*RV interacts with circular DNA. The inhibition constant of pUC8 binding to CL-*Eco*RV is as measured by cleavage of the 20mer substrate $K_I = 3\times10^7$ M^{-1} under these conditions. Interestingly, linear DNA is not a better competitor than circular DNA ($K_I = 3\times10^7$ M^{-1}) in cleavage experiments with the 20mer and CL-*Eco*RV. Analogous experiments with uncrosslinked *Eco*RV resulted in very similar competition curves as measured for CL-*Eco*RV (Figure 5C) with a competition constant for circular DNA of $K_I = 3\times10^7$ M^{-1} and for linear DNA of $K_I = 1\times10^7$ M^{-1}, although uncrosslinked *Eco*RV can bind to the plasmid at the internal binding site that is not accessible for circular DNA molecules in CL-*Eco*RV. Taken together, these experiments demonstrate that circular DNA can bind to CL-*Eco*RV. Such binding could only take place at the surface of the enzyme. The finding that circular plasmid DNA can inhibit oligonucleotide cleavage by CL-*Eco*RV demonstrates that the plasmid can bind to the outer binding site of CL-*Eco*RV and thereby prevent binding of the oligonucleotide to the internal site. Thus, binding to both sites is mutually exclusive. The affinity of non-specific DNA to *Eco*RV is almost identical regardless of whether crosslinked or uncrosslinked enzyme and circular or linear DNA are employed. These data demonstrate that the binding affinity of the surface binding site for non-specific DNA cannot be much lower than that of the internal binding site which means that under

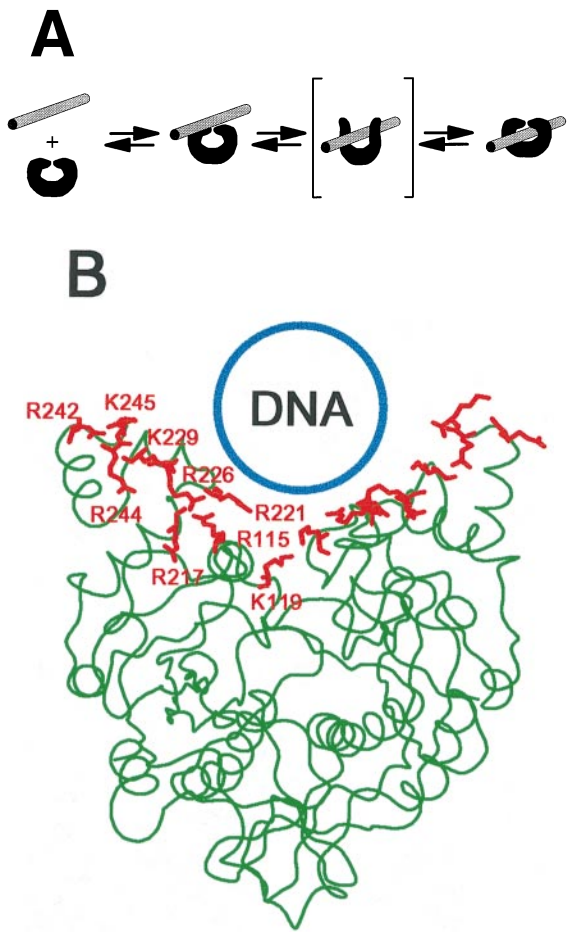

Fig. 6. (**A**) Schematic drawing of the proposed two-step association mechanism of *Eco*RV to DNA. First, the DNA associates to the outer binding site of *Eco*RV. Then, the arms of the enzyme open transiently and allow entry of the DNA to the internal binding site. (**B**) Structure of the *Eco*RV apoenzyme (Winkler *et al*., 1993), showing a wide shallow groove at the outer surface of the arms. The surface of this groove is decorated by 18 basic amino acid residues (shown only for one subunit). The diameter of B-DNA is drawn to scale for comparison.

equilibrium conditions DNA bound to *Eco*RV occupies the external as well as the internal binding site.

Mutagenesis of amino acid residues assumed to be involved in initial binding of DNA

Our data suggest that DNA initially can bind to the outer side of the arms of *Eco*RV. This assumption appears reasonable, as several basic amino acid residues are located on the outer side of the arms of *Eco*RV (Figure 6B). Beside Lys119, Arg221 and Arg226, these residues are not in contact with the DNA in the specific complex (Winkler *et al*., 1993). All of these amino acid residues were individually exchanged for alanine by site-directed mutagenesis. The enzyme variants were purified and their DNA-binding properties characterized (Table I). All of the variants retain at least 40% of the activity of wild-type *Eco*RV, with the exception of the K119A and R226A variants, in which amino acid residues are substituted that contact the DNA in the specific complex and are very important for indirect readout (Wenz *et al*., 1996). The K119A, R221A and R226A variants have a reduced non-specific affinity for DNA, but their specific DNA-binding constants are reduced even more. R242A and K245A behave like wild-type *Eco*RV. However, the non-specific DNA-binding constants of R115A, R217A, K229A and R244A are significantly more reduced than the specific binding constants, leading to an increased value of $K_{Ass,\ specific}/K_{Ass,\ non\text{-}specific}$. This result suggests, that these residues are important for non-specific DNA binding. As they do not interact with DNA bound at the internal binding site, it supports the existence of an outer binding site.

Discussion

A common feature of many enzymes which interact with DNA is that the DNA is completely encircled by the protein. The architecture of a closed complex obviously provides important advantages such as access to both grooves of the DNA, high complex stability and the ability to locate target sites by linear diffusion. However, association to DNA becomes more complicated than in an open complex: it can occur either via the ends of the DNA by threading them into the protein or it requires conformational changes of the protein. These conformational changes of the protein can play an important role in DNA recognition and enzymatic activity (Spolar and Record, 1994). Here, we have addressed experimentally these questions using the restriction enzyme *Eco*RV as a model system, which is a dimeric protein that encircles the DNA with two arms. Both subunits of the enzyme were crosslinked across the DNA-binding site using bivalent cysteine-specific chemical crosslinkers. If crosslinking occurs while a circular plasmid DNA is bound to *Eco*RV, the enzyme is catenated with the DNA (Figure 1B). This interaction is irreversible as both molecular rings (circular DNA and crosslinked protein) are topologically interconnected unless the protein subunits are dissociated (which requires protein denaturation) or the DNA is cleaved (which requires Mg^{2+} ions and the presence of an *Eco*RV-recognition site). The properties of such a chemically modified enzyme allow us to draw important conclusions as to the dynamics of *Eco*RV during its interaction with DNA and the mechanism of DNA binding.

Flexibility of the arms of EcoRV

The crosslinking yields of *Eco*RV with four crosslinkers of different length are similar, suggesting that the arms of *Eco*RV are flexible enough to accommodate different distances between the reactive maleimid groups. In agreement with this interpretation, crosslinking yields decrease at lower temperatures, where some of this flexibility is lost. Crosslinking yields were significantly larger if *Eco*RV is bound non-specifically to DNA than if it were free or in specific complex with DNA. These results are in good agreement with results of X-ray structure analyses which show that parts of the arms of *Eco*RV (amino acid residues 222–229) are poorly ordered in all complexes indicating conformational flexibility (Perona and Martin, 1997). Whereas these loops retain a residual structure in the apoenzyme, as well as in specific *Eco*RV DNA complexes, they could not be modelled if *Eco*RV is bound to a non-target DNA (Winkler *et al*., 1993). Thus, the high crosslinking yield of the non-specific *Eco*RV–DNA com-

Table I. Catalytic activities and DNA-binding affinities of *Eco*RV variants carrying an amino acid substitution in the arms of the enzyme

Variant	Specific activity [U/μg $_{protein}$][a]	$K_{Ass,\ non\text{-}specific}$ [M^{-1}][b]	$K_{Ass,\ specific}$ [M^{-1}][c]	$K_{Ass,\ specific}/K_{Ass,\ non\text{-}specific}$
Wild-type *Eco*RV	700	4.7×10^6	1×10^{10}	2100
R115A	300	$<1\times10^5$	2.7×10^9	>27 000
K119A	50	0.9×10^6	1×10^7	11
R217A	800	$<1\times10^5$	1.3×10^{10}	>125 000
R221A	600	3.6×10^6	9×10^8	250
R226A	80	0.5×10^6	1×10^7	20
K229A	800	1.3×10^6	1.1×10^{10}	8500
R242A	600	6.1×10^6	1.2×10^{10}	1900
R244A	600	0.8×10^6	1.6×10^{10}	20 000
K245A	500	2.3×10^6	7.4×10^9	3200

Binding constants are valid ± 30%, specific activities within a factor of two.
[a]Determined using bacteriophage λ-DNA as substrate.
[b]Determined with the non-specific 20mer in the presence of Mg^{2+}.
[c]Determined with the specific 382mer in the presence of Ca^{2+}.

plex is correlated to the high degree of conformational flexibility of the arms of *Eco*RV in this complex.

Kinetics of threading DNA into and out of the DNA-binding tunnel of CL-EcoRV

CL-*Eco*RV can bind to and cleave linear DNA. This means that the ends of a linear DNA can be threaded into the artificially created DNA-binding tunnel of CL-*Eco*RV. Yet, the rate of DNA cleavage of linear substrates (0.04 min^{-1}) is much slower than the DNA-cleavage rate observed for uncrosslinked *Eco*RV (k_{cat} = 2.9 min^{-1}; Wenz *et al*., 1996). This rate difference cannot be due to the catalytic step *per se*, as shown by the high activity of CL-*Eco*RV towards internally bound plasmids. Most likely, under multiple turnover conditions the rate of DNA cleavage by CL-*Eco*RV is limited by the rate of substrate binding. As in our experiments 1 μM DNA ends were present, the bimolecular rate constant of threading a DNA into the DNA-binding tunnel of CL-*Eco*RV can be estimated to be 4×10^4 M^{-1} min^{-1}. DNA binding by CL-*Eco*RV, therefore, is by five orders of magnitude slower than binding of uncrosslinked *Eco*RV to DNA (7.2×10^9 M^{-1} min^{-1}; Erskine *et al*., 1997) which is mediated by opening the arms to allow direct association of the substrate into the DNA-binding cleft of *Eco*RV. Thus, threading DNA ends into *Eco*RV is possible but slow. It certainly does not contribute to the usual catalytic mechanism of the enzyme. However, there are other nucleases known, such as bacteriophage T5 5′-exonuclease or calf 5′→3′ exo/endonuclease, that use DNA threading as the only mechanism of association to their substrate (Murante *et al*., 1995; Ceska *et al*., 1996). The rate of dissociation of CL-*Eco*RV from a bound DNA molecule (k_{off} = 0.1 min^{-1}) again is much slower than dissociation of uncrosslinked *Eco*RV from non-specific DNA (k_{off} = 1.2 s^{-1}; Erskine *et al*., 1997). These numbers show that product dissociation usually does not occur via the ends of the DNA (unless the mechanism of end-dissociation of uncrosslinked *Eco*RV would be different from that of CL-*Eco*RV), a result which confirms our kinetic finding that *Eco*RV has a low probability to fall off at the ends of a linear DNA molecule (Jeltsch and Pingoud, 1998).

Mechanism of DNA association to EcoRV

In the crystal structure the *Eco*RV apoenzyme has a closed conformation (Winkler *et al*., 1993; Perona and Martin, 1997). This would prevent association to DNA. Thus, a conformational change of the enzyme is required prior to or in the course of DNA binding. One could speculate that free *Eco*RV exists in an equilibrium between an open and closed conformation. Then, the open conformation must be populated significantly to explain the fast association of *Eco*RV to DNA, because otherwise many unproductive encounters between *Eco*RV in the closed conformation and DNA would occur. However, there is no experimental evidence for a populated open state of free *Eco*RV in solution. We have shown here that the Stokes' radii of free CL-*Eco*RV and uncrosslinked *Eco*RV are identical, suggesting that the average solution conformation is not altered by the crosslink. As an open conformation is not accessible for CL-*Eco*RV, this result suggests that uncrosslinked *Eco*RV also predominantly exists in a closed conformation. Moreover, it has been shown previously that the radii of gyration of *Eco*RV, and *Eco*RV in complex with specific DNA as determined by neutron scattering are identical within the limits of error (J.Langowski and A.Pingoud, unpublished results). Although these observations do not rule out the existence of an open conformation, they provoke one to think of an alternative association pathway of *Eco*RV to DNA following a two-step process (Figure 6A): in this model, free *Eco*RV is in a closed conformation. The DNA first docks to the outer parts of the arms of *Eco*RV at the surface of the protein. It stays bound there until the arms open up and it can enter the DNA-binding cleft. One could even speculate that the interaction of the DNA with the outer parts of the arms might induce the opening of the gate. According to the two-step association mechanism (Figure 6A) each *Eco*RV–DNA encounter could result in the formation of an *Eco*RV–DNA complex at the surface of *Eco*RV which could explain the fast association rate constant of *Eco*RV to DNA. It should be noted that this model also provides a reasonable pathway for the dissociation of *Eco*RV from DNA, which is rate limiting for cleavage of macromolecular DNA by *Eco*RV.

The two-step binding model is strongly supported by our finding that CL-*Eco*RV (which can only exist in a closed conformation) can bind to circular DNA. This result implies that a DNA-binding site at the surface of *Eco*RV must exist which is a prerequisite for the two-step binding model. Most probably, this binding site is located at the outer side of the arms of *Eco*RV in the closed conformation. There a wide, shallow groove exits, large enough to accommodate B-DNA. The surface of this groove is decorated with 18 basic amino acid residues (Arg115, Lys119, Arg217, Arg221, Arg226, Lys229, Arg242, Arg244 and Lys245 of each subunit) (Figure 6B). We have shown that in particular Arg115, Arg217 and to a lesser extent Arg244 and Lys229 are required for non-specific binding, but not for specific complex formation. It is not clear at present how exactly the DNA is bound in this groove, if the protein undergoes conformational changes upon binding DNA to the outer binding site and whether the amino acid residues shown to be important for non-specific binding make direct contacts to the DNA or contribute to an attractive electrostatic field.

Although *Eco*RV has been studied intensively over the last 10 years, both biochemically and structurally, this extra binding site for DNA had not been found so far [ironically, F.K.Winkler, after solving the structure of the *Eco*RV apoenzyme, speculated that the DNA-binding site of *Eco*RV might be located at the outside of the arms, an idea that was not followed up after solving the cocrystal structures (personal communication)]. Our data, without reasonable doubt, demonstrate the existence of this extra site which we assume to be an entry site which is occupied as an intermediate in each catalytic cycle. Non-specific binding of DNA to *Eco*RV can occur at the outer binding site, as shown by the ability of CL-*Eco*RV to bind to circular DNA, and at the internal binding site, as shown by the crystal structure of the non-specific *Eco*RV–DNA complex and the finding that non-specifically bound circular DNA can be catenated with the enzyme by performing the crosslinking reaction in the presence of DNA. As both binding modes are almost isoenergetic, non-specific DNA may change its position more or less frequently.

Coupling of specific DNA binding to catalysis

Following non-specific binding of *Eco*RV to DNA the enzyme scans the sequence of the DNA by linear diffusion searching for GATATC sites (Jeltsch *et al*., 1996; Jeltsch and Pingoud, 1998). If specific sites are encountered, the catalytic center of the enzyme is activated by a conformational change of the *Eco*RV–DNA complex. Whereas details of this conformational change are unknown, it is presumably associated with a transition of the loose non-specific complex with the DNA fluctuating between the external and the internal binding site to a tightly closed form in which the DNA is exclusively bound at the internal site, as seen in the different specific *Eco*RV–DNA complexes (Figure 1A) (Winkler *et al*., 1993; Kostrewa and Winkler, 1995; Perona and Martin, 1997). We have shown here that CL-*Eco*RV in which both arms are permanently joined to each other is more active than uncrosslinked *Eco*RV. The Arrhenius energy of activation of DNA cleavage at canonical sites is reduced by 180 kJ/mol in CL-*Eco*RV compared with native *Eco*RV. Interestingly, ground state DNA binding is barely influenced by the crosslink. In addition, CL-*Eco*RV is less accurate in cleaving DNA than uncrosslinked *Eco*RV. This means that the transition state energy for cleavage of a star-site by CL-*Eco*RV is much lower than for cleavage by uncrosslinked *Eco*RV. Taken together, these results show that crosslinked *Eco*RV is frozen in a conformation which is closer to the conformation of the transition state of catalysis than the average conformation of uncrosslinked *Eco*RV—an unusual outcome of chemical modification experiments. It means, that bringing together the arms of *Eco*RV and holding the DNA in the internal binding site makes the structure more transition state-like. These findings reinforce the conclusion drawn from crystal-structure analyses that large domain movements do occur in *Eco*RV during DNA cleavage (Perona and Martin, 1997). Furthermore, they show that these conformational changes are strongly linked to catalysis.

Materials and methods

Site-directed mutagenesis and purification of EcoRV and EcoRV variants

Site-directed PCR mutagenesis was carried out essentially as described previously (Roth *et al*., 1998). Expression and purification of the enzyme variants were performed by chromatography on Ni-NTA–agarose and phosphocellulose as described previously (Wenz *et al*., 1994; Jeltsch and Pingoud, 1998). Specific activity was determined using λ-DNA as substrate as described previously (Wenz *et al*., 1994).

Crosslinking of EcoRV

For the cysteine-specific chemical crosslinking experiments an *Eco*RV C21S/T222C variant was prepared by site-directed mutagenesis that bears only one single cysteine residue per subunit at a position well-suited for intramolecular crosslinking. To obtain an *Eco*RV preparation in which all cysteine residues are reduced but that does not contain free SH-reagents, 500 μg of the purified *Eco*RV mutant were incubated with 10 mM dithioerythritol (DTE) for 2 h at 0°C in 2 ml 20 mM Tris–HCl pH 7, 0.25 mM EDTA, 150 mM NaCl, 38% (v/v) glycerol. The solution was dialyzed twice against 20 mM Tris–HCl pH 7, 50 mM NaCl to remove DTE. The SH-specific crosslinking reactions were carried out using a series of bivalent-SH specific crosslinkers consisting of two reactive maleimide groups linked by three, five, six or seven methylene groups, synthesized and purified as described (Franke and Pingoud, 1998). 1,6-bis(maleimido)hexan containing six methylene groups is commercially available (Pierce chemicals).

For the analytical crosslinking reactions *Eco*RV (1 μM) was incubated in 20 mM Tris–HCl pH 7.5, 50 mM NaCl containing either 10 mM $MgCl_2$, 10 mM $CaCl_2$ or 2 mM EDTA in the absence or presence of oligonucleotide (5 μM, 5′-GAGGAAAGATATCTTTCCTC-3′) for 30 min. The crosslinkers were added to a final concentration of 2.5 mM and after 3 min incubation the reaction was stopped with 13 mM DTE. For preparative crosslinking reactions 500 μg *Eco*RV were incubated with 500 μg pAT153 in 5 ml 20 mM Tris–HCl pH 7, 50 mM NaCl, 10 mM $CaCl_2$ for 20 min at room temperature. The crosslinking reaction was started by the addition of the crosslinker that contains six methylene groups to a final concentration of 50 μM. After 5 min the reaction was stopped by addition of DTE to a final concentration of 100 μM.

Purification of crosslinked EcoRV in complex with DNA

The solution obtained after the preparative crosslinking reaction was diluted 1:3 with buffer N (30 mM potassium phosphate pH 7.6, 0.1 mM DTE, 0.01% Lubrol, 0.5 M NaCl, 10 mM imidazole) and applied onto a small column filled with 2 ml Ni-NTA–Agarose (Qiagen) equilibrated with buffer N. In contrast to uncrosslinked *Eco*RV, CL-*Eco*RV in complex with DNA does not bind to Ni-NTA. The flowthrough containing CL-*Eco*RV was concentrated to a final volume of 500 μl using Centriplus 10 columns (Amicon) and dialyzed against 20 mM Tris–HCl pH 7.5, 50 mM NaCl, 2 mM EDTA (buffer A). The sample was applied onto a Mono Q column (5.5×0.6 cm, Pharmacia) equilibrated with buffer A. The elution was carried out with a gradient of 0–50% buffer B (20 mM Tris–HCl pH 7.5, 2 mM EDTA, 2 M NaCl) in 80 min. After a concentration step using a Centriplus 10 column, the *Eco*RV preparation

was dialyzed against storage buffer [30 mM potassium phosphate pH 7.6, 0.5 mM EDTA, 0.1 mM DTE, 0.01% Lubrol, 0.3 M NaCl, 77% (v/v) glycerol].

Purification of crosslinked EcoRV without DNA

*Eco*RV was crosslinked in the presence of plasmid DNA as described. After purification using Ni-NTA–agarose, 1000 U of *S.marcescens* nuclease were added to the flowthrough and incubated for 10 h at 4°C in 20 mM Tris–HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$ to digest the DNA. Then, NaCl was added to a final concentration of 1 M. Under these conditions, *Eco*RV does not bind to DNA. CL-*Eco*RV was separated from the DNA and the *S.marcescens* nuclease using a Ni-NTA–agarose column as described by Wenz *et al*. (1994).

Analytical ultracentrifugation experiments

Sedimentation velocity runs with CL-*Eco*RV and uncrosslinked *Eco*RV were carried out at 20°C in 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, at 40 000 r.p.m. in a Beckman Optima SL-A centrifuge equipped with absorption optics and an An-50 8-place rotor. The protein concentration was 1 μM. Sedimentation velocity data were evaluated with the program package AKKUPROG by fitting the time-dependent concentration profiles calculated with Lamm's differential equation for a simple sedimenting species to the measured data.

DNA-binding analyses

DNA binding was analyzed by nitrocellulose-filter binding experiments in a dot blot apparatus (Bio-Rad). The nitrocellulose membrane (Schleicher and Schuell) was equilibrated in washing buffer (20 mM Tris–HCl pH 7.5, 50 mM NaCl, 10 mM $CaCl_2$) for 30 min. After 30 min incubation at ambient temperature the samples containing labelled DNA and enzyme were sucked through the nitrocellulose membrane and washed twice. For all binding reactions, CL-*Eco*RV without DNA was used. For specific DNA-binding experiments a radioactively labelled 382 bp fragment containing a single *Eco*RV site was prepared as described (Jeltsch *et al*., 1995). Forty picomolar 382mer was titrated with increasing amounts of either CL-*Eco*RV or uncrosslinked *Eco*RV in binding buffer [20 mM Tris–HCl pH 7.5, 50 mM NaCl, 10 mM $CaCl_2$, 100 μg/ml bovine serum albumin (BSA)]. Binding to a non-specific oligonucleotide was analyzed using 2 mM of the 20mer oligonucleotide 5′-GATCGACGAGCTCGTCGATC-3′ in cleavage buffer (20 mM Tris–HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) with 100 μg/ml BSA. The oligonucleotide was radioactively labelled with [γ-^{32}P]dATP and T4 polynucleotide kinase. To measure binding to circular plasmid DNA, pAT153 was radioactively labeled by nick-translation with [α-^{32}P]dATP and DNA polymerase I. Binding experiments were carried out in washing buffer using 0.7 pM labeled DNA. The detection and quantification of bound DNA was performed using an InstantImager system (Canberra Packard). To calculate K_{Ass}-values, the bound DNA versus c_{enzyme} data obtained in the binding experiments were fitted to the equation characterizing a bimolecular binding equilibrium.

To determine the rate constant for dissociation of *Eco*RV from DNA (k_{off}) 100 nM CL-*Eco*RV was incubated with 4 nM of radioactive 382 bp DNA fragment in cleavage buffer for 30 min to allow complex formation. Then the sample was diluted 1:50 with cleavage buffer containing 12 nM unlabelled DNA fragment and incubated for a defined period of time. The fraction of DNA still bound by *Eco*RV was analyzed by nitrocellulose-filter binding as described above. Dissociation kinetics were fitted to a mono-exponential decay curve.

DNA-cleavage experiments with plasmid DNA

CL-*Eco*RV (100 nM containing 5 nM pAT153 internal plasmid, which means that ~20 *Eco*RV molecules are bound to the plasmid in this preparation) was incubated in cleavage buffer. After defined time intervals the reaction was stopped by addition of agarose gel loading buffer containing 250 mM EDTA and the amount of DNA cleavage was analyzed by agarose gel electrophoresis. For the kinetic experiments a quenched flow apparatus (SFM-3, Bio-logic, Claix, France) was used. The DNA-cleavage reaction was started by mixing a solution of 80 nM CL-*Eco*RV containing 4 nM pAT153 in 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 10 mM NaCl with an equal volume of 20 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$ and stopped by adding the same volume of 50 mM EDTA. Experiments were carried out under the same conditions with uncrosslinked *Eco*RV.

Oligonucleotide-cleavage experiments

For oligonucleotide-cleavage experiments we used 0.5 μM radioactively labeled specific oligonucleotide (5′-GAGGAAAGATATCTTTCCTC-3′) and 100 nM CL-*Eco*RV (containing 5 nM pAT153 internal plasmid) in cleavage buffer. Prior to the addition of the oligonucleotide substrate CL-*Eco*RV was pre-incubated for 30 min at ambient temperature in cleavage buffer to allow for digestion of the internal plasmid. After defined time intervals, aliquots were withdrawn from the reaction mixture, spotted onto a DEAE–cellulose plate (Macherey-Nagel, Düren, Germany) and subjected to homochromatography. Detection and quantification of the separated substrates and products were achieved using an InstantImager system (Canberra Packard). To analyze the interaction of CL-*Eco*RV with circular DNA, oligonucleotide cleavage experiments were carried out using 100 nM CL-*Eco*RV (containing 5 nM pAT153 internal plasmid) in the presence of 0–90 nM circular pUC8 (2665 bp) as a competitor DNA. pUC8 was used, because it does not contain an *Eco*RV site. Similar experiments were performed with uncrosslinked *Eco*RV under identical conditions using 20 nM *Eco*RV in the presence of 5 nM linear pAT153.

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