Engineering of DNA binding proteins into site-specific cutters: reactivity of Trp repressor–1,10-phenanthroline chimeras

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Introduction

DNA-binding proteins of known 3D structure have been engineered into site-specific scission reagents by a procedure involving site-directed mutagenesis and chemical modification (Bruce et al., 1991; Sutton et al., 1993; Pan et al., 1994b; Pendergast et al., 1994; Pfau et al., 1994b; Shang et al., 1994) (Figure 1). The first step is the identification of a residue in the amino acid sequence which is proximal to the minor groove of DNA but not directly involved in high-affinity binding. Accessibility to the minor groove is essential since the C1-H of the deoxyribose, which is the site of oxidative attack by the untargeted 2:1 1,10-phenanthroline–cuprous complex ([OP]2–Cu1) (Goyne and Sigman, 1987), is the likely target of reaction by the protein–1,10-phenanthroline chimeras. In the second step, an amino acid residue which satisfies these criteria is then converted into a cysteine using site-directed mutagenesis, followed by alkylation with 5-iodoacetamido-1,10-phenanthroline or an analog thereof (Sigman et al., 1991). Any other cysteine residue is preferably converted into a serine or alanine in order to permit the preparation of structurally homogeneous cleavage reagents and facilitate the interpretation of the scission data in terms of structural models based on X-ray crystallography and nuclear magnetic resonance (NMR) (Pendergast et al., 1994).

In addition to providing a chemical method for auditing proposed structures, these chimeras, which generally recognize DNA sequences 12–16 bp long, could serve as rare cutters that would be useful for digestion of chromosomal DNA into larger fragments than would be possible with available restriction enzymes which usually have 6–8 bp recognition sequences. In conjunction with hydrolytic restriction enzymes, these site-specific scission reagents could be used for the linear ordering of cloned DNA fragments. For these goals to be achieved, efficient double-stranded scission must be obtained.

The Trp repressor of *Escherichia coli*, which is responsible for the tryptophan-dependent regulation of the *trpEDCBA*, *aroH* and *trpR* operons (Gunsalus and Yanofsky, 1980; Yanofsky, 1981; Gunsalus et al., 1986), has been transformed into a double-stranded DNA scission reagent which cleaves its *E.coli* binding sites with yields approaching 90% in solution based on fraction of parent band remaining (Sutton et al., 1993; Pfau et al., 1994b). The initial chimera was prepared from the 108 amino acid homodimer by alkylation of the Trp repressor E49C with 5-iodoacetamido-1,10-phenanthroline. Its tryptophan-dependent binding to TrpR-regulated operators is approximately 7×10−9 M, equivalent to that observed with the wild-type protein under comparable conditions (Sutton et al., 1993). The scission pattern by E49C–OP has provided unambiguous evidence for the positioning of the TrpR on the trpEDCBA operator proposed in the co-crystal structure (Schevitz et al., 1985; Otwinowski et al., 1988; Haran et al., 1992). It has also strongly supported the tandem model of repressor binding to the *E.coli* trp R, aro H and trp EDCBA operators. Initially proposed by Gunsalus and colleagues (Kumamoto et al., 1987) and based on methylation interference and DNase I footprinting, the tandem binding model has recently received additional support from X-ray studies (Lawson and Carey, 1993).

Since the chimera derived from TrpR is remarkable for its efficiency as an engineered site-specific nuclease, it could serve as the starting point for the design of a family of chimeric restriction endonucleases based on its 3D structure if the DNA binding specificity of this protein could be altered either by site-directed mutagenesis or random mutagenesis followed by screening. An altered binding mutant, 179K, isolated by phage challenge methodology (Bass et al., 1988), in fact has been transformed into a new nuclease activity which reflects this new specificity (Pfau et al., 1994b). Because of this initial success, we have investigated structural and chemical aspects which influence the efficiency of the nuclease activity of Trp
repressor-OP chimeras including the amino acid position at which the 1,10-phenanthroline is attached, the affinity of binding of the chimera to the protein and the pH of the cleavage reaction. In examining the reactive properties of the chimera prepared from the wild-type repressor, we have discovered two important features of repressor binding. First, we have shown that the corepressor tryptophan is not only essential for high-affinity repressor binding and scission but also inhibits non-specific binding by the apo Trp repressor. Second, we have been able to determine the relative binding affinities of the three subsites of the trp EDCBA operator for TrpR using the targeted scission of the chimeras.

Materials and methods

Generation of TrpR mutants
TrpR E49C and A77S, E49C were generated by PCR based mutagenesis from the parent vector pPY2000 which was kindly provided by P. Youderian. This vector is based on pBR322 but contains an Ml3 origin of replication at the Dra I site of pBR322 and carries a deletion from Sal I to Ava I to make the Sal I site in trpR unique. The vector was made available with the wild-type gene or A77S. E49C was introduced in a two-step PCR mutagenesis and combination with the wild-type gene or A77S. E49C, A77S.

Protein purification
Cells (DH5a) were grown in 0.5 l of LB-ampicillin and were induced with 1 mM IPTG at OD 0.58 for 2 h. The precipitated cells were resuspended and sonicated in 10 ml of 50 mM Tris (pH 8.5), 1 mM DTT. The suspension was centrifuged twice in small aliquots in a microcentrifuge for 5 min each and the clear supernatant was filtered and applied to a MonoQ column. Protein was eluted in a gradient from 0 to 1 M NaCl over 2 h (1 ml/min). TrpR eluted at ~280 mM NaCl and was identified by SDS-PAGE. An estimate of protein purity is hard to obtain from these gels since substantial overloading is required to stain TrpR both by Coomassie and silver staining. However, less concentrated samples seem to indicate a purity of at least 70–80%. The correct fractions were further identified by gel-shift analysis on radio-labeled trpEDCBA operator fragments. The identified fraction was frozen with 15% glycerol at a TrpR concentration of ~10–50 nM.

Derivatization
For a standard derivatization, 200 µl of protein (~37 mM Tris (pH 8.5), 0.75 mM DTT) were incubated for 10 min at 4°C with fresh DTT at a final concentration of 5 mM. The mixture was diluted with eight equivalents of phosphate buffer (12.5 mM, pH 7.8) and KCl (375 mM) and one volume equivalent of 5-iodoacetamido-1,10-phenanthroline (IAAOP) in DMF (final IAAOP concentration 2.5 mM) (Sigman et al., 1991). A precipitate forms immediately after the addition of IAAOP. The reaction was incubated overnight at 4°C. After a 5 min centrifugation in an Eppendorf centrifuge at maximum speed, the clear supernatant was purified on 1 ml G50 spin columns in batches of 200 µl. The final storage buffer for the protein was 10 mM phosphate buffer (pH 7.8), 300 mM KCl.

Gel-shift conditions
Gel retardations were performed in 10% non-denaturing polyacrylamide (30:1) gels with a recirculated running buffer of 10 mM NaH2PO4 (pH 6.0) and 0.5 mM tryptophan. The gel was run at 300 V constant voltage and 4°C.

Scission in the gel slice
After identification of the gel-shifted complex by autoradiography, a narrow slice was cut out of the gel (~50–100 µl volume). The slice was incubated in a final volume of ~200 µl of 20 mM sodium phosphate buffer (pH 7.2) containing 1 mM tryptophan and 10 µM cupric sulfate. After 5 min, scission was activated with 15 µl of 50 mM 3-mercaptopropionic acid (MPA) plus H2O2 [7 µl H2O2 to 1 ml (50 mM) MPA just before use]. After 30 min, the reaction was stopped with 2 mM 2,9-dimethyl-1,10-phenanthroline. The products were eluted overnight and analyzed on a sequencing gel.

Scission in solution
Scission reactions typically contained 0.1 pmol of linearized plasmid (trpEDCBA containing pUC vector) and approximately

Fig. 1. Outline for the conversion of a DNA binding protein into a site-specific scission reagent through derivatization with 5-iodoacetamido-1,10-phenanthroline (IAAOP). After the identification of a suitable attachment point on the protein and removal of secondary cysteines where applicable a cysteine residue is introduced through site-directed mutagenesis. Copper ion is not part of the derivatization reaction and is shown in this scheme only to demonstrate the distance from the alpha carbon of cysteine to the end of the scission reagent. The scission chemistry is briefly outlined below with mercaptopropionic acid (MPA) and H2O2 as the cofactors.

Outline for the conversion of a DNA binding protein into a site-specific scission reagent through denvatization with 5-iodoacetamido-1,10-phenanthroline (IAAOP)
stoichiometric concentrations of TrpR-OP in a final reaction volume of 40 μl. Plasmid and protein were first mixed in 20 μl of binding buffer [100 mM Tris (pH 7.5), 200 mM KCl, 6 mM MgCl₂] for 10 min at room temperature. An equal volume of scission mixture (10 μM CuSO₄, 1 mM tryptophan) was added and the scission reaction was activated after 1 min by the addition of 3 μl H₂O₂ and mercaptopropionic acid (this mixture was prepared freshly every time just before use by adding 3 μl of H₂O₂ to 1 ml of 50 mM MPA). The reaction was stopped with 3 μl of 0.1 M EDTA and analyzed on a 0.8% agarose gel.

Results

Kinetic mechanism of scission by protein–OP chimeras

In enzymatic reactions, substrate binding and catalysis are intimately related; mutational changes in the substrate binding site invariably lead to changes in both k_cat and K_m (Hackney, 1991). In contrast for the chimeric nucleases discussed here, the chemistry underlying DNA scission is not coupled to DNA binding. The 1,10-phenanthroline–copper redox system is active whether or not the Trp repressor is tightly bound to its target sequence. As a result, the net reaction scheme (Scheme 1) includes several steps which are not directly on the reaction pathway. For example, during preparation or storage, a chimera could be oxidatively inactivated (Scheme 1, k_3'). In this case, incomplete scission will be observed if high-affinity DNA binding is retained since the cleavage competent and the cleavage incompetent forms of the Trp R protein would compete for the same binding site.

The yield of site-specific scission will depend on the concentration of the cleavage-competent chimera (P–OP) and the partitioning of the central intermediate, DNA–P-OP [k_1/(k_1 + k_2 + k_3)], where k_1 is the dissociation of the essential intermediate back to starting material, k_3 self-inactivation within the protein–DNA complex (DNA–P–OP) and k_2 productive scission. High-affinity binding (low k_1) is clearly favorable for efficient scission. However, genetic or chemical alterations which increase the absolute affinity of binding (e.g. decreased k_1) are only useful if they do not increase non-specific binding as well (low k_d/k_3). At protein concentrations that favor specific over non-specific binding [i.e. K_d(spec.) < [P–OP] < K_d(non-spec.)], the yield of site-specific scission is governed by the magnitude of k_2 relative to both k_1 and k_3. Self-inactivation in solution (e.g. k_3') during the course of the cleavage reaction will also diminish specific scission by decreasing P–OP.

Optimizing the orientation of the reaction center

The most effective method for enhancing the efficiency of scission is to increase the rate constant for productive scission, k_2. The best way to enhance this rate constant would be to optimize the orientation of the OP relative to the oxidatively sensitive bond. Initial examination of the Trp repressor crystal structure indicated that two sequence positions, 46 and 49, were candidates for the site of attachment of 1,10-phenanthroline. Residue 46 is an aspartate (D) and residue 49 is a glutamate (E). Both residues are located near the dyad axis of the protein and adjacent to the oxidatively sensitive minor groove. After initially focusing our efforts on TrpR E49C–OP, Trp repressor D46C–OP has now also been prepared to determine if it might provide a more robust scission reagent. Although this chimera binds trpEDCBA with affinity comparable to the wild-type repressor, as measured by gel retardation analysis, its DNA cleavage pattern differs markedly from that obtained with E49C–OP, even though extensive cleavage of the parent band is observed. In lieu of the sharply focused set of two or three cleavage sites characteristic of E49C–OP, which can be used to position TrpR on the dyad axis, multiple sites of scission are apparent with D46C–OP when the DNA scission is carried out within the acrylamide matrix following separation of the protein–DNA complexes by a mobility shift assay (Figure 2).

Fig. 2. Comparison of the scission of TrpR D46C–OP and TrpR E49C–OP in the gel-slice. The DNase I footprint confirms the tryptophan-dependent (W) binding on the operator sequence. Gel retardations were performed in 10% non-denaturing polyacrylamide gels with a recirculated running buffer of 10 mM NaH₂PO₄ (pH 6.0) and 0.5 mM tryptophan. The scission reaction on the gel-shifting complex shown in lanes a and b was carried out in 20 mM sodium phosphate buffer (pH 8.0) containing 1 mM tryptophan, 10 μM cupric sulfate and 3 mM H₂O₂ and mercaptopropionic acid for 30 min at 25°C. The reaction was stopped with 2 mM 2,9-dimethyl-1,10-phenanthroline. The products were eluted overnight and analyzed on a sequencing gel.
of attacking multiple deoxyribose moieties within the minor groove. This explanation is inconsistent with the restricted sites of cleavage obtained with TrpR E49C-OP and OP chimeras prepared with the factor for inversion stimulation (Fis) protein (Pan et al., 1994a) and the cyclic AMP receptor protein (CAP) (Ebright et al., 1990; Pendergrast et al., 1994). In addition, product analysis (Kuwabara et al., 1986; Goyne and Sigman, 1987) and physical organic studies (Johnson and Nazhat, 1987) have indicated that the 1,10-phenanthroline-copper system does not generate diffusible hydroxyl radicals like ferrous-EDTA, despite its formal similarity to Fenton chemistry (Tullius, 1988). The second possible explanation is that the tethered phenanthroline–copper system does not generate diffusible hydroxyl radicals like ferrous-EDTA, despite its formal similarity to Fenton chemistry (Tullius, 1988). The second possible explanation is that the tethered phenanthroline can assume multiple orientations within the minor groove and as a result cleave at several sequence positions. This explanation has been advanced to account for the multiple cleavage sites observed with the OP chimera prepared from the lambda phage Cro protein (Bruice et al., 1991).

Molecular modeling supports the latter interpretation. The primary difference between the model generated for the D46C-OP chimera relative to that obtained with the E49C-OP construct is that the OP moiety extends along the phosphodiestere backbone in the former case but is held in a restricted orientation in the latter (Figure 3). These results suggest that minor perturbations in the structure of these chimeras can have a significant impact on their reactivity. The E49C-OP construct was the subject of our further work because its focused reactivity is more useful than that obtained with D46C-OP for the various applications of these chimeric scission reagents.

Distribution of TrpR between alternative binding sites

Comparison of the DNA binding affinity of TrpR E49C-OP with wild-type TrpR using gel retardation reveals that both proteins bind E. coli regulated operons with comparable affinity. Within a small range of TrpR concentrations, two retarded bands are observable after titration of trp EDCBA with TrpR (Figure 4a). The faster moving protein–DNA complex is formed when DNA is in excess of Trp R and therefore is likely to have a stoichiometry of 1 mol of TrpR per trp EDCBA. Upon a slight increase of the protein concentration, a second retarded band (second shift) is observed. Since the scission chemistry can be activated by the addition of 3-
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Fig. 4. Gel-shift of trpEDCBA and single-stranded DNA scission by TrpR-OP. (a) Tryptophan-dependent gel shift of a trpEDCBA containing end-labeled fragment by TrpR E49C-OP. Lanes 1 and 2, no protein (duplicated for scission purposes); lane 3, retardation by TrpR, gel-shift conditions as described for Figure 2. The binding behavior of TrpR E49C-OP is essentially unchanged compared with undervalutized protein (Sutton et al., 1993). The lower band (1st shift) converts into the second, slower migrating band upon modest increases in the protein concentration and is not always observed. (b) Two alternative models for TrpR binding to the trpEDCBA operator sequence in the two complexes observed in (a). The general outline of the operator is adopted from Sutton et al. (1993) and Kumamoto et al. (1987). The first gel shift (1st), corresponding to the middle band in lane 3 in (a), is presented in both cases as the dominant species with one TrpR dimer bound to the central site. The second band (2nd) could either represent a population of operators with TrpR dimers at two positions (model I) or fully saturated operator (model II). The position of the 5' end-label relative to the binding sites is indicated by an asterisk. (c) Scission of gel-shifted trpEDCBA in the gel slice by TrpR E49C-OP or A77S, E49C-OP. Lane 1 shows single-stranded DNA scission on the faster migrating band (1st gel shift) by TrpR E49C-OP. The yield of single-stranded scission on the slower moving band (2nd shift) with E49C-OP (lane 2) represents the maximum yield of scission observed under the conditions described for Figure 2. In lane 3 the complex was treated in an identical manner but scission was carried out at pH 7.2. Lane 4 represents the same experiment (pH 7.2) carried out with TrpR A77S, E49C-OP.

mercaptopropionic acid (MPA) and cupric ion within the acrylamide matrix containing the retarded band, the cleavage sites within the two separable protein-DNA complexes can be determined.

The Trp E49C-OP in the faster moving gel-shifted band must be bound at the subsite with the highest affinity for the repressor. The scission pattern indicates that in the 1:1 TrpR E49C-OP the protein binds preferentially to the central site (Figure 4c, lane 1). High-affinity binding of the protein at this central site is consistent with the co-crystal structure previously solved by Sigler and colleagues (Otwinski et al., 1988). In these studies, the protein was positioned at the principal dyad (*) of the palindromic operator sequence:

5'-GTACTAGTT *AACTAGTAC-3'
3'-CTTGATCAA *TTGATCATG-5'

which differs from the naturally occurring sequence only at the highlighted position:

5'-GAATAGTT *AACTAGTAC-3'
3'-CTTGATCAA *TTGATCATG-5'

This conclusion had been challenged on the basis of mobility-shift assays which asserted that the principal binding sites of the dimeric TrpR should be centered at positions indicated (\(\wedge\)) below (Staacke et al., 1990):

5'-GTACT\(\wedge\)AGTT AACT\(\wedge\)AGTAC-3'
3'-CATGA\(\wedge\)TCAA TTGA\(\wedge\)TCATG-5'

No evidence is provided from the targeted scission data for this binding geometry.

The slower moving band in the gel mobility shift assay is formed when protein is in excess of DNA. Since it is composed of more than one TrpR per operator, the observed scission pattern is more complex (Sutton et al., 1993). In previous studies, we have demonstrated that three discrete foci of cleavage sites were observed when the gel retardation analysis
was carried out in the presence of excess protein (Sigman et al., 1993; Sutton et al., 1993; Pan et al., 1994b). These three envelopes of scission sites provide unambiguous evidence for the presence of these subsites within the Trp EDCBA operator but they do not necessarily argue that the slower complex has a 3:1 TrpR:trp EDCBA stoichiometry. If the binding of three TrpR was very cooperative, as would be required for the 1:1 gel-shifted band to convert directly to 3:1, then the three envelopes of scission should be observed at all protein concentrations because cleavage at any one of the three sites should lead to protein dissociation. On the other hand, if the slower moving retarded band is due to a mixture of TrpR bound at either sites B and C or A and B (Figure 4b) with a 2:1 stoichiometry, the cleavage patterns can be readily explained. At increased TrpR concentrations, all the operator fragments will be saturated with two TrpR dimers binding at sites B and C or A and B. Although the complex with TrpR at B and C will lead to cleavage at the central B site and the C site, the latter product will be unobservable since the product will not be labeled. Similarly, TrpR bound at A and B will cleave at sites A and B but only the product at A will be visible. This model of isoenergetic dimers explains the concentration dependence of the scission and accounts for the change in the relative ratio of scission products as a function of extent of reaction. The tendency of TrpR to bind to adjacent subsites in the operator is consistent with the propensity of TrpR dimers to interact and form tetramers, as has been demonstrated in solution through crosslinking studies (Martin et al., 1994). Moreover, the co-crystal structure of TrpR with operator half-sites reveals tandem binding of the repressor (Lawson and Carey, 1993).

Optimizing the scission yield with TrpR E49C-OP

Two methods for enhancing the rate of DNA scission are illustrated in cleavage reactions on the slower moving band (second shift) which was isolated from mobility shift assays (Figure 4a). Single-stranded DNA scission of the trpEDCBA operator obtained from the slower moving band (second shift) by E49C-OP within the acrylamide gel matrix (Figure 4c, lane 2) approaches 90%, the maximum yield of scission obtained under the conditions described in Figure 2. Improvement of the scission yield can be obtained by decreasing the pH of the phosphate buffer in which the cleavage reaction is carried out from 8.0 to 7.2. The scission yield increases from 80–90% to 98–99%, as indicated by the loss of the parent band. The virtually quantitative cleavage with the drop in pH can attributed to a decrease in $k_-$ since lower pHs stabilize the protein–DNA complex (Carey, 1988). The fact that quantitative scission is achieved also indicates that derivatization of the protein was complete and that the self-inactivating oxidative reaction governed by $k_3$ is not a major factor in limiting the scission yield. Additional evidence for a more robust cleavage reaction is provided by the shift of the product bands to the smallest scission product as would be expected if TrpR bound to the A site can cleave Trp EDCBA after the chimera has cleaved at site B.

The second method for improving scission (lane 4) is genetic and relies on increasing binding affinity of the repressor by mutation. This can be implemented using the double mutant E49C-OP, A77S instead of E49C-OP. The A77S mutant shows 2–3-fold tighter binding relative to the wild-type operator (Youderian et al., 1983; Bass et al., 1987, 1988; Pfau et al., 1994a,b). The A77S mutant apparently decreases $k_-$ and increases the stability of the protein–DNA complex. Cleavage of the slower moving band of the gel retardation assay of the A77S E49C-OP chimera binding to trp EDCBA indicates that the parent band completely disappears and that the yield of products corresponding to cleavage at A increases relative to that observed at B. These results indicate that measurable increases of scission can be achieved by (a) carrying out the reaction between pH 7.2 and 7.5 rather than pH 8.0, which had previously been used because of our prior studies with the Cro-OP chimeras, and (b) using high-affinity mutants such as A77S.

Cleavage of linearized plasmid DNA

The Trp A77S, E49C-OP sample used in the scission of trp EDCBA within the gel matrix was also used to cleave trp EDCBA within a linearized pUC vector in which the operator was cloned near an Eco RI restriction site. The quantitative cleavage of the operator within the gel matrix indicated that the repressor had been completely chemically modified by 5-iodoacetamido-1,10-phenanthroline. The kinetics of scission obtained in a 100 mM Tris (pH 7.5) binding buffer in the presence or absence of tryptophan are presented in Figure 5. If the reaction was carried out in phosphate buffer in solution, a higher background rate of scission was observed. Secondary restriction with Eco RI provides convenient molecular weight markers for the products of TrpR A77S, E49C-OP cleavage. Approximately 75% double-stranded scission by this chimera is observed after 120 min in this tryptophan-dependent reaction.

Tryptophan not only promotes specific scission but also lowers background scission. In its absence, background scission is observed after 30 min and progresses with time. This cleavage must reflect non-specific binding of the repressor at multiple sites on the plasmid. Tryptophan therefore not only increases the affinity of the repressor for the trp operator but also destabilizes the binding of the Trp repressor for ‘non-specific protein–DNA complexes’. The inhibition is not due to a substoichiometric amount of TrpR–OP because increasing the amount of protein to five times the concentration of operator binding site does not lead to the random cleavage observed in the absence of tryptophan.

A notable feature of the data summarized in Figure 5 is that...
scission reaches a maximum at ~30 min and little additional cleavage is observed after that time. A possible explanation for this residual uncleaved target plasmid DNA when cleavage is carried out in solution is that the OP moiety on unbound TrpR E49C-OP is rapidly damaged once the scission chemistry is activated while DNA-bound TrpR E49C-OP is not susceptible to self-inactivation and is able to cleave DNA. In the experiments which couple gel retardation with targeted scission, the only TrpR present is bound to DNA and therefore more efficient scission is observed.

Discussion
Several factors contribute to the efficient scission of trp operator sequences by Trp repressor--OP. The first design question to address in the engineering of these chimeric cleavage reagents is the sequence position at which the 1,10-phenanthroline should be tethered. A residue crucial for DNA recognition cannot be mutated, nor can the protein--DNA interaction be affected by the sterically demanding phenanthroline moiety. On the other hand, the copper chelated to the tethered phenanthroline must be able to approach the minor groove within 3-4 Å of the C=H of the deoxyribose and be relatively constrained to this orientation. Few amino acid residues satisfy these criteria and the optimal site must be established by experiment.

The attachment of OP to two sequence positions, 46 and 49, has now been investigated. Both these positions are adjacent to the oxidatively sensitive minor groove and seem to satisfy the imposed distance requirements. However, Trp D46C-OP cleaves DNA with a very different pattern than TrpR E49C-OP. Multiple sites of scission are observed rather than the two distinct cleavage sites characteristic of TrpR E49C-OP. Although this pronounced difference in the cutting properties would have been hard to predict, molecular modeling does reveal that the OP of D46C-OP can align itself with the minor groove whereas the OP in the E49C-OP construct is more rigidly restricted. The cleavage pattern of the Trp D46C-OP is reminiscent of the pattern obtained with Cro A66C-OP in which the OP is linked to the C-terminal amino acid residue (Bruce et al., 1991). These studies showed that Cro A66C-OP cleaves the O3 site at the sequence positions protected from scission by (OP)2Cu+ footprinting. However, within the 17 bp O3 site, Cro A66C-OP cut DNA at a low yield in a pattern virtually identical with that of free (OP)2Cu+. These results indicate that the C-terminal arm of Cro is highly flexible, allowing the two Cro-linked OPs (one on each monomer) to contact and cleave all 17 nucleotides within the O3 site. The structure of the Cro-O3 complex, which has been solved at 3.9 Å resolution (Brennan et al., 1986, 1990), is consistent with the localization and flexibility (as judged by comparison of alternative Cro-O3 structures) of the carboxyl terminus suggested by the scission results.

The highly efficient scission of TrpR A77S, E49C-OP indicates that the development of a family of 12--16 bp DNA cleavers based on the 3D structure of the Trp repressor is feasible. This projected application requires that the cleavage of the chimeras reflect the new specificities and properties of altered binding mutants. Two results have indicated that this criterion is fulfilled. In a previous study, we have demonstrated that Trp repressor I79K, E49C-OP cleaved DNA with the specificity which had been established for the I79K mutant (Pfau et al., 1994b). In this communication, we demonstrate that the high-affinity mutant E49C-OP, A77S binds to the trp operator tighter and as a result cleaves DNA more efficiently than the wild-type protein. These findings suggest that both a cysteine at 49 and a serine at 77 should be incorporated in all chimeric cleavage reagents based on TrpR. The retention of cysteine at 49 is desirable because OP attached at this position in the dimers is optimally oriented for double-stranded scission. The A77S mutation is useful because it increases the affinity of the TrpR--DNA interaction.

The high yield of scission found for TrpR can be attributed to the stability of the protein--DNA complex (Kd = 10-9 M), the shielding of OP from solution and its rigid orientation relative to the minor groove of the DNA. Clearly, the rate of productive scission can be enhanced by carrying out the cleavage reaction at lower pHs where the DNA--protein interaction is more stable. One direction for possibly further increasing the scission yield may be through modification of the phenanthroline moiety.

The corepressor L-tryptophan markedly alters the cleavage specificity of TrpR A77S, E49C-OP. In its absence, the chimera randomly causes double-stranded breaks in plasmid DNA containing a high-affinity trp operator sequence. In its presence, efficient scission of the plasmid is observed and background cutting is suppressed. These results indicate that the apoprotein associates randomly to double-stranded DNA at protein concentrations at which it binds to its specific operator sequences in the presence of tryptophan. Therefore, L-tryptophan binding to the apoprotein has two effects: first, it increases the affinity of TrpR for its operator sequence; and second, it dramatically decreases the affinity of the holoprotein relative to the apoprotein for non-specific DNA.

This effect of tryptophan on scission by TrpR A77S, E49C-OP is of interest in the light of the structural studies on TrpR. Analysis of the apo- and holorepressor by crystallography and NMR (Zhang et al., 1987; Zhao et al., 1993) shows a decrease in disorder within the DNA reading heads upon binding of L-tryptophan This ligand-induced conformational change of the protein has two effects. On the one hand, the reading heads are likely to have a better match towards operator sequences while maintaining enough flexibility to adjust to a family of related sites (Lawson and Sigler, 1998). On the other hand, the loss of flexibility is likely to reduce its ability to bind non-specific DNA (Lavoie and Carey, 1994). In the plasmid test system (Figure 5), low-affinity, non-specific and tryptophan-independent binding as well as scission are probably driven by the high concentration of non-specific sites compared with the operator sites. When bound non-specifically, neither the reading heads nor the OP will be rigidly positioned. The non-specific scission pattern indicates that Trp R will be randomly bound to DNA at low tryptophan concentrations in the cell and not freely soluble in the cytoplasm.

In conclusion, the Trp R E49C-OP and A77S, E49C-OP and 179K, E-49C-OP chimeras have proven to be highly efficient, specific double-stranded scission reagents. These results indicate that the Trp repressor structure should serve as a convenient starting point for the design of a family of DNA scission reagents with a recognition sequence of about 15 bp. Screening methods are being explored which may permit the isolation of repressor mutants with binding specificities that differ substantially from the wild-type protein.

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