Transforming the *Escherichia coli* Trp Repressor into a Site-Specific Nuclease†

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ABSTRACT: The *Escherichia coli* Trp repressor has been converted into an operator-specific nuclease by alkylating cysteine-49, inserted by site-directed mutagenesis, with 5-(iodoacetamido)-1,10-phenanthroline. In the presence of copper ion and thiol, high yields (>50%) of double-stranded breaks of DNA are observed after a 20-min reaction. The high cleavage efficiency of this derivatized protein (Trp repressor E49C-OP) can be attributed to the proximity of cysteine-49 to the minor groove, the site of the C-1H of the deoxyribose and the target of the oxidative nuclease activity of (1,10-phenanthroline)copper. Since sequence position 49 is close to the protein's C_2 dyad axis and adjacent to the minor groove, Trp repressor E49C-OP reacts with the operator DNA near the binding site of this symmetry locus of the protein. The patterns of scission of the *trpR*, *aroH*, and *trpEDCBA* operators (a) confirm the orientation of the repressor to the operator predicted from the X-ray study of a cocrystal (Otwinowski et al., 1988) and (b) support the model for tandem binding of the repressor to the *trpR*, *aroH*, and *trpEDCBA* operators based on DNase I footprinting and methylation interference (Kumamoto et al., 1987). There are one, two, and three binding sites for the repressor on the *trpR*, *aroH*, and *trpEDCBA* operators, respectively. In addition to providing a novel approach to studying the interactions of DNA binding proteins, 1,10-phenanthroline-derivatized proteins such as Trp repressor E49C-OP may be useful as rare cutters in the analysis of high molecular weight DNAs, especially if their binding specificities can be altered.

Site-specific DNA scission reagents can be prepared by targeting the reactivity of a chemical nuclease using a ligand with high affinity for a characteristic feature of DNA structure (Sigman, 1986, 1990; Dervan, 1986; Francois et al., 1989; Sigman & Chen, 1990; Ebright et al., 1990; Bruice et al., 1991). The efficiency and specificity of these chimeric catalysts are a function of the binding properties of the carrier ligand, site of attachment, reactivity, and underlying chemistry of the cleavage reagent. In addition to generating nucleases of novel specificities, these new molecules can be used to probe the binding of the carrier ligands.

In this paper, we report an efficient site-specific nuclease based on the Trp repressor whose design has been guided both by the structure of the *Escherichia coli* Trp repressor (Schevitz et al., 1985; Otwinowski et al., 1988) and by the reaction mechanism of the chemical nuclease activity of (1,10-phenanthroline)copper (Goyne & Sigman, 1987). Glutamate-49 has been converted into a cysteine residue and then modified with 5-(iodoacetamido)-1,10-phenanthroline, generating the new scission reagent Trp repressor E49C-OP. Glu-49 is near the C_2 dyad axis of the wild-type protein and close to the protein dimer interface (Schevitz et al., 1985; Otwinowski et al., 1988). In addition, it is held rigidly near the minor groove, which contains the C-1H of the deoxyribose, the target of the oxidative nuclease activity of (1,10-phenanthroline)copper (Goyne & Sigman, 1987; Kuwabara et al., 1986). The length of the DNA recognition site of this engineered protein exceeds that of naturally occurring restriction enzymes because it makes double-stranded breaks within a 17-bp recognition sequence. Its catalytic activity addresses two controversial issues in the mechanism of Trp repressor binding to its regulated operators: (a) the biological relevance of the cocrystal structure of the Trp repressor with a truncated version of the *trpEDCBA* operator (Staacke et al., 1990; Carey et al., 1991) and (b) the validity of the tandem binding model of Trp repressor to the *trpR*, *aroH*, and *trpEDCBA* operators of *E. coli* (Kumamoto et al., 1987).

EXPERIMENTAL PROCEDURES

Materials. 5-(Iodoacetamido)-1,10-phenanthroline was prepared as previously described (Sigman et al., 1991). The following reagents were obtained commercially: 1,10-phenanthroline and 2,9-dimethyl-1,10-phenanthroline (GFS, Columbus, OH), 3-mercaptopropionic acid (Aldrich), cupric sulfate pentahydrate, and Tris (Sigma).

Restriction fragments containing the *aroH* and *trpEDCBA* operators were prepared as previously described (Chen & Sigman, 1987). The *trpR* operator was prepared by digesting pRPG5 with *Sau*3A (Kumamoto et al., 1987). The 187-bp fragment was isolated following purification by gel electrophoresis and then either 3′-labeled with [α-^{32}P]dGTP or 5′-labeled with calf intestinal phosphatase, T4 polynucleotide kinase, and [γ-^{32}P]ATP. A secondary digest with *Sal*I followed by gel electrophoresis yielded the uniquely 3′- or 5′-labeled fragment.

Site-Directed Mutagenesis. The gene for the wild-type Trp repressor protein sequence was excised from pRPG47 (Arvidson et al., 1987) and placed into M13mp18 for mutagenesis. Oligonucleotide-directed mutagenesis was performed as described by Kunkel et al. (1985). The gene for the mutant protein (Trp repressor E49C) was then returned to its original vector where its sequence and orientation were verified.

Preparation of Trp Repressor E49C-OP. Cells transformed with the plasmid containing the gene for the Trp repressor E49C mutant were pelleted, resuspended in 1 mL of buffer containing 25 mM Tris, pH 8, 10 mM EDTA, 50 mM glucose, and 0.1 mM β-mercaptoethanol, and sonicated. Cell debris

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was removed by centrifugation, and the supernatant was subjected to streptomycin sulfate at a final concentration of 0.5% for 10 min at 85 °C and recentrifuged. Then, 100 μL of supernatant was diluted to 1 mL with the above resuspension buffer lacking thiol. Twenty microliters of 50 mM 5-(iodoacetamido)-1,10-phenanthroline was added and the reaction mixture incubated at 0 °C. After 1 h, 10 μL of 3-mercaptopropionic acid was added for an additional 30 min to inactivate any residual 5-(iodoacetamido)-1,10-phenanthroline. Aliquots of the derivatized protein were made 10% in glycerol and stored at −80 °C.

Gel Retardation. For gel retardation, a 10% nondenaturing polyacrylamide gel described by Carey (1988) was used. For each binding reaction, 50 000–100 000 cpm of operator fragment was dissolved in a buffer containing 100 mM Tris, pH 7.5, 200 mM KCl, 6 mM $MgCl_2$, 11 mM L-tryptophan, and 0.1 mM poly(dI-dC) in a final volume of 10 μL. Trp repressor (200 nM) was added and the resulting mixture incubated for 10 min at 25 °C. Five microliters of 40% sucrose/0.01% bromophenol blue was added. The entire reaction volume was then loaded on the polyacrylamide gel. The gel was run at 300 V for 5 min and 200 V for 4 h with recirculation of the 10 mM NaH_2PO_4, pH 6/0.5 mM L-Trp buffer; the gel was then exposed to film, and the protein–DNA complexes were localized. The gel matrix containing the complexes was excised with a razor blade.

Scission Reaction. Most reactions (e.g., results of Figures 2 and 3) were carried out on Trp repressor–DNA complexes within the gel matrix. Gel slices containing the DNA–protein complex were incubated in 200 μL of a 2× scission buffer containing 20 mM NaH_2PO_4, pH 8, 1 mM L-Trp, and 10 μM $CuSO_4$ for 15 min at 25 °C. Both H_2O_2 and 3-mercaptopropionic acid (3 mM each) were then added, and the reaction mixture was incubated for 10 min at 25 °C. To quench the reaction, 2,9-dimethyl-OP was added to a final concentration of 2 mM. The same reaction conditions were used when the scission chemistry was carried out in solution with restriction fragments containing the three operators. To carry out the scission of a linearized plasmid, the reaction mixture was incubated for 60 min.

Analysis of Products. For reactions carried out within the acrylamide matrix, the gel slice was crushed into very small pieces and soaked in 330 μL of PAGE elution buffer (1 M ammonium acetate, pH 7, 1 mM EDTA) in a 37 °C heat block overnight in order to elute the reaction products from the gel slice. The following day, the eluted products were ethanol precipitated, washed with 70% ethanol, and dried in a Speed-vac.

For the analysis of single-stranded nicks, the products were dissolved in 15 μL of a denaturing loading buffer (80% deionized formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, and 1 mM EDTA). The reaction products were electrophoresed on a 10% denaturing gel (10% polyacrylamide, 1× TBE, and 7 M urea). The gel was run at 55 W for 2 h after loading. Autoradiography was performed overnight at −80 °C with an intensifying screen.

For the analysis of the double-stranded breaks, the products were dissolved in 15 μL of 40% sucrose and 0.1% bromophenol blue and electrophoresed on an 8% nondenaturing polyacrylamide gel. The molecular weights were calibrated using ^{32}P-labeled molecular weight standards prepared by a *Hae*III digest of pBR322 (Boehringer-Mannheim).

RESULTS

Sequence position 49 was chosen as the site of attachment of the 1,10-phenanthroline because it was adjacent to the minor groove, the target of the nuclease activity of (1,10-phenanthroline)copper. In order to serve as an accurate reporter of interactions of the wild-type Trp repressor, Trp repressor E49C-OP must bind with similar efficiency and stoichiometry as the wild-type protein. Since Glu-49 is remote from the helix–turn–helix DNA recognition motif of the Trp repressor, alkylation with the bulky 5-(iodoacetamido)-1,10-phenanthroline moiety should have minimal effect on the protein's high-affinity DNA binding. Retention of DNA binding activity was confirmed by gel retardation assays (Carey, 1988) and in situ footprinting of the retarded band with (1,10-phenanthroline)copper (OP-Cu) (Kuwabara & Sigman, 1987). Gel retardation assays (Figure 1A) demonstrated that the partially purified mutant Trp repressor (E49C) that had been alkylated with 5-(iodoacetamido)-1,10-phenanthroline binds to either the *trpEDCBA* or *aroH* operators. Furthermore, the OP-Cu footprint of the retarded complex of Trp repressor E49C-OP was very similar to that of the wild-type repressor (e.g., lane 3, Figure 1B).

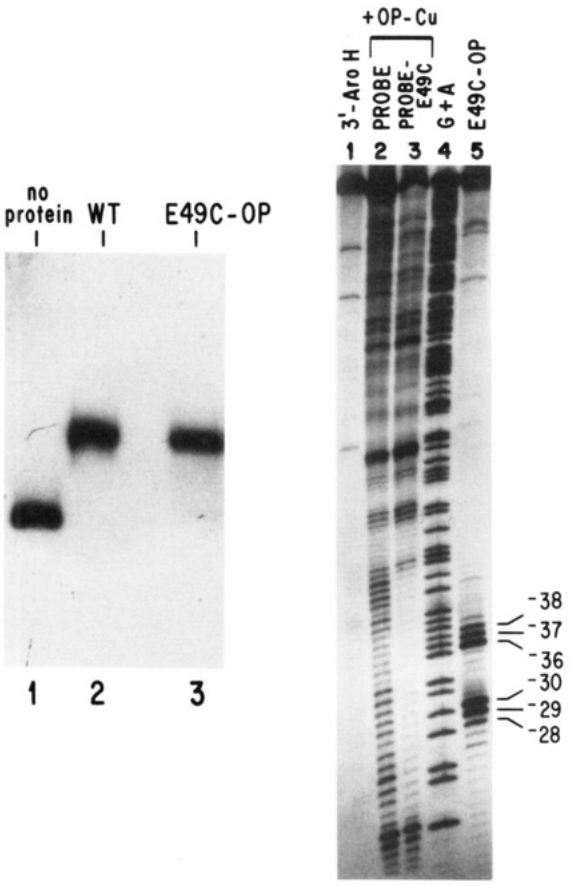


FIGURE 1: (A, left) Gel retardation of wild-type and E49C-OP mutant Trp repressor bound to the *trpEDCBA* operator (5′-labeled). Gel retardation was performed as described by Carey (1988). All lanes contained the restriction fragment of the *trpEDCBA* operator. The template strand is 5′-labeled. Lanes: (1) probe alone; (2) complex with wild-type Trp; (3) complex with Trp repressor E49C-OP. (B, right) Binding of Trp repressor E49C-OP to the *aroH* operator (3′-labeled on the nontemplate strand). All scission chemistry was carried out within the gel matrix. Lanes: (1) nontemplate strand of the *aroH* operator (3′-labeled); (2 and 3) OP-Cu scission of the operator fragment (lane 2) or operator fragment bound to the E49C mutant Trp repressor (lane 3) (the footprint generated is from the region −21 to −47); (4) Maxam–Gilbert G+A chemical sequencing lane; (5) scission of the *aroH* operator by bound E49C-OP Trp repressor.

Most scission reactions were carried out within the gel matrix on DNA–protein complexes isolated by gel retardation assays under nondenaturing conditions (Bruice et al., 1990, 1991). In addition to ensuring that the cleavage reaction is being carried out on an electrophoretically homogeneous species,

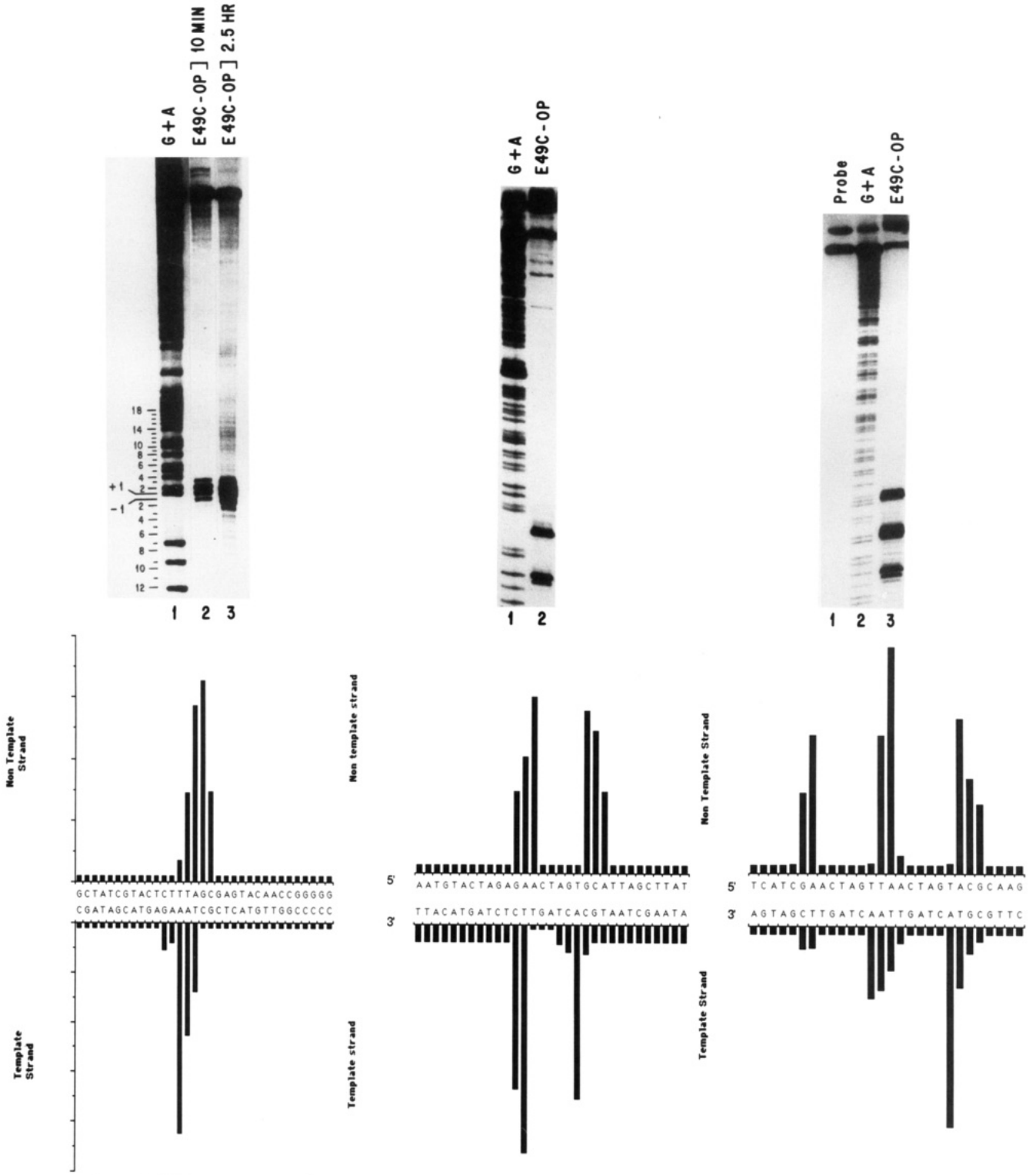


FIGURE 2: (A, top left) Scission pattern of the nontemplate strand of *trpR* within the gel matrix. (B, bottom left) Histogram showing cleavage of template (lower) and nontemplate (upper) strands of the *trpR* operator by the Trp repressor E49C-OP. (C, top middle) Scission pattern of the template strand of *aroH* within the gel matrix (the scission pattern of the nontemplate strand is presented in Figure 1). (D, bottom middle) Histogram showing cleavage of template (lower) and nontemplate (upper) strands of the *aroH* operator by the Trp repressor E49C-OP. (E, top right) Scission pattern of the nontemplate strand of *trpEDCBA* within the gel matrix. (F, bottom right) Histogram showing cleavage of template (lower) and nontemplate (upper) strands of the *trpEDCBA* operator by the Trp repressor E49C-OP.

the separation accomplished by the gel retardation also purifies the complex from other proteins in the bacterial extract, excess Trp repressor E49C-OP, and any residual 5-(iodoacetamido)-1,10-phenanthroline. To activate scission, the slice of polyacrylamide containing the Trp reprssor E49C-OP–operator complex was submerged in a solution containing cupric ion, 3-mercaptopropionic acid, and hydrogen peroxide (Bruice et al., 1991). L-Tryptophan is also included in the scission mixture to maintain high-affinity, site-specific binding. As is apparent from lane 5, Figure 1B, scission is restricted to the binding site defined by OP-Cu footprinting.

Kinetics of Scission. The observed first-order rate constant for the appearance of all single-stranded breaks (i.e., the sum of the first-order rate constants at all scission sites) was approximately 0.003 s^{-1} at 25 °C in the presence of 3 mM thiol and 3 mM hydrogen peroxide for the *aroH* operator. This apparent first-order rate constant was approximated by determining the ratio of products to starting material at a

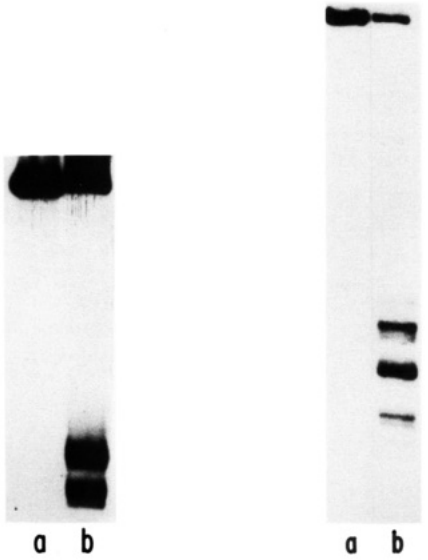


FIGURE 3: Double-stranded scission of the *aroH* and *trpEDCBA* operators by the Trp repressor E49C-OP. (A, left) Separation of the products of double-stranded scission of *aroH* labeled at the 3′- end of the nontemplate strand using an 8% nondenaturing polyacrylamide gel. Lanes: (a) probe alone; (b) scission products. (B, right) Separation of the products of double-stranded scission of the *trpEDCBA* operator labeled at the 3′-end of the nontemplate strand using an 8% nondenaturing polyacrylamide gel. Lanes: (a) probe alone; (b) scission products.

single, fixed time point. Assuming a dissociation constant of 5×10^{-10} M for the Trp repressor–operator complex (Carey, 1988), the estimated second-order rate constant for scission (i.e., 0.003 $s^{-1}/5 \times 10^{-10}$ M) is 6×10^6 M^{-1} s^{-1}. Decreasing the concentrations of thiol, hydrogen peroxide, and cupric ion simultaneously by a factor of 10 slows the reaction rate by only 10%. Extensive site-specific scission of the recognition sequences is observed in a 12-h incubation even in the absence of added thiol and hydrogen peroxide. Since the reaction is blocked by catalase, this scission chemistry must be dependent on hydrogen peroxide with reducing equivalents provided by the organic matrix of the acrylamide gel.

Single-stranded nicks and double-stranded breaks are observed with comparable efficiency if the reaction is carried out in solution in the presence of added 3-mercaptopropionic acid and hydrogen peroxide. Unlike the scission reaction in the gel slice where reducing equivalents can be provided by the acrylamide gel matrix, no reaction is observed in solution in the absence of these two components.

Patterns of Single-Stranded and Double-Stranded Breaks. Products can be analyzed either as single-stranded nicks or double-stranded breaks. When single-stranded nicks are assayed on sequencing gels, a single pocket of scission is apparent for *trpR* on both strands (Figure 2A,B), two pockets of scission are observed for the *aroH* operator (Figure 2C,D), and three pockets of scission are observed for the *trpEDCBA* operator (Figure 2E,F). When double-stranded breaks are assayed under nondenaturing conditions using DNA labeled at only a single end, two fragments were detected for *aroH* (Figure 3A) and three were detected for *trpEDCBA* (Figure 3B). The first-order rate constant for production of all double-stranded scission products of *aroH* is 4×10^{-2} min^{-1} in a reaction mixture containing 3 mM MPA and 3 mM hydrogen peroxide. Verification of double-stranded breaks is provided by calibration with a labeled *Hae*III digest of pBR322 and the bisection of a linearized plasmid containing an *aroH* insert. The rapid production of double-stranded breaks could result from the focused reactivity of 1,10-phenanthroline of each subunit near the dyad axis. The robust double-stranded scission catalyzed by Trp repressor E49C-OP is remarkable in comparison to DNA scission by other chemical nucleases (Sigman & Chen, 1990; Sigman, 1990).

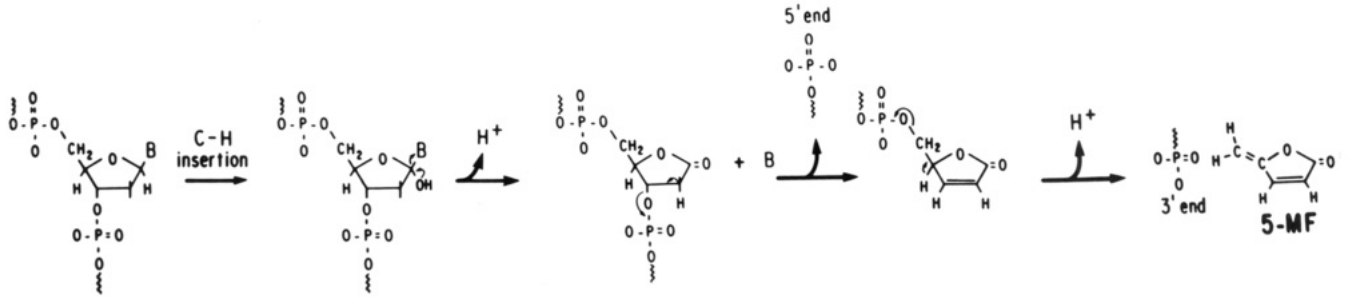
Chemical Structure of Termini. Analysis of the chemical structure of the termini is consistent with the occurrence of oxidative scission of the phosphodiester backbone under all reaction conditions. Previous studies with the nuclease activity of (1,10-phenanthroline)copper have indicated that the primary reaction pathway involves oxidative attack at the C-1 hydrogen of the deoxyribose followed by the production of 5′- and 3′-termini by a series of elimination reactions (Scheme I). Because of the restricted number of termini produced by Trp repressor E49C-OP, it is very challenging to demonstrate the production of 5-methylenefuranone. However, it is possible to examine the chemical structure of the termini by comparing the migration of the products generated to those produced by DNase I (5′-phosphates and 3′-hydroxyl groups) (Laskowski, 1971), micrococcal nuclease (5′-hydroxyl and 3′-phosphates) (Anfinsen et al., 1971), and Maxam–Gilbert chemistry (Maxam & Gilbert, 1980). In Figure 4, 3′-labeled DNA has been used to investigate the chemical nature of the 5′-ends. The products derived from Trp repressor E49C-OP comigrate with those generated by Maxam–Gilbert cleavage and DNase I digestion but do not migrate equivalently with those produced by micrococcal nuclease. By these criteria, the oxidative nuclease of Trp repressor E49C-OP produced 5′-phosphorylated termini (e.g., Figure 4). Comigration of the scission products of 5′-labeled DNA by Trp repressor E49C-OP with those of Maxam–Gilbert scission chemistry indicates that 3′-phosphate termini are formed. These findings are consistent with, but not proof of, the chemistry outlined in Scheme I.

DISCUSSION

The procedure used to convert the *E. coli* Trp repressor into an operator-specific nuclease is general and in principle can be readily adapted to many DNA binding proteins or peptides. First, a sequence position is identified, which is accessible to the minor groove but does not contribute to the binding affinity or specificity. Ideally, this choice is guided by a high-resolution structure. Then, this position is converted into a cysteine residue either by chemical synthesis or site-directed mutagenesis. This introduced cysteine residue is then alkylated by 5-(iodoacetamido)-1,10-phenanthroline. The nuclease activity of (1,10-phenanthroline)copper is useful in generating chimeric catalysts because of its minor groove reactivity. Since many DNA binding proteins require major groove contacts for recognition (Brennan & Matthews, 1989b), a chemical nuclease reacting in the minor groove will neither interfere with binding of the protein nor be blocked from its primary site of reaction.

The λ phage cro protein has previously been converted into a nuclease by the same methodology (Bruice et al., 1990, 1991). In the case of the cro protein, the cysteine was inserted at the C-terminus, which is accessible to the minor groove but a very flexible part of the structure (Brennan & Matthews, 1989a; Hubbard et al., 1990; Brennan et al., 1990). In contrast, the structural model of the Trp repressor indicates that sequence position 49 is adjacent to the minor groove and is held rigidly near the protein's dyad axis (Otwinowski et al., 1988). This preorganization of Trp repressor E49C-OP most likely accounts for its impressive efficiency and in turn provides chemical evidence for central features of the structural model. In one case of the successful transformation of a DNA binding protein into a site-specific nuclease, a unique cysteine of the *E. coli* catabolite gene activator protein (CAP) within the helix–turn–helix domain was alkylated by 5-(iodoacetamido)-

Scheme I



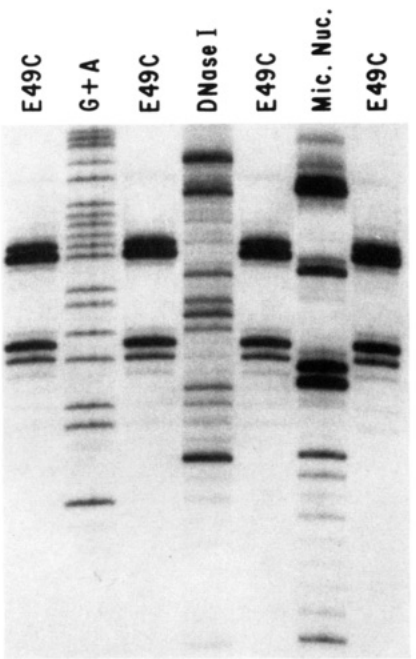


FIGURE 4: Analysis of the 5′-termini using 3′-labeled *aroH*. Denaturing polyacrylamide gel (20%) comparing the relative migrations of oxidative reaction products from the nontemplate strand (3′-labeled) of the *aroH* operator with chemical and enzymatic reaction products. Information about the 5′-end is deduced. (Left to right) Lanes 1, 3, 5, and 7 show the reaction product from the oxidative scission with the E49C mutant derivatived with OP. Lane 2 shows a Maxam–Gilbert G+A sequencing lane with 5′-phosphomonoesters. Lane 4 shows a DNase I digestion with 5′-phosphomonoesters. Lane 6 shows a micrococcal nuclease digestion which produces 5′-hydroxyl ends.

1,10-phenanthroline (Ebright et al., 1990). The resulting chimeric protein in which the chemical nuclease was held rigidly near the minor groove was also an efficient and specific nuclease although the modification reaction decreased the binding of the chimeric protein 3 orders of magnitude relative to the wild-type protein.

The mechanism of Trp repressor binding to its operators embodies many central questions relevant to protein–DNA interactions. The structure of the Trp repressor/*trpEDCBA* cocrystal has stimulated debate because it suggests that the DNA binding protein can recognize DNA by sensing subtle differences in the geometry of the nucleotide sequence (indirect readout) rather than from specific amino acid–base interactions (direct readout) (Otwinowski et al., 1988). The structure of the cocrystal has indicated that the protein binds to DNA primarily by making hydrogen bonds to the phosphodiester backbone directly or mediated through hydrogen bonds to water.

The conclusions of the cocrystal study have been challenged by Staacke et al. (1990), who claim that the deoxyoligonucleotide (I) used in those studies does not bind the Trp repressor with high affinity even though it was derived from the

TGTACTAGTTAACTAGTAC
CATGATCAATTGATCATGT
(I)

trpEDCBA operator by making a neutral A-T to T-A transversion (Bass et al., 1987). These authors suggest that the conditions of crystallization may have led to the formation of a stable, nonspecific complex. They further claim that, under physiological conditions, the protein would not be centered at the dyad axis of the recognition sequence but would instead be centered at a pseudodyad axis of the GTAC sequence. Consistent with this binding mode, they report that the deoxyoligonucleotide (II) is tightly bound by the repressor.

TAGCGTACTAGTACGCTA
ATCGCATGATCATGCGAT
(II)

The contention that I is not bound tightly by the Trp repressor has been contradicted by Marmorstein et al. (1991), using a novel assay of protein–nucleic acid involving alkaline phosphatase, and by Carey and co-workers (1991), using a gel retardation assay. These two groups have shown convincingly that the deoxyoligonucleotide used in the crystallographic study is tightly complexed by the protein. Brennan and Matthews (1989a) have suggested that although I does contain a high-affinity binding site for the repressor, the DNA–protein complex is nonspecific.

The present study cannot address the validity of the "indirect" or "direct" mechanism of readout of DNA sequence information by the Trp repressor. However, since amino acid 49 is adjacent to the dyad axis of the protein structure, it provides a novel approach to characterize the high-affinity binding sites of the Trp repressor on the *trpR*, *aroH*, and *trpEDCBA* operators in dilute solution. Our results are consistent with the cocrystal structure because the major site of scission of *trpEDCBA* is at the principal dyad axis predicted by the crystallographic study. In the scission of this operator, as well as *aroH* and *trpR*, scission is *never* observed at the pseudodyad axis of the GTAC as proposed by Staacke et al. (1990). We conclude that the positioning of the Trp repressor on *trpEDCBA* as determined by X-ray crystallography is biologically relevant and not an artifact of the crystallization conditions.

However, the scission patterns by Trp repressor E49C-OP also indicate that the Trp repressor can bind at one site in the *trpR* operator, at two sites in the *aroH* operator, and at three sites in the *trpEDCBA* locus. This conclusion has been independently reached by Gunsalus and colleagues using DNase I and dimethyl sulfate footprinting (Kumamoto et al., 1987). On the basis of their experiments, they proposed the binding models summarized in Figure 5 for the three operators studied here. The guanines blocked from alkylation by dimethyl sulfate, the sequence positions protected from scission by DNase I and/or OP-Cu footprinting, and the scission sites

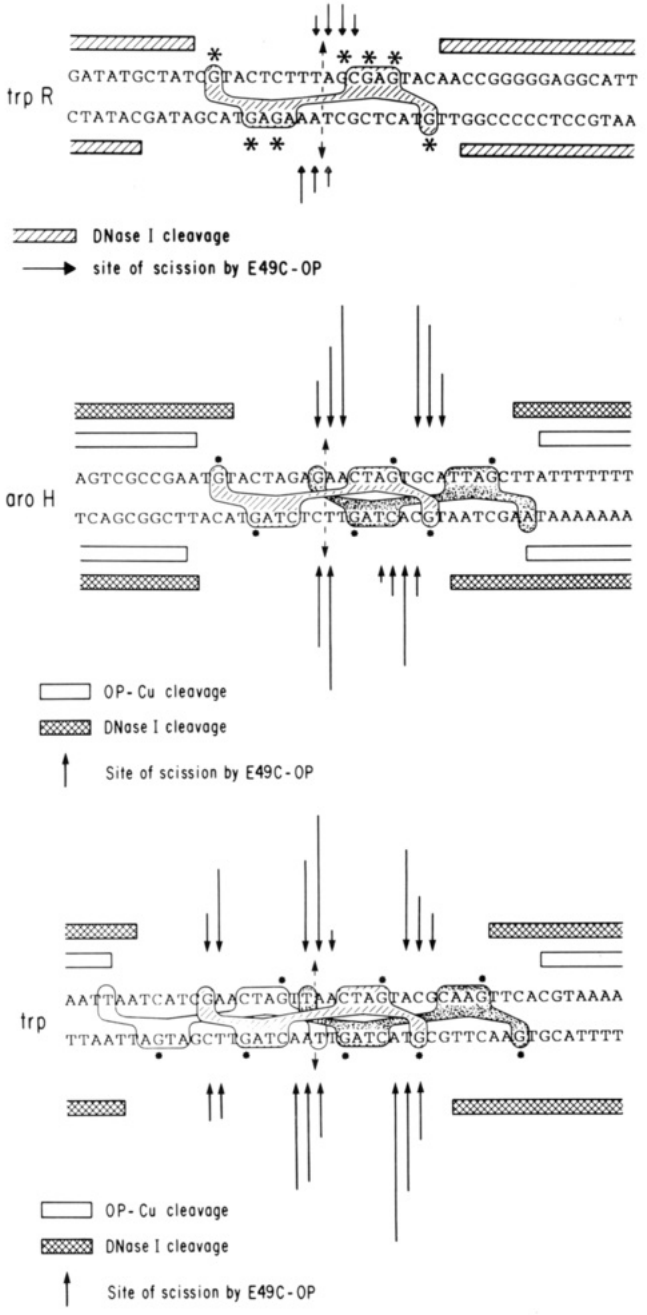


FIGURE 5: Models of binding of Trp repressor to its operator sequences (Kumamoto et al., 1987): (A, top) *trpR*, (B, middle) *aroH*, and (C, bottom) *trpEDCBA*.

of Trp repressor E49C-OP are all indicated. The nucleotides at the dyad axis are precisely those attacked with Trp repressor E49C-OP. These diverse experimental methodologies agree on the number and position of the binding sites for Trp repressor for the three operators. Variation of the Trp repressor E49C-OP concentration in the binding reaction prior to a gel retardation assay has never resulted in more than one detectable protein–DNA complex for any of the operators examined. Our results are consistent with the isomerization of the protein between isoenergetic binding sites within the 1:1 complex isolated in the gel retardation assays in the case of the *trpEDCBA* and *aroH* operators.

In summary, the insertion of a cysteine residue at a sequence position that is held rigidly adjacent to the minor groove in the case of Trp repressor E49C-OP generates a DNA cleavage agent of impressive efficiency. Analysis of the folding of the Trp repressor has shown that sequence position 49 is part of the structural core of the protein contributing to the stability of the dimers (Tasayco & Carey, 1991). Since the stability of the protein structure does not depend on the two helices responsible for protein recognition, it should be possible to generate a family of nucleases on the basis of the Trp repressor by altering the DNA reading helix–turn–helix domain while still retaining the reactivity characteristic of Trp repressor E49C-OP.

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