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Restriction Endonuclease *Eco*RI Alters the Enantiomeric Preference of Chiral Metallointercalators for DNA: An Illustration of a Protein-Induced DNA Conformational Change†

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ABSTRACT: A conformational change in the DNA plasmid $ColE_1$ appears to occur upon specific binding of the restriction endonuclease *Eco*RI. Enzyme association alters the chiral discrimination found in binding metallointercalators to DNA sites. The complexes tris(1,10-phenanthroline)ruthenium(II), $Ru(phen)_3^{2+}$, tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II), $Ru(DIP)_3^{2+}$, and tris(4,7-diphenyl-1,10-phenanthroline)cobalt(III), $Co(DIP)_3^{3+}$, in general, bind stereoselectively to DNA helices, with enantiomers possessing the Δ configuration bound preferentially by right-handed B-DNA. In the presence of *Eco*RI, however, this enantioselectivity is altered. The chiral intercalators, at micromolar concentrations, inhibit the reaction of *Eco*RI, but for each enantiomeric pair it is the Λ enantiomer, which binds only poorly to a B-DNA helix, that inhibits *Eco*RI preferentially. Kinetic studies in the presence of Λ-$Ru(DIP)_3^{2+}$ indicate that the enzyme inhibition occurs as a result of the Λ enantiomer binding to the enzyme–DNA complex as well as to the free enzyme. Furthermore, photolytic strand cleavage experiments using $Co(DIP)_3^{3+}$ indicate that the metal complex interacts directly at the protein-bound DNA site. Increasing concentrations of bound *Eco*RI stimulate photoactivated cleavage of the DNA helix by Λ-$Co(DIP)_3^{3+}$, until a protein concentration is reached where specific DNA recognition sites are saturated with enzyme. Thus, although Λ-$Co(DIP)_3^{3+}$ does not bind closely to the DNA in the absence of enzyme, specific binding of *Eco*RI appears to alter the DNA structure so as to permit the close association of the Λ isomer to the DNA helix. Mapping experiments demonstrate that this association leads to photocleavage of DNA by the cobalt complex at or very close to the *Eco*RI recognition site. This study provides evidence that in solution, under enzymatic conditions, a DNA-binding protein may distort the DNA helical structure and further illustrates how small molecular probes of DNA conformation might be used in examining the structure of protein-bound DNA sites.

Proteins that recognize specific DNA base sequences play a major role in the regulation of DNA expression, in DNA replication and transcription, and in repair processes. The structural basis for this macromolecular recognition is a subject of considerable interest. The crystal structures of several DNA binding proteins have been reported (Anderson et al., 1981, 1982; Frederick et al., 1984; McKay & Steitz, 1981; Pabo & Lewis, 1982; Steitz et al., 1982; Tanaka et al., 1984). On the basis of the complementarity of these structures to B-form DNA, also characterized crystallographically (Wing et al., 1980), models for protein–DNA recognition sites have been proposed. While several workers have suggested non-B conformations for DNA bound to proteins (Crick & Klug, 1975;

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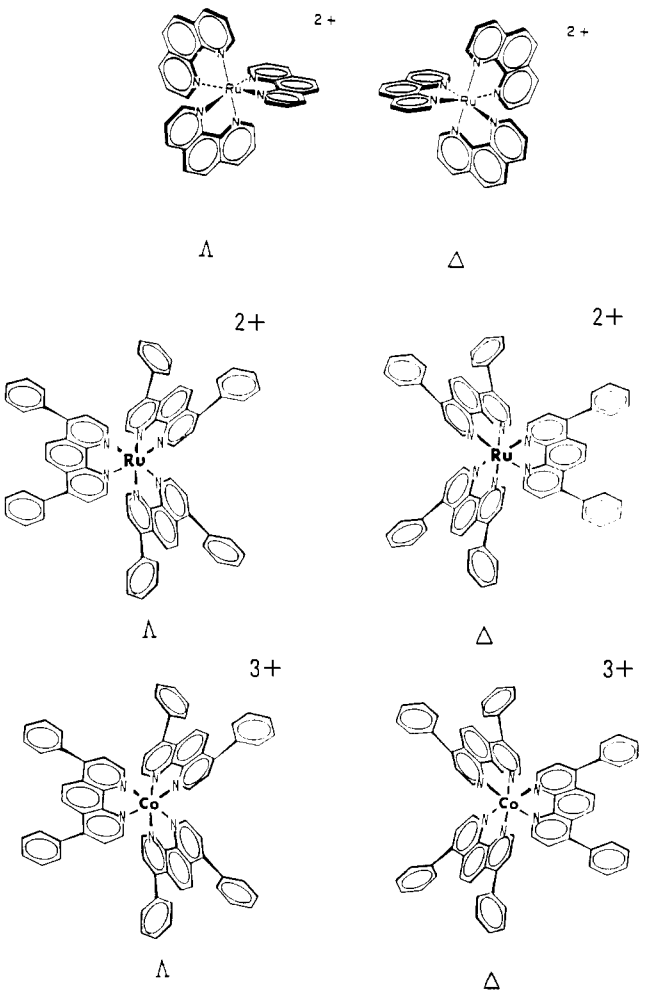

FIGURE 1: Chiral metallointercalators (top to bottom) $Ru(phen)_3^{2+}$, $Ru(DIP)_3^{2+}$, and $Co(DIP)_3^{3+}$.

Kim et al., 1984; Klug et al., 1979; McKay & Steitz, 1981), for the most part a necessary assumption in the model building has been that the B-DNA duplex conformation is largely unperturbed by protein binding (Sauer et al., 1982). More recent studies, however, indicate that DNA unwinding, bending, and kinks may be associated with protein binding (Frederick et al., 1984; Kim et al., 1984; Wu & Crothers, 1984). The structure of the restriction endonuclease *Eco*RI cocrystallized with its specific oligonucleotide reveals a torsional kink in the DNA as well as other departures from a B-form helix which cause DNA unwinding (Frederick et al., 1984).

We have been examining the interactions of chiral tris(phenanthroline)metal complexes with DNA (Barton et al., 1982, 1984b) and have developed spectroscopic probes (Barton et al., 1984a) and photoactivated cleaving agents (Barton & Raphael, 1984, 1985) that are specific for right- or left-handed DNA helical conformations. Figure 1 shows the structures of Λ and Δ isomers of phenanthroline complexes used in this study. Complexes possessing the Δ configuration may bind by intercalation easily to a right-handed helix with one ligand stacked between the base pairs and the two nonintercalating ligands disposed along the right-handed helical groove. The Λ isomer is precluded from similar intercalative binding to the right-handed helix owing to steric repulsions between the diphenylphenanthroline ligands and the DNA–phosphate backbone. Both isomers bind to left-handed Z-DNA, which contains a wide and very shallow major groove. In binding to right-handed B-DNA, stereoselectivity becomes evident even with tris(phenanthroline) complexes, but strong enantiospecificity is apparent for the bulkier diphenylphenanthroline (DIP)[1] complexes.

In this study we examine the utility of the chiral intercalators as probes for DNA structure in a protein-bound complex. Intercalating agents have been found to inhibit cleavage by the *Eco*RI restriction enzyme (Kania et al., 1976; Nath & Azzolina, 1981; Nosikov et al., 1976). *Eco*RI recognizes and binds to double-stranded DNA having the sequence GAATTC and in the presence of a divalent metal ion cofactor (e.g., Mg^{2+}) cleaves the DNA on both strands to give product (Greene et al., 1974; Jack et al., 1981; Modrich, 1979; Modrich & Zabel, 1976). Several base-specific and nonspecific DNA intercalators have been shown to alter the digestion patterns of restriction endonucleases, presumably as a result of interactions of the small molecule with the DNA restriction site or flanking sequences. Differential enzyme inhibition and binding by the chiral intercalators appeared to us to be an interesting approach to investigate any changes in DNA structure that could occur in binding *Eco*RI to its specific DNA substrate. We report here that *Eco*RI binding alters the enantiomeric preference of the conformation-specific intercalators $Ru(phen)_3^{2+}$, $Ru(DIP)_3^{2+}$, and $Co(DIP)_3^{3+}$ for DNA. The result provides evidence under solution conditions that binding by the restriction enzyme causes a conformational change in the DNA helix.

Experimental Procedures

Materials. The ruthenium complexes $[Ru(phen)_3]Cl_2$ and $[Ru(DIP)_3]Cl_2$ were prepared as described by Lin et al. (1976), and enantiomers were resolved and characterized as described elsewhere (Barton et al., 1984a,b; Barton & Nowick, 1984). The synthesis and resolution of $[Co(DIP)_3](tartrate)_3$ diastereomers was also conducted as previously described (Barton & Raphael, 1984). The enzyme *Eco*RI was prepared in large quantity and purified to homogeneity (Modrich & Zabel, 1976) by using the overproducing strain M5248/pSCC2 (Cheng, 1983) kindly provided to us by Dr. P. Modrich. The plasmid DNA $ColE_1$ (P-L Biochemicals and Sigma) and λ-phage (Bethesda Research Laboratory) DNA were dialyzed against assay buffer before use. For the cleavage experiments at 315 nm, DNA samples were first incubated with topoisomerase (BRL) in the presence of ethidium (Singleton & Wells, 1982) to produce closed-circular plasmids having a uniform superhelical density of −0.05.

Methods. All assays were performed in assay buffer (0.1 M Tris, pH 7.5, 0.05 M sodium chloride, 1% glycerol, 0.01% Triton) at 37 °C. For the inhibition studies, plasmid $ColE_1$ DNA (7 nM) was incubated with *Eco*RI (0.05–3 nM) in buffer containing either Δ or Λ isomers of $Ru(phen)_3^{2+}$, $Ru(DIP)_3^{2+}$, or $Co(DIP)_3^{3+}$ at various concentrations, and magnesium chloride was added to a concentration of 5 mM to commence the reaction. After 1–15 min, the reactions were quenched with the addition of EDTA (final concentration of 100 mM) and 0.025% bromophenol blue in 50% sucrose solution and then cooled to 0 °C. Reaction mixtures with high concentrations of metal complexes were quenched with ethanol in order to precipitate the DNA. DNA was recovered after centrifugation (4 °C, 8000 rpm, 7 min) and dissolved in assay buffer before the addition of dye. For kinetic studies DNA concentrations ranging from 3 (sites) to 12 nM were used at

[1] Abbreviations: DIP, diphenylphenanthroline; $Ru(DIP)_3^{2+}$, tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II); $Ru(phen)_3^{2+}$, tris(1,10-phenanthroline)ruthenium(II); $Co(DIP)_3^{3+}$, tris(4,7-diphenyl-1,10-phenanthroline)cobalt(III); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

different fixed values of Λ-$Ru(DIP)_3^{2+}$. The enzyme and the Mg^{2+} concentrations used were 0.1 nM and 5 mM, respectively. Incubations were conducted at 37 °C for 2 min. The reaction was quenched and the DNA isolated as mentioned before. Cleavage experiments with $Co(DIP)_3^{3+}$ were conducted similarly but without the addition of magnesium ion. For experiments conducted at 254 nm, the mixture was irradiated from above (10 cm) for 30 min at 37 °C by using a 4-W mercury lamp. When the cleavage reaction was performed using 315-nm light, the sample was irradiated for 1 min at room temperature in a horizontal position 50 cm away from a 1000-W mercury–xenon lamp. A parallel set of experiments were always performed without irradiation. After reaction, all samples were electrophoresed at 50–55 V for 6 h in electrophoresis buffer (0.05 M Tris, 0.02 M sodium acetate, pH 7.0, 0.18 M sodium chloride) through 1% agarose gels. For experiments where more than stoichiometric amounts of enzyme were used, SDS was added (final concentration ~1%) to the samples before electrophoresis to improve resolution by denaturing the enzyme. The gels were stained with ethidium bromide solution overnight and then photographed under UV light by using polaroid type 605 positive/negative film. Negatives were scanned at 500 nm by using the gel scanning accessory to the Varian Cary 219 spectrophotometer. Controls had previously established the range of linearity of negative exposures with sample concentrations. From the resulting densitometer peaks, the percentages of supercoiled form I DNA, nicked form II DNA, and linear form III DNA in each sample were determined. For kinetic studies, the weight of linear DNA formed was calculated by using the percentages. The initial velocity was taken as the concentration of linear DNA formed in 1 min.

In order to determine the site at which the metal complex $Co(DIP)_3^{3+}$ photolytically cleaves the DNA in the presence of *Eco*RI, the following procedure was used: Closed-circular plasmid $ColE_1$ DNA was first linearized by using the restriction enzyme, *Sma*I (BRL). Five units of enzyme was used per 1 μg of DNA in order to achieve complete linearization. The DNA was then isolated by phenol extraction and ethanol precipitation. During ethanol precipitation, 1 mM EDTA in assay buffer was used to dissolve the DNA and effectively remove the residual Mg^{2+} that may have been bound to DNA. Photoactivated cleavage of DNA was then accomplished with 7 nM DNA sites in assay buffer containing 800 μM EDTA, in the presence and absence of either Δ- or Λ-$Co(DIP)_3^{3+}$ (10 μM) and in the presence of each enantiomer and *Eco*RI (7 nM), in the absence of Mg^{2+}. The samples were then irradiated for 8 min at 315 nm as described previously. Appropriate controls were done to determine the level of photodamage in the presence and absence of enzyme alone and any nicking activity without light. The cleaved DNA was then isolated by ethanol precipitation and digested at single-stranded regions with S_1 nuclease as follows. The DNA in each sample was dissolved in 8 μL of electrophoresis buffer (pH 4.5), followed by the addition of $Zn(NO_3)_2$ solution to a final concentration of 5 mM and 1.6 units of S_1 nuclease. After incubation for 5 min at 37 °C, the samples were quenched with 100 mM EDTA and electrophoresed as described before. Positions of the resulting bands were determined by comparison with a molecular weight marker, the authentic *Eco*RI fragments obtained by first digesting $ColE_1$ DNA with *Sma*I followed by *Eco*RI.

Results and Discussion

Enzyme Inhibition by the Chiral Intercalators. The tris(phenanthroline)metal complexes, like other DNA-intercalating agents, inhibit the action of *Eco*RI. The metal complexes inhibit *Eco*RI cleavage of both supercoiled $ColE_1$ DNA (Figure 2A) and linear λ phage DNA (Figure 2B). Relative activities of the enzyme on the closed-circular substrate were determined through measurements of the formation of linear DNA product, since the plasmid $ColE_1$ contains a single *Eco*RI restriction site. The presence of the tris(phenanthroline)metal complexes in the incubation mixture reduces cleavage to the linear form and at surprisingly low concentrations of the metal. Figure 2 shows the gel electrophoretic patterns of both $pColE_1$ and linear λ phage DNA after digestion with *Eco*RI in the presence of micromolar concentrations of either Λ-$Ru(DIP)_3^{2+}$ or Δ-$Ru(DIP)_3^{2+}$. Both enantiomers inhibit the digestion of the DNA substrate, and in fact, the Λ enantiomer inhibits somewhat more. Moreover the complexes are quite potent inhibitors. Effects are evident with 3 μM ruthenium, and at a metal concentration of 10 μM, more than 50% inhibition of the enzyme is observed.

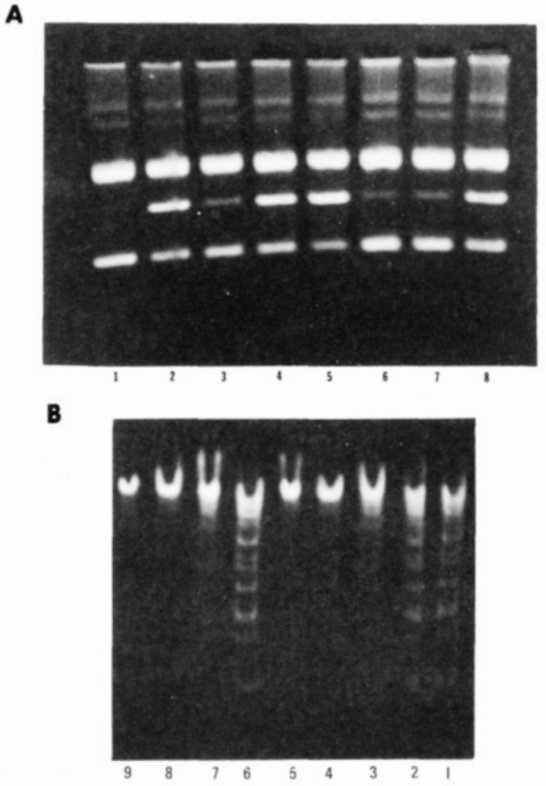

FIGURE 2: Inhibition of the *Eco*RI cleavage reaction by $Ru(DIP)_3^{2+}$ enantiomers. (A) Agarose gel electrophoresis of $ColE_1$ plasmid DNA before (lane 1) and after reaction with *Eco*RI in the absence of metal (lane 2) and in the presence of various concentrations (10, 5, and 3 μM from right to left) of Δ-$Ru(DIP)_3^{2+}$ (lanes 3–5) and Λ-$Ru(DIP)_3^{2+}$ (lanes 6–8). (B) Electrophoresis pattern of λ phage DNA before (lanes 4, 5, 8, and 9) and after partial digestion with *Eco*RI in the absence of metal (lane 1) and in the presence of 1 or 5 μM Δ-$Ru(DIP)_3^{2+}$ (lanes 2 and 3) and Λ-$Ru(DIP)_3^{2+}$ (lanes 6 and 7). Incubations were performed at 37 °C for 3 min wth 7 nM DNA and 0.9 nM enzyme in assay buffer with and without the intercalator.

The presence of the metal complex appears simply to reduce the rate of double-stranded cleavage by the enzyme rather than selectively modifying the activity in some fashion. As is seen in Figure 2A, there is no increased formation of singly cleaved form II DNA. Thus, the metal complexes do not promote a buildup and early release of a nicked intermediate. The metal complexes also do not affect the recognition site specificity or even the hierarchy of recognitiion sites on a given DNA strand. The digestion by *Eco*RI of λ phage DNA, which possesses five recognition sites with a gradient of cleavage efficiencies (Rubin & Modrich, 1980; Thomas & Davis, 1975), was examined

Table I: Inhibition of *Eco*RI Activity by Chiral Metallointercalators

complex	concn (μM)	% inhibition[a] Δ	Λ
$Ru(DIP)_3^{2+}$	10	48	77
	5	24	41
	1	0	18
$Ru(phen)_3^{2+}$	20	4	15
$Co(DIP)_3^{3+}$	15	61	43
	10	12	31
	6	6	12

[a] The percent inhibition is defined as the percent decrease in the ratio of form III $ColE_1$ DNA found in the presence of metal compared to that found in the absence of metal, [1 − (amount linear with metal)/(amount linear without metal)] × 100, after reaction with *Eco*RI for 3 min at 37 °C in assay buffer.

under conditions that yield partial fragmentation. Figure 2B shows this fragmentation pattern with *Eco*RI in the presence and absence of $Ru(DIP)_3^{2+}$ enantiomers. It is clear that increasing concentrations of metal complex enhance the inhibition, but no new bands of intermediate molecular weight resulting from a change in relative site specificity or secondary bands of low molecular weight due to a reduction in specificity are evident with either metal complex. Sequences outside the canonical recognition site then do not appear to be primarily involved in this inhibition. It is noteworthy here as well that the inhibition does not require the topological constraints of a supercoil. Here inhibition is seen with both enantiomers on a linear DNA substrate.

The DNA cleavage inhibition by Δ-$Ru(DIP)_3^{2+}$ is easily understandable since Δ-$Ru(DIP)_3^{2+}$ binds by intercalation to the double-stranded B-DNA substrate with high affinity ($K = 10^5$ in 50 mM NaCl at 25 °C). Λ-$Ru(DIP)_3^{2+}$ does not, however, bind to right-hand B-DNA (Barton et al., 1984a; Kumar et al., 1985) owing to steric constraints, yet this isomer inhibits the *Eco*RI reaction and in fact more effectively than the Δ enantiomer. Table I indicates the degree of enzyme inhibition in the presence of several chiral intercalators. Although Δ-$Ru(DIP)_3^{2+}$ does inhibit *Eco*RI, no decreased linear DNA formation is apparent at 1 μM levels. For 1 μM Λ-$Ru(DIP)_3^{2+}$, however, an 18% decrease in linear DNA formation is obtained. Thus, as with binding to B-DNA, there is a strong enantiomeric selectivity observed in *Eco*RI inhibition. But here the enantiomeric preference is reversed. *The observed stereoselectivity in inhibition of EcoRI activity by the chiral intercalators is opposite to that found for DNA binding.* A greater response is obtained with the Λ isomer.

This reversal of enantiomeric preference is found consistently with the metal complexes inhibiting the *Eco*RI reaction as shown in Table I. While both isomers of $Co(DIP)_3^{3+}$ inhibit *Eco*RI, at 6 μM cobalt more inhibition is seen with the Λ isomer. Similarly greater inhibition is obtained with Λ-$Ru(phen)_3^{2+}$ compared to Δ-$Ru(phen)_3^{2+}$. It is interesting, however, that the degree of stereoselectivity seen in inhibition parallels that found in binding to the DNA helix. For $Ru(phen)_3^{2+}$, a small enantiomeric preference ($K_\Delta/K_\Lambda \approx 1.3$) in binding to B-DNA is observed, but for the bulky $Ru(DIP)_3^{2+}$ complexes binding to B-DNA is completely enantiospecific. Accordingly, a small differential inhibition is found for $Ru(phen)_3^{2+}$ enantiomers, and greater stereoselectivity in the enzyme inhibition, although not enantiospecifically, is apparent for the large diphenylphenanthroline complexes.

Other features of Table I are noteworthy. Tris(phenanthroline)metal complexes in binding to calf thymus DNA show association constants of at least 1 order of magnitude lower than those of Δ isomers of diphenylphenanthroline complexes (Barton et al., 1984a). Consistent with these observations here

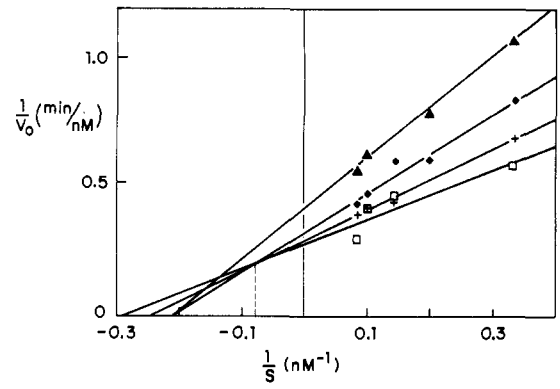

FIGURE 3: Kinetics of inhibition of the cleavage reaction of $ColE_1$ DNA by *Eco*RI by Λ-$Ru(DIP)_3^{3+}$. Lineweaver–Burk plot of DNA site/substrate concentration vs. initial velocity (V_0) at different fixed levels [0 (□), 4 (+), 6 (♦), and 10 (▲) μM] of inhibitor. The enzyme concentration used was 0.04 μM.

we find that higher concentrations of $Ru(phen)_3^{2+}$ are necessary to achieve inhibition than of $Ru(DIP)_3^{2+}$. The inhibitory effect is not simply a reflection of binding constants to DNA helices, however. With 5 μM ethidium under identical conditions, we find no inhibition of *Eco*RI and for B-DNA $k(\text{Eth})/k[Ru(phen)_3^{2+}] \approx 100$. Higher concentrations of the tricationic cobalt complexes appear to be needed to achieve inhibition comparable to that of $Ru(DIP)_3^{2+}$ isomers, although more highly charged species bind the DNA polyanion more closely. Perhaps this finding reflects the presence of the positively charged center in the enzyme active site.

The inhibition results suggest a chiral discrimination that differs from that observed in simple DNA binding. Some properties, e.g., relative affinity and relative enantioselectivity, resemble those found in binding to DNA in the absence of enzyme. From these results we may infer that either the protein alters the DNA conformation in some way, and in so doing also the binding characteristics of the chiral metal complex, or the enzymatic inhibition reflects metal association with the protein, not the DNA, and that association coincidently yields enantiomeric selectivity comparable to DNA binding.

We tested these ideas further by determining the kinetics of the inhibition reaction of *Eco*RI by Λ-$Ru(DIP)_3^{2+}$. Figure 3 shows the inhibitory effect of this isomer on the cleavage reaction of plasmid $ColE_1$ DNA by *Eco*RI. The double-reciprocal plot of concentration of DNA sites vs. initial reaction velocity at varying levels of inhibitor indicates mixed inhibition where the metal complex binds to both the free enzyme and the enzyme–substrate complex with different affinities (Cornish-Bowden, 1976). Each set of data was analyzed to give the best linear least-squares fit with high correlation (>0.9). The apparent dissociation constant (K_M) is 3.5 nM for the uninhibited cleavage reaction under the assay conditions used here. This value is well within the range of reported values of 3 (Woodhead & Malcolm, 1980) and 8 nM (Modrich & Zabel, 1976) for the plasmid $ColE_1$ DNA substrate. Therefore, the reaction obeys Michaelis–Menten kinetics under the assay conditions employed by us. However, as can be seen from Figure 3, the K_M increases from 3.5 (for the uninhibited reaction) to 13 nM in the presence of the inhibitor showing that the binding affinity of the enzyme to substrate is decreased in the presence of the inhibitor. Also the maximum velocity (V_{max}) of the reaction decreases as the inhibitor concentration is increased as seen by the intercepts on the y axis. A decrease in V_{max} results in a decrease in catalytic rate of reaction owing to inhibitor binding to the enzyme–substrate complex.

Table II: Plasmid DNA Cleavage by $Co(DIP)_3^{3+}$ Enantiomers after Photolysis in the Ultraviolet

	relative nicking activity[a] at [enzyme]/[DNA sites]		
enantiomer	0	1	2
neither	0.0	0.2	
Δ	1.0	1.6	1.6
Λ	0.2	2.0	1.5

[a] Nicking activity is expressed as the ratio of form II DNA produced for a given sample vs. that found with 6 μM Δ-$Co(DIP)_3^{3+}$ in the absence of enzyme and with irradiation. Subtraction of the small amount of form II DNA produced in irradiated samples lacking cobalt, due to photodamage, has been made. Samples were irradiated at 254 nm for 30 min at 37 °C in assay buffer in the presence or absence of 6 μM cobalt.

Stimulation of DNA Cleavage by Chiral Cobalt Complexes. In order to demonstrate that the metal complexes interact directly with the DNA helix, photoactivated DNA cleavage reactions by the chiral cobalt complexes in the presence of enzyme were examined. We found earlier (Barton & Raphael, 1984) that, upon irradiation, the photoreduction of only Δ-$Co(DIP)_3^{3+}$ promotes single-strand cleavage of $ColE_1$ plasmid (for superhelical densities ≥ −0.065); the Λ enantiomer, although able to undergo photoreduction in solution, does not promote cleavage of this plasmid, presumably since no non-B-DNA sites are recognized and tightly bound. Since an intimate association with the helix is necessary for cleavage, the assay in the presence of enzyme provides a unique means to establish whether the inhibition described above results from direct metal complex binding to DNA.

In the absence of a divalent cation such as Mg(II) or Mn(II), *Eco*RI binds tightly to its recognition site but does not cleave (Greene et al., 1979; Modrich, 1979). Thus, *Eco*RI and $ColE_1$ plasmid were incubated in assay buffer lacking magnesium but in the presence of micromolar concentrations of either Λ-$Co(DIP)_3^{3+}$ or Δ-$Co(DIP)_3^{3+}$. Upon irradiation with UV light at 254 or 315 nm single-strand DNA cleavage was found with either isomer at low concentrations. Without light, no cleavage was evident, and with irradiation but without enzyme under identical conditions, significant cleavage was found only with 6 μM Δ-$Co(DIP)_3^{3+}$. The results of one trial using a 4-W ultraviolet light source at 254 nm are summarized in Table II. Figure 4 shows the results of several trials at various enzyme/DNA ratios conducted by using high-intensity irradiation at 315 nm. The metal concentration range selected was that where inhibition had been obtained. DNA concentrations were used which maximized detection, and *Eco*RI was added in the range of stoichiometric levels. Note that, for the experiment in Table II, conditions were identical with those used to measure *Eco*RI activity, in other words, in assay buffer and at 37 °C. The enzyme is therefore specifically bound to its recognition site. For the experiment depicted in Figure 4, samples were irradiated at 25 °C for 1 min. These data show that *the presence of EcoRI stimulates photocleavage by* Λ-*Co(DIP)*$_3^{3+}$.

Several aspects of this experiment require comment. First, in this experiment the DNA nicking detected results from reaction with the cobalt center, not with the enzyme. No cleavage is found without photoactivation. Interestingly this trivalent cation, which is large and coordinatively saturated, does not catalyze the endonuclease reaction. Similarly, *Eco*RI cleavage of DNA is not found with either 50 μM $Co(phen)_3^{3+}$ or 50 mM $Co(NH_3)_3^{3+}$. Second, the quantum efficiency for photoreduction of the cobalt center in solution should be the same for both isomers, and if anything, the efficiency would be decreased in the presence of enzyme due to light absorption.

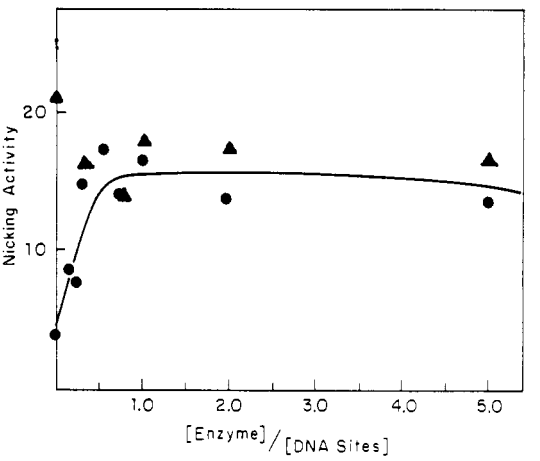

FIGURE 4: Photoactivated cleavage of $ColE_1$ DNA by Δ- (▲) and Λ-$Co(DIP)_3^{3+}$ (●) (6 μM) in the presence of increasing concentrations of *Eco*RI. Enzyme/DNA ratios are expressed as *Eco*RI monomer units per DNA cleavage site. Nicking activity is given as the difference between the percentage of form II DNA of samples irradiated at 315 nm for 1 min at ambient temperature and the unirradiated control.

The differential photoactivated cleavage by the isomers therefore reflects differential binding to the DNA by these isomers. In the absence of protein, nicking over background levels occurs only with 6 μM Δ-$Co(DIP)_3^{3+}$. In the presence of protein, there is a marked increase in the level of cobalt-promoted cleavage. This is best seen in the plot given in Figure 4 where cleavage by Λ-$Co(DIP)_3^{3+}$ is found to increase with the increasing ratio of enzyme added to DNA. It can be argued that, according to the results of the kinetic experiments, photocleavage is brought about by both inhibitor-bound enzyme and enzyme–DNA complex. However, at high enzyme–DNA site ratios above saturation, cleavage remains constant when the same concentration of metal complex is used. If it is the metal–enzyme complex that is effective in causing strand cleavage, then lower levels of DNA cleavage in the presence of competing enzyme concentration would be found. Interestingly and consistent with the notion that cleavage is stimulated at the enzyme-bound site, increased cleavage was observed with increasing enzyme until the DNA recognition sites were near saturation. Cobalt cleavage levels off at an enzyme monomer/DNA site ratio of 1/1. Therefore, the ternary protein–DNA–metal complex rather than a metal–protein complex seems to be the effective agent during photocleavage. Finally, it is noteworthy that the Δ isomer may bind anywhere along the plasmid and promote a nick, yet in the presence of protein, comparable cleavage is seen with both isomers. The cleavage results therefore all support a reversal of chiral selectivity at the enzyme-bound DNA site.

Mapping the Cobalt Cleavage Site in the Presence of Enzyme. That the protein-stimulated cleavage by Λ-$Co(DIP)_3^{3+}$ occurs at or near the *Eco*RI recognition site is seen finally in a mapping experiment. The strategy used is depicted in Figure 5A. The topological constraints on the DNA substrate are first removed by linearization of the $ColE_1$ DNA substrate. This linearization effectively prevents interactions that might occur between Λ-$Co(DIP)_3^{3+}$ and non-B-DNA conformations inherent in a closed-circular plasmid. We sought to determine whether Λ-$Co(DIP)_3^{3+}$ cleavage was in the vicinity of the *Eco*RI recognition site. If so, then photolysis of the *Sma*I linearized DNA in the presence of $Co(DIP)_3^{3+}$ and *Eco*RI under conditions where *Eco*RI does not itself cleave should still yield *Eco*RI digestion fragments, i.e., a 5351 base pair fragment and a 1295 base pair fragment. As seen from Figure 5B, photocleavage by Λ-$Co(DIP)_3^{3+}$ in the presence of *Eco*RI

Table III: Fragmentation of ColE$_1$ DNA by Λ-Co(DIP)$_3^{3+}$ at *Eco*RI Sites

sample	Co-(DIP)$_3^{3+}$ [a]	*Eco*RI	integrated intensity: intact linear 6646 bp	integrated intensity: 5351 bp fragment	ratio 5351 bp fragment/linear DNA
irradiated[b] DNA	–	–	885	48	0.054
	Δ	–	387	62.5	0.161
	Δ	+	312	47.5	0.152
	Λ	–	542	28	0.053
	Λ	+	430	52	0.122
unirradiated DNA	Λ	+	1199	15	0.012

[a] Experimental details are described under Methods. [b] Samples were irradiated at 315 nm.

does yield a band corresponding to the 5351 base pair fragment. Therefore, *cobalt cleavage and binding must occur at or near the EcoRI recognition site.*

Table III shows the extent of photocleavage of linear ColE$_1$ DNA by each enantiomer in the presence of *Eco*RI. It is clear that the Λ isomer which causes no photocleavage of DNA in the absence of enzyme shows enhanced reaction in the presence of *Eco*RI. This result is an indication that the nicking activity of Λ-Co(DIP)$_3^{3+}$ depends upon the presence of the enzyme. Since the metal complex must bind closely for productive cleavage, it is very likely that the enzyme binding causes the conformational change necessary for the association of the Λ isomer. Also, as would be expected, Δ-Co(DIP)$_3^{3+}$ is seen to cleave DNA with photoactivation irrespective of the presence or absence of enzyme. This is in accordance with previous results indicating that Δ-tris(phenanthroline)metal complexes bind preferentially to right-handed B-DNA.

In summary, then, *Eco*RI stimulates photoactivated DNA cleavage by Λ-Co(DIP)$_3^{3+}$. The cleavage results indicate direct binding of the tris(diphenylphenanthroline)cobalt complexes to the DNA site. Table IV summarizes the changes in metal–DNA associations found in the presence of *Eco*RI. Consistent with the enzyme inhibition results described above, *Eco*RI appears to change the enantiomeric preference of the chiral intercalators in binding to the DNA helix. Protein binding must induce a DNA conformational change that alters the binding characteristics of the chiral metal complex.

DNA Conformation in the Presence of EcoRI. The inhibition and cleavage results with the chiral probes indicate that in solution under enzymatic conditions a change in DNA conformation occurs with protein binding. Details of this DNA conformation cannot be easily inferred from the results, however. Relative binding by these probes does suggest that several conformations are unlikely. In the presence of *Eco*RI, the DNA site cannot maintain a regular B-DNA conformation. B-DNA binds only Δ-tris(diphenylphenanthroline) complexes. The inhibition and stimulated cleavage results are not consistent also with an opening of a DNA segment into a single-stranded region. Binding of the chiral intercalators to this conformation is extremely low, and no significant cleavage has been detected (Barton & Raphael, 1985). Furthermore, electrostatic association of the metal along an extended DNA groove rather than some form of intercalation

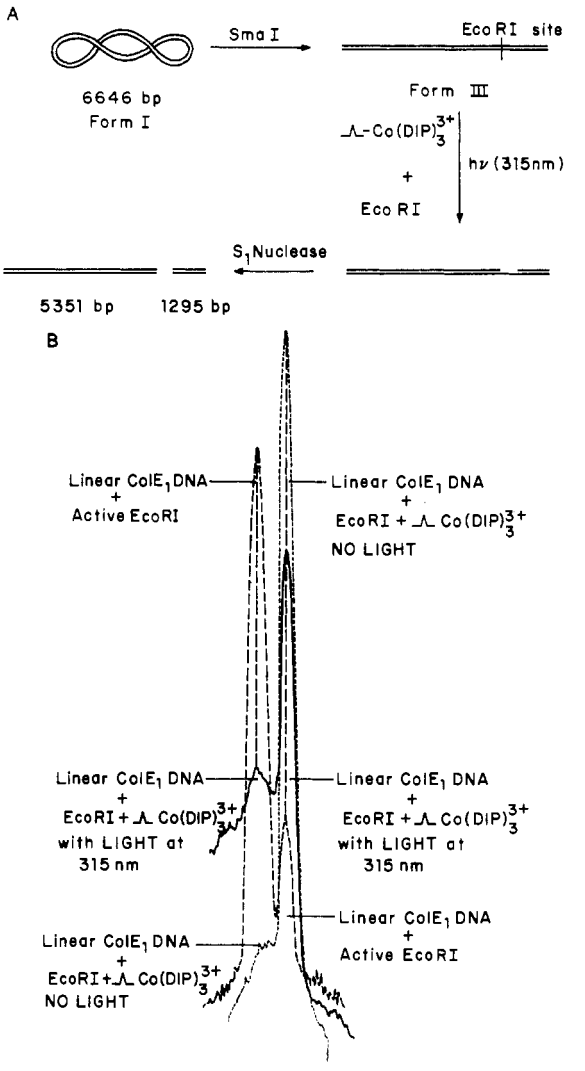

FIGURE 5: (A) Strategy for mapping site-specific cleavage of linear ColE$_1$ DNA by Λ-Co(DIP)$_3^{3+}$ in the presence of *Eco*RI and light (315 nm). (B) Densitometer scan indicating the formation of the 5351 bp fragment (left) from intact ColE$_1$ DNA (right) due to specific cleavage by Λ-Co(DIP)$_3^{3+}$ in the presence of *Eco*RI and 315-nm light: (- - -) linear ColE$_1$ DNA + *Eco*RI + Mg^{2+}; (···) linear ColE$_1$ DNA + Λ-Co(DIP)$_3^{3+}$ + *Eco*RI without light; (—) linear ColE$_1$ DNA + Λ-Co(DIP)$_3^{3+}$ + *Eco*RI + 315-nm light. The presence of *Eco*RI and light stimulates cleavage by Λ-Co(DIP)$_3^{3+}$ in the vicinity of the *Eco*RI recognition site, producing a 5351 bp fragment identical with that seen by *Eco*RI cleavage in the presence of magnesium.

is unlikely since this binding does not yield cleavage, and the presence of bound protein should minimize ionic metal–DNA interactions. Our chiral probes provide sensitive assays for the left-handed Z-DNA structure since both tris(diphenylphenanthroline) complexes favor Z form and without substantial chiral discrimination; the results would therefore not be inconsistent with a left-handed conformation. Indeed any stacked conformation that locally untwists and significantly unwinds the duplex could account for our results (unwinding to the point of a left-handed twist being an extreme).

The kinked DNA conformation described the basis of X-ray diffraction data (Frederick et al., 1984) for the crystalline *Eco*RI–DNA complex may provide a basis for some specu-

Table IV: Alterations in DNA–Metal Interactions with *Eco*RI

without enzyme	with enzyme
Λ enantiomers bind poorly to B-DNA	Λ enantiomers preferentially inhibit *Eco*RI; the inhibition is mixed and involves binding to the protein–DNA complex
Λ-Co(DIP)$_3^{3+}$ does not cleave B-DNA upon photoactivation	*Eco*RI stimulates DNA cleavage by Λ-Co(DIP)$_3^{3+}$ with light; cleavage occurs in the vicinity of the *Eco*RI-bound recognition site

lation. The 25° helical unwinding and 4-Å displacement of the DNA–phosphate backbone at the kink (defined as neo-1) would permit binding of both enantiomers. Perhaps more likely due to accessibility, an unwound helical structure at the secondary kink may provide a binding site for the intercalators. On the basis of the reported structure of the protein–DNA complex, it is difficult, however, to understand the effects of protein binding on metal association with the helix, with respect to either enhancements or inhibition. Furthermore, there is no obvious structural explanation for the preferential binding of the Λ enantiomer, unless some protrusion into the groove, of either the protein or DNA, that is not evident in the crystal structure accompanies helical unwinding at the kink. Thus, while several conformations may be ruled out on the basis of our results, in a positive sense, we can conclude only that protein binding does indeed cause an alteration of helix conformation in solution. Results by Kim et al. (1984) have also supported definite distortions of the DNA in solution in the specific recognition complex of *Eco*RI.

Utility of the Chiral Metal Complex as a Probe of Protein-Bound DNA Sites. This study serves as an illustration of how small DNA-binding agents may be used to probe chemically the structure of a protein-bound DNA site. A practical concern in the more usual methods of analysis is that any effects detectable at the protein-bound recognition site are severely diluted by effects detected in the remaining unbound DNA sites, which can constitute as much as 99.9% of total DNA. The assays here of inhibition of enzyme activity and photoactivated cleavage in particular sensitively monitor the protein–DNA-bound site. Taken together, these experiments focus specifically on the DNA structure at the active site and provide some controls for the effects of the probe interactions with protein. Caution must be exercised, however. Structural detail is not available, and really more utility rests in ruling out possible conformations than in elucidating one in particular. Metallointercalators, however, can provide useful spectroscopic and electron dense probes for macromolecules, and both these features should improve the quality of information obtainable. Most importantly from these data we can examine changes in DNA conformation as a function of protein binding *under enzymatic conditions* in solution. These experiments illustrate one protein-induced DNA conformational change. The structure of DNA can be sufficiently heterogeneous that in general changes in conformation resulting from protein binding need to be considered.

Registry No. Λ[Ru(phen)$_3^{2+}$], 24162-09-2; Δ[Ru(phen)$_3^{2+}$], 25747-34-6; Λ[Ru(DIP)$_3^{2+}$], 91237-66-0; Δ[Ru(DIP)$_3^{2+}$], 91176-72-6; Λ[Co(DIP)$_3^{3+}$], 89460-94-6; Δ[Co(DIP)$_3^{3+}$], 89460-93-5; restriction endonuclease *Eco*RI, 80498-17-5.

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