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The Restriction Endonuclease Eco RI: Its Intrinsic Metal Center and Specific Interactions with a DNA Recognition Site

by

Shanthi R. Paranawithana

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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ABSTRACT

The Restriction Endonuclease Eco RI: Its Intrinsic Metal Center and Specific Interactions with a DNA Recognition Site

by

Shanthi R. Paranawithana

Advisor: Professor Jacqueline K. Barton

Protein-nucleic acid interactions play an important role in life processes such as replication, transcription and regulation of gene expression. In an attempt to characterize the nature of these interactions, the restriction enzyme Eco RI was examined for its metal content. This site-specific DNA cleaving enzyme contains one tightly bound zinc atom per subunit. Removal of the zinc ion results in loss of endonuclease activity indicating that the metal is an integral component of the protein. Addition of the zinc ion back to the inactive protein restores the catalytic activity. The replacement of the native zinc ion with cobalt produces active endonuclease having the same site-specificity for DNA. The enzyme containing increasing amounts of substituted cobalt (0-80%), shows a gradual increase in its apparent dissociation constant (4.3-36 nM) for the substrate Col E_1 DNA. The maximal velocity of the reaction is increased from 0.63 for the native enzyme to 3.3 nM/minute for the cobalt enzyme. These kinetic studies indicate the direct participation of the metal ion in enzyme activity. Chelators having high affinity for zinc inhibit the enzyme. Some of these ligands are competitive

inhibitors implying that they inhibit the reaction by coordinating to zinc ion. This observation again suggests that the metal is necessary for Eco RI activity. The enzyme-bound zinc ion may also be replaced by cobalt ion *in vivo*, indicating that the metal ion is intrinsic to the protein. The visible absorption spectrum of Co-Eco RI resembles that of Co-Zn RNA polymerase, showing two intense peaks between 375-475 nm and a much weaker peak at 590 nm. The former bands were perturbed in the presence of substrate DNA implying that the metal may be required for substrate binding. The structure of the DNA site in the bound protein-DNA complex was also probed using chiral metal complexes which are DNA conformational probes. Site-specific cleavage reaction with the metal complexes indicated that a structural change of DNA occurred near the enzyme-bound site. Thus the results illustrate in solution the alteration in DNA conformation as a result of the site-specific association of Eco RI.

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CHAPTER 1

INTRODUCTION

Presence and requirement of zinc in biological systems:

Zinc has long been considered to be an essential element that is required in trace amounts for the healthy continuation of life. The importance of zinc in biological and physiological processes is well established. It is now known that zinc is present in relatively high concentrations in the cells of most organisms. For example, 70kg of human body weight contains 2-3g of zinc (1) as compared to a soil zinc level of 50mg/kg (2). Levels of zinc in individual tissues can vary from 10-20μg per gram of wet weight (1). In fact among the $3d^n$ elements, its cellular concentration is exceeded only by that of iron (3).

The discovery of the relative abundance of zinc in living matter prompted many investigations regarding the metal's role in life processes. These studies have been conducted on many aspects of biology such as nutrition, pathology, enzyme action and gene expression. They have been useful in understanding the importance of zinc in living systems and how the metal influences various metabolic processes.

On a nutritional level, investigations show that zinc affects the development, growth and differentiation of various tissues. Zinc deficiency results in retardation of normal animal growth, impaired bone development, slow maturation and improper

function of reproductive organs (4) and decreased learning capacity (5). Pathological studies indicate that zinc deficiency can lead to various diseases which sometimes occur spontaneously or can be induced experimentally. Reduced zinc levels cause dermatitis and loss of hair in vertebrates and chlorotic mottling and formation of abnormal leaves in plants (6). As a therapeutic agent, zinc is thought to be helpful in the process of wound healing and in the treatment of atherosclerosis (3). Furthermore, effects of zinc on regulation of gene expression have been demonstrated recently. For example, zinc deficient cells of the simple eukaryote Euglena gracilis showed decreased overall rates of RNA synthesis (7). These cells contained chromatin that was more compactly arranged than the normal chromatin of zinc sufficient cells. The abnormal chromatin structure observed in these cells was attributed to the association of an unusual protein with the chromatin material. The overall structure of chromatin was such that it hindered the translational processing of zinc depleted cells (8). Another instance where zinc affects gene expression is reported for the 5 S RNA gene of Xenopus. Xenopus 5 S RNA genes contain an intragenic control region which is required for accurate initiation of transcription. The transcription regulatory protein, factor A, activates transcription of Xenopus 5S RNA genes by binding to this control region. It has been demonstrated that Xenopus transcription factor A contains zinc and that the metal is required for the

binding of the protein to the 5 S RNA gene (9). Lastly, biochemical studies show that zinc is an integral component of many proteins. Examination of these metalloproteins has contributed much to the understanding of the influence this metal exerts on life processes.

Zinc metalloenzymes:

The first zinc containing enzyme, carbonic anhydrase, was discovered in 1940 by Keilin and Mann (10) in mammalian erythrocytes. They demonstrated that the protein contained 0.33% zinc and found that this zinc is necessary for catalytic activity. Fifteen years later, the enzyme carboxypeptidase A was found to contain an essential zinc atom (11). With the development of assays capable of detecting concentrations of zinc as low as $5x10^{-14}g$ (12), the discovery of zinc enzymes was accelerated. It is now known that zinc ion is an integral component of many proteins isolated from organisms as diverse as bacteria, fungi, higher plants, animals and humans. Over 200 zinc enzymes have been identified so far, and they belong to all six enzyme classes, with hydrolases comprising the majority (12).

A zinc metalloenzyme is generally defined as one that contains a high affinity site for zinc. This definition classifies two groups of proteins, zinc metalloenzymes and metal activated enzymes. The latter represents more loosely bound zinc

enzyme complexes (12). For several zinc metalloenzymes, the dissociation constants of the metal protein complex have been found to be in the order of 10^{-10}M or smaller (13-16). These proteins can be isolated and purified as zinc protein complexes. As a consequence, levels of zinc associated with protein seem to increase as purification proceeds. This increase indicates that the purified protein is able to bind zinc tightly. Furthermore, it is important to demonstrate that this tightly bound zinc atom is necessary for catalytic activity.

Demonstration of the functional importance of zinc to an enzyme system:

A tightly bound zinc atom is present as an intrinsic component of a protein molecule. Hence, metal removal inactivates the enzyme. But a zinc atom with a high affinity for a protein cannot be easily removed. Therefore metal chelating agents are used to remove the metal to obtain apo enzyme. A chelator such as o-phenanthroline which has a high affinity for zinc (affinity constant, log K = 6.4) is often used to remove the metal from the protein. As can be expected, the ease with which the apo protein is obtained depends on the tertiary structure of the enzyme and the binding constant of the metal-enzyme complex. Nevertheless, the removal of intrinsic zinc and the demonstration that the resulting apo enzyme is catalytically inactive are very important in establishing the close association of the metal with

the protein (12). Sometimes the addition of zinc can reconstitute the stable inactive apoenzyme thereby fully restoring its activity. Removal of zinc with the resulting loss of catalytic activity, and addition of metal leading to the restoration of activity of a protein, provide strong evidence for the functional importance of the metal in catalysis (12).

Use of metal chelators to investigate the role of zinc:

The involvement of the zinc ion in the catalytic mechanism of a zinc enzyme is difficult to examine in many ways because the d^{10} zinc ion is spectroscopically invisible. Early investigations have therefore been carried out through the use of metal chelators as inhibitors of reactions catalysed by zinc enzymes. Claude Bernard (17) was the first to employ ligands such as azide, cyanide, carbon monoxide and sulfide as inhibitors of enzymes containing transition metal and II B elements. Warburg used these compounds to investigate the catalytic reactions of copper, iron and zinc enzymes (18). In recent years, a large number of chelating agents have been used as potent enzyme inhibitors which complex with the metal, in examining the reactions catalysed by zinc enzymes. Ethylenediamine tetraacetic acid (EDTA), imidazole, 1,10-phenanthroline, mercaptoethanol, sulfonilamide and α-α' bipyridyl are a few of the chelating agents that are commonly used in enzyme inhibition studies (12). A study of the kinetics of inhibition by a metal chelator is

often helpful in elucidating the catalytic mechanism of a metalloenzyme. The investigation of the mechanistic details of carbonic anhydrase catalytic activity is a good example of an extensive use of metal chelators as inhibitors in kinetic studies (19).

Cobalt substitution and spectroscopy in characterization of zinc enzymes:

One of the significant features of the study of metalloenzymes has been the use of physicochemical methods which take advantage of the chemistry of the metal ion. Many properties of the metal ion such as the energies and optical activity of its d-d transitions, ESR transitions, the effect of the metal ion on NMR transition of neighboring nuclei, are all influenced by the surrounding ligands and/or other protein structure. Hence, measurements of these properties afford a way of learning about the structure of an active center. These transitions involve the presence of a partially filled d shell of a metal ion. But zinc (II) has a complete d shell and may be considered spectroscopically silent, in contrast to the other first row transition metals which are valuable probes of protein structure. It has been possible, however, to exchange the zinc(II) in many of these proteins for a first row transition metal ion.

Cobalt(II) has proved particularly advantageous because this metal, which is d^7, has a paramagnetic center and a visible spectrum which is sensitive to the ligand environment. Association of substrate or inhibitors is revealed by perturbations of the cobalt spectrum of the free enzyme (20). Furthermore, what is generally observed is that cobalt restores catalytic activity approaching or sometimes exceeding that of the native enzyme. The chemistry of the cobalt(II) therefore resembles that of zinc(II) sufficiently to be enzymatically useful. Vallee et al (21) pioneered metal substitution experiments using carboxypeptidase A. They removed the native zinc atom using a chelator and restored activity by the addition of cobalt. Substitution of transition metals such as cobalt has been done on many zinc enzymes since then. Spectral studies of cobalt-substituted enzymes show that the metal center has an irregular and in some cases nearly tetrahedral environment (21). Irregular geometry at the catalytic metal center could be an advantageous feature associated with enzymes when one considers their catalytic capacity. A distorted active site may allow further structural changes more easily than a stable fixed structure, in binding to a substrate. In fact, for thermolysin, x-ray crystallographic studies carried out in the presence of inhibitors suggest that the catalytic zinc atom which is tetracoordinated in the free enzyme probably goes through a pentacoordinate intermediate during catalysis (22).

Properties of the metal center:

Zinc metalloenzymes catalyse a variety of different reactions. Therefore the catalytic mechanism and the active site architecture can be expected to vary depending on the reaction and the protein concerned. There are about seven well characterized zinc enzymes. The following discussion of the properties is based on the information available on those enzymes.

The amino acid residues that can be protonated are considered to be the most likely ligands of zinc in metalloproteins. These include carboxyl groups of aspartyl and glutamyl residues, the N-terminal α-amino acid group of the peptide chain, the ε-amino group of lysyl residues, the imidazole nitrogen of histidyl residues, the hydroxyl group of tyrosyl residues, the SH group of cysteinyl residues and the guanidinium group of arginyl residues (5). Out of these only three have been identified as Zn(II) ligands in zinc metalloproteins. For example, carboxypeptidase A has a zinc atom coordinated to two imidazole moieties of histidines and γ-carboxyl group of a glutamyl residue (23). Thermolysin, a bacterial endopeptidase, has three imidazole ligands of histidines coordinated to its intrinsic zinc atom (24). In both, the fourth coordination position of a distorted tetrahedron is occupied by a water molecule. In carbonic anhydrase, the catalytic zinc atom is bound to three histidine imidazole groups and either a water

molecule or a hydroxide ion (25).

The nature of the coordination sites at the active centers of the metalloenzymes differ from small metal coordination complexes which have flexible ligands capable of assuming a geometry around the metal depending on the d-orbitals of the metal ion. In a protein molecule, however, the positions of the amino acid ligands may be relatively inflexible owing to the tertiary structure of the protein. Therefore the geometry around the metal site may be determined by the stereochemistry of the protein ligands as well as by the electron distribution of the metal ion orbitals. Highly distorted metal environments seen in enzyme active sites presumably are stabilized by the structure of the rest of protein molecule (5). This type of accomodation may not be possible for some of the first row transition metals with partially filled d orbitals, owing to the lack of ligand field stabilization energy which contributes greatly towards stabilizing first transition metal complexes. Zinc ion with a filled d shell and no ligand field stabilization may be able to assume a distorted geometry without destabilization.

In postulating a catalytic function to an active site zinc atom, one significant factor has been its ability to act as a Lewis acid withdrawing electrons from susceptible groups in the substrate. In the case of caboxypeptidase A, the x-ray structure of crystalline glycyl-L-tyrosine complexed with the enzyme shows coordination of the zinc ion to the carbonyl oxygen of the

susceptible peptide bond (26). The mechanism proposed for carbonic anhydrase which catalyses a reversible reaction suggests that the active site zinc ion is coordinated to the oxygen of either an active OH^- or bicarbonate depending on whether the enzyme is catalysing the forward or the backward reaction (27,28). It is not clear whether a single Lewis acid catalysis is applicable to all the reactions catalysed by zinc metalloenzymes (5).

Interaction of zinc with nucleic acid binding proteins and nucleic acids:

The zinc dication seems to be associated with many enzymes that interact with nucleic acids. Many nucleotidyl transferases from a variety of sources contain zinc as an essential component of the enzyme (29, 30,31). Nucleotidyl phosphoesterases, 5' nucleotidase from bacteria (32) and human lymphoblast plasma (33) and snake venom phosphodiesterase (34) contain zinc as part of the active protein. Among the enzymes that are specific for ribonucleic acids, four of the transfer RNA synthetases contain tightly bound zinc (35,36). One of the most studied enzymes, DNA dependent RNA polymerase, from Escherichia coli, contains two tightly bound zinc atoms, one of which is implicated in promotor recognition and binding (37), orientation of initiating nucleotide (38) and structural stabilization (39). Nuclear magnetic resonance studies carried out with the catalytically

active cobalt-substituted RNA polymerase suggest that the metal is directly coordinated to the base moiety of the initiating ATP molecule (38). The ability of this enzyme to bind to nucleotides through the metal center suggests that perhaps zinc in DNA binding proteins may be involved directly in coordination to nucleic acids. Numerous investigations carried out on metal nucleic acid interactions suggest that this could well be a possibility (40-46).

Nucleic acids are formed by the condensation of 5'-phosphates with 3'-hydroxide groups of individual nucleotides. The presence of phosphate groups along the polymer makes them long polyanionic molecules. Nucleic acids contain many potential sites for metal interactions. Klug et al (40,41), using isomorphous x-ray diffraction methods on a modified yeast $tRNA^{Phe}$, found many metal binding sites. Manganese(II), cobalt(II) and osmium(VI) complexes bind to N-7 of a guanine base with the ligands of the complex forming hydrogen bonds to the neighboring oxygen and nitrogen atoms. Furthermore, Mn(II), Sm(III) and Lu(III) prefer regions rich in phosphate groups and some of these regions are found to be the same as Mg(II) binding sites in native tRNA. Interaction of Ag(I) with nucleic acids occurs primarily at guanosine-cytidine (G-C) region of DNA (42). It has been suggested that Ag(I) can bind to two adjacent cytosines through O-2 and N-3 of the two bases (42).

Binding of metal ions can have an effect upon the nucleic acid structure depending on their relative ability to bind phosphate or bases. Phosphate binding of metals stabilizes double helical DNA by neutralizing the negative charges and hence reducing the repulsive forces between adjacent phosphate groups. Base binding by metals such as copper usually destabilizes the ordered double helix (44). The zinc ion, however, has been shown to behave in a somewhat different manner by Shin and Eichhorn (45), who discovered that this metal is able to reversibly unwind and rewind double helical DNA by heating and cooling respectively. The process can be carried out through many cycles and indeed on cooling DNA is renatured. They attributed this to the ability of zinc to hold two strands together by binding to bases during denaturation as well as binding to phosphates at low temperature thereby stabilizing the double helix. In contrast, copper did not bring about renaturation on cooling, under similar experimental conditions.

The remarkable nature of metal-nucleic acid interactions and the fact that many DNA binding proteins contain zinc, makes it worthwhile to investigate the metal sites in these proteins. Fascinating as it may seem, the complexity of many DNA binding enzymes as well as the reactions they catalyse makes it a difficult field to study. Working under metal free conditions is a requirement, in investigating any zinc metalloenzyme. Besides the fact that careful control of metal contamination is required

owing to the universal presence of zinc in the environment, the polyanionic DNA substrate which has an affinity for metals, adds yet another source of contamination. Keeping these in mind one could look for a system where at least some of these complications are minimized. Therefore, we looked into the possibility of examining a type II restriction endonuclease for its zinc content in order to facilitate our study of protein-DNA interactions through a metal center. Since these enzymes are among the simplest DNA binding proteins known, they afford a means of investigating a relatively uncomplicated system.

Type II Restriction Endonucleases:

Type II restriction endonucleases are sequence-specific DNA cleaving enzymes and are commonly referred to as restriction enzymes (46). In contrast, type I enzymes which recognize DNA site-specifically do not cleave the substrate at a specific site. (47). The type II enzymes cleave the double stranded DNA at specific sites (4-8 base pairs) by hydrolysing the phosphodiester bonds. The first type II restriction enzyme was isolated by Smith (48) from the bacterium Haemophilus influenzae. Now over 200 restriction enzymes are known from a variety of bacterial cells.

Smith and Nathans (49) established the nomenclature of restriction enzymes using the names of the genus and the species of the bacteria from which they are isolated. As an example, Bam

HI was the first enzyme activity reported from Bacillus amyloliquefaciens strain H.

Structurally, most restriction enzymes have two equal subunits with molecular weights of 20,000-25,000 or single polypeptides with molecular weights of 30,000-35,000 d (46). More than one restriction enzyme recognize the same sequence, but each enzyme recognizes only one unique sequence (46). Cleavage of DNA by all type II restriction enzymes known so far produces fragments with a 5'-terminal phosphate and a 3'-terminal hydroxyl residue. Some of the restriction enzymes are present in the bacterial cell along with a modification methylase enzyme. Both methylase and endonuclease from one system could recognize a similar sequence but methylase modifies the DNA by adding a methyl group to one of the bases thus inhibiting the activity of the restriction enzyme. These systems are assumed to be involved in presenting barriers to the invasion of the bacterial cell by foreign DNA (50). Foreign DNA entering a bacterial cell seem to be inactivated by restriction enzymes through cleavage. The cell's own DNA is protected by methylation. Restriction enzymes are widely used in molecular biology as reagents for site specific cleavage of DNA molecules. DNA sequencing, mapping and cloning are very much dependent on the action of restriction endonucleases. Even though their use as an important research tool is very much recognized, most of these enzymes are not well-characterized in terms of their catalytic mechanisms, DNA binding

and recognition and hydrolytic activity.

Eco RI: A restriction enzyme:

Among the many restriction endonucleases known, endodeoxyribonuclease Eco RI (EC 3.1.23.13) from E. coli is one of the most studied. It has a monomer molecular weight of 31,000 and requires magnesium (II) for its activity. It recognizes the palindromic sequence 5' G↓AATTC and cleaves as
CTTAA↑G 5'
indicated by arrows to generate 5'-phosphoryl and 3'-hydroxyl ends (50).

The reaction catalysed by the Eco RI enzyme is kinetically simple. Reaction velocities are first order with respect to endonuclease concentration and the kinetics are reported to be Michaelis-Menten (51,52,53). The stable form of the endonuclease at catalytic concentrations is the dimer (52,54). Kinetic analysis of the single turnover of Eco RI endonuclease with $ColE_1$ DNA suggests that the rate limiting step of the reaction is the release of the cleavage products from the enzyme (55). Using chiral phosphorothioate groups at the cleavage site of Eco RI, Connolly et al. (56) showed that the hydrolysis reaction proceeds with inversion of configuration at the phosphorus. They suggested a direct enzyme catalyzed nucleophilic attack of H_2O at phosphorus and the absence of a covalent enzyme intermediate during catalysis. As determined by equilibrium binding assays,

the affinity of the enzyme for its specific site is about five orders of magnitude higher than that of the non-specific sites (57). It is also speculated that at least *in vitro* the enzyme finds its recognition sequence by a facilitated diffusion path along its DNA substrate (57).

Potential DNA contacts for the enzyme within the Eco RI sequence, as determined by base analog substitution and alkylation procedures, reveal that the enzyme contacts the bases both from the minor and the major grooves. The nitrogen N-7 of the guanine and 6-NH_2 of adenine both of which occur in the major groove and the N-3 of adenine in the minor groove are thought to be important as Eco RI contact sites (58,59). The crystal structure of Eco RI characterized in the presence of its recognition sequence indicates that the binding of the enzyme causes a torsional kink in the DNA which can lead to unwinding of the duplex (60).

Chiral metal complexes as probes for DNA conformation:

Another approach to the study of protein-nucleic acid interactions is to investigate the changes in DNA structure, that might occur upon binding of a protein. Chiral metal complexes developed in this laboratory (61,62,63) are sensitive probes of the local conformations of DNA along the helix. In an attempt to probe the structure of the Eco RI specific DNA site, the binding of the enzyme to DNA was examined in the presence of chiral

probes. Any deviations from the regular right handed B structure are detected by the stereoselective binding of the metal complexes. Hence, these compounds allow the determination of DNA conformational changes that could occur as a result of Eco RI endonuclease binding to its cognate DNA.

Objectives:

We chose to investigate the site-specific DNA cleavage reaction catalysed by the restriction enzyme Eco RI. This enzyme was found to contain one zinc atom per enzyme monomer subunit (Appendix 1). The metal chelator o-phenanthroline inhibits the site-specific cleavage reaction of Eco RI. In contrast, the non-chelating analog m-phenanthroline was unable to inhibit the cleavage reaction. The tightly bound metal ion could be removed by dialysis against o-phenanthroline leading to the loss of catalytic activity of the enzyme.

In this dissertation I describe further work carried out on the restriction endonuclease Eco RI in an attempt to characterize its metal center. The essentiality of the zinc ion to the function of the enzyme was demonstrated by enzyme reconstitution experiments where the metal ion was removed to obtain the inactive apo protein which was reactivated by the addition of zinc. Participation of the metal during the catalytic reaction was examined by inhibitory kinetics studies using metal chelators that have high affinity for zinc. Additionally, as a first step

towards spectroscopic investigations of the metal center, the intrinsic zinc ion was replaced with cobalt(II) ion both in vitro and in vivo. The catalytic activities of the native and the cobalt-substituted enzymes were compared to determine whether the cobalt substitution produced enzymatically active protein. Furthermore, the kinetics of the DNA cleavage reaction of the two enzymes was compared in order to assess the role of the metal ion.

The site-specific binding and cleavage reaction of DNA by Eco RI was investigated using metal complexes not intrinsic to the enzyme but instead developed as probes that interact with DNA stereoselectively. A change in conformation in the DNA that can occur as a result of enzyme binding is seen as a change in stereoselectivity of metal complexes. Therefore these studies provide a means of observing the local changes in the DNA conformation brought about by the site-specific interaction of the enzyme.

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CHAPTER 2

IN VITRO SUBSTITUTION OF THE ZINC ION

INTRODUCTION

Zinc ion can affect enzyme activity in many ways. For example, the presence of zinc both inhibits and activates the catalytic activities of sheep liver lysyl and phenylalanyl t-RNA synthetases. These enzymes are inhibited during the aminoacylation reaction but activated to synthesize diadenosine tetraphosphate (Ap_4A)(1). Similarly, dinucleotide tetraphosphatase from rat liver, which specifically cleaves Ap_4A to ATP and AMP is inhibited by zinc(II) concentrations as low as 5μM (2). These enzyme reactions, however, require the addition of zinc to the reaction mixture as an effector or an inhibitor. In contrast, zinc ion can be an integral component of many enzymes which are classified as zinc metalloenzymes. DNA dependent RNA polymerase from *E. coli* is one example of a well studied zinc enzyme (3).

A zinc metalloenzyme differs from a protein which is regulated by the addition of zinc to the reaction mixture, even though the activities of both are very much affected by zinc. Unlike an effector ion, intrinsic zinc in a metalloprotein is tightly bound to form a metal-protein complex (dissociation

constants in the order of 10^{-10}M are common). Therefore zinc free "apo protein" is usually obtained by dialysis against buffer containing chelators such as EDTA or o-phenanthroline which have high affinities for zinc. Removal of zinc invariably leads to the inactivation of protein. Furthermore, addition of the metal reactivates the enzyme, but again, specific conditions for metal addition may be required in order to regain catalytic activity. Nevertheless, reconstitution of an inactive apo protein with zinc, to obtain active protein supports the fact that the zinc atom is an intrinsic part of the enzyme.

A zinc ion when present as part of an active protein serves one or more purposes. A catalytic zinc atom found at the active site is involved in the reaction as part of the enzyme. Also, the metal center may serve a structural role when it is stabilizing the higher orders of structure of a protein. A regulatory zinc ion helps in the assembly of proper protein subunits to produce active enzyme. A study of the ligand environment of the metal inside the enzyme is often useful in assessing the role of the metal. Zinc is usually found coordinated to four ligands forming a complex having a distorted tetrahedral geometry. Metal that is required for structural integrity of a protein generally contains four amino acid residues coordinated to it whereas a catalytic zinc atom often contains a solvent water molecule as the fourth ligand in addition to the three amino acid residues. For example, in liver

alcohol dehydrogenase, x-ray crystallographic data show that the " structural zinc" is bound to four sulfur atoms from four cysteine residues in a pseudotetrahedral arrangement and is apparently not accessible to solvent (4). But the "catalytic zinc" of the same enzyme is coordinated to two sulfur atoms from two cysteine residues and a nitrogen atom from a histidine. The fourth ligand is supposed to be a water molecule (4).

Besides x-ray crystallography there are other ways to examine the metal site in a protein. One of the more commonly used techniques is the characterization of the zinc enzyme through metal substitution. Zinc(II) is a d^{10} ion and therefore is diamagnetic and colorless. These properties render zinc proteins unsuitable for electron paramagnetic resonance studies or electronic spectroscopy. Therefore other metals such as Co(II), Cu(II), Mn(II) and Ni(II) which are paramagnetic and/or colored are substituted for the native zinc atom and the artificial metalloprotein thus obtained is studied using spectroscopy. Co(II) is often selected for replacing zinc because the substituted enzyme in some cases has a catalytic activity comparable to or more than that of the native enzyme. Therefore, it is speculated that cobalt(II) substituted enzymes are structurally more similar to the zinc enzyme than any other transition metal substituted enzyme. Since the d^7 Co(II) ion is paramagnetic and forms complexes that are colored at relatively high concentrations (Extinction coefficients on the order of 100-

1000 $M^{-1}cm^{-1}$), Co(II) substitution has the advantage that the ligand environment of a protein can be determined using spectroscopy. Information obtained in this way is then extended by inference to the native zinc enzyme. Additionally, metal substitution allows comparison of two proteins through kinetic studies. Determination of parameters such as catalytic rate and apparent binding constant of the enzyme to substrate can provide useful information about the participation of the metal during substrate binding and catalysis.

The in vitro replacement of zinc by another metal is frequently achieved in two ways. One way is to dialyse the native enzyme against "metal free" buffer containing a chelating compound with high affinity for zinc, to effect removal of the metal. The apo protein is reactivated using the metal of choice and assayed for activity in comparison with the zinc enzyme. If the metal free apo enzyme tends to be unstable, dialysis of native enzyme against a large excess of a second metal (usually cobalt) is carried out. This method has the definite advantage, in that one does not have to prepare the apo enzyme which demands extreme care in order to prevent contamination by traces of free zinc ion. Extraneous metal present after direct exchange is removed by dialysis against metal free buffer.

In this chapter the reconstitution experiments carried out with the apo restriction endonuclease Eco RI, and the metals, zinc and cobalt are described. Furthermore, kinetic

investigations carried out using the native and the cobalt substituted Eco RI are reported here. These experiments provide necessary information to determine whether the metal is an integral part of the protein or a cofactor. Additionally, a comparison of kinetics of the native zinc enzyme and the cobalt substituted enzyme permits a way of determining the role of the metal during catalysis.

EXPERIMENTAL PROCEDURES

Materials

DNA: Plasmid Col E_1 DNA was obtained from Sigma Chemical Co. and pBR322 DNA and lambda phage DNA were purchased from BRL. DNA was stored at -20°C after dialysis against assay buffer containing 0.1M Tris base (pH 7.5 at 25°C), 0.05M NaCl, 1% glycerol and 0.01% Triton x 100.

Bacterial strains: Two bacterial strains JC4588/λ^+(pSCC2) and M5248 were received from Prof. P. Modrich. Plasmid pSCC2 contains the genes for Eco RI endonuclease and methylase with the λ P_L promotor. The λ^+ lysogen represses the λ P_L promotor of pSCC2 so that P_L is not deleted. M5248 contains a crytic λ lysogen which allows the thermal induction of pSCC2 (5). Cells were grown as M5248(pSCC2) after transformation and the restriction endonuclease isolated as described later.

Enzymes: Enzyme Eco RI restriction endonuclease was either

purchased from Boehringer Mannheim or prepared in our laboratory (vide infra) using the overproducing strain M5248(pSCC2). Commercial enzyme was dialysed against metal free assay buffer and stored at -20°C in 50% glycerol.

Metals: Zinc metal (99.9999%) was obtained as a powder from Aldrich Chemical Co. Puratronic cobaltous chloride (99.999%) was from Alfa Chemical Co.

Other materials: Phosphocellulose (Celex P) for column chromatography was purchased from Bio-rad laboratories. O-phenanthroline, Tris base, streptomycin sulfate, ammonium sulfate, bovine serum albumin and lysozyme were obtained from Sigma Chemical Co. Other chemicals used were of the purest grade available.

Dialysis tubing was stored "metal free" in 50% ethanol in water after heating with EDTA and washing with distilled deionized water. Prior to use each tube was soaked in o-phenanthroline solution followed by washing with deionized water. All dialyses were done exclusively in plasticware made metal free by soaking in o-phenanthroline solution and cleaning with deionized water.

For dispensing small volumes (µl), Pipetman pipetters with metal free plastic tips (Bio-rad) were used. All enzyme assays were carried out in 1.5ml plastic microcentrifuge tubes (VWR) prewashed with deionized water.

Methods

Overproduction of Eco RI and its isolation and purification: The baterial strain JC4588/λ^{+}(pSCC2) was grown in culture media containing L-broth [per liter: 10g of tryptone, 10g of NaCl, 5g of yeast extract, 5mM potassium phosphate (pH 7.0)] at 37°C overnight. The cells were collected by centrifugation at 2500rpm using the SS 34 rotor for 30 minutes and plasmid pSCC2 was isolated as follows. A cell pellet obtained from 500ml of growth media was suspended in 9ml of 20mM Tris base (pH 8.0) with 0.14M NaCl was treated with 1ml solution of 5mg/ml lysozyme in 50mM Tris base (pH 8.0) containing 10% sucrose, 5M NaCl, 0.2M EDTA and 10% SDS. The cells were gently shaken so that the plasmid DNA was separated from the partially lysed cells containing chromosomal DNA. Supernatant obtained after centrifugation was treated with RNAse to hydrolyse the RNA and extracted with chloroform and isoamyl alcohol (95:1) to obtain DNA which was purified by treating it with a mixture of phenol and chloroform (1:1) to remove proteins. Plasmid DNA thus isolated contained no chromosomal DNA and RNA as judged by agarose gel electrophoresis and was stored at 4°C for use.

Strain M5248 was grown at 30-32°C in L-broth overnight and the cells were harvested and suspended in SOB media (2% tryptone, 0.5% yeast extract, 10mM NaCl, 10mM $MgSO_4$, 2.5mM KCl, 10mM $MgCl_2$) and grown to an OD_{550} of 0.45 and harvested. The cells were

resuspended in TE buffer [10mM MES (pH 6.3), 100mM RbCl and 100mM KCl, 45mM $MnCl_2$, 10mM $CaCl_2$ and 3mM $Co(NH_3)_6Cl_3$ and transformed using plasmid pSCC2 by the procedure of Hanahan (6).

Transformed strain M5248/(pSCC2) was grown at 30-32°C in L-broth until OD_{590} reached 1. Cell cultures were then shifted to 42°C and incubated for 4 hours before harvesting. All the following operations were carried out at 4-10°C. Cell pellets (150g) were suspended (0.32g/ml) in 0.02M potassium phosphate buffer (pH 7.4) containing 15mM 2-mercaptoethanol, 1mM EDTA and sonicated in an ice bath. Sonication was done until the cells appeared to be broken when examined under the microscope. The extract was cleared by centrifugation and the supernatant was adjusted to an A_{260} of 200 using the same buffer. Freshly prepared aqueous streptomycin sulfate (25% w/v), (0.2ml per ml of extract) was added and stirred for 45min and the suspension was centrifuged (3000rpm, 20min.). The supernatant had a ratio of A_{280}/A_{260} of about 0.7. Solid ammonium sulfate was added to the supernatant (70% saturation) over a period of 30 min, while stirring, and the stirring was continued for an additional 60 min. The precipitate was collected by centrifugation and dissolved in about 200ml of 0.02M potassium phosphate(pH 7.4), 1mM EDTA and 5mM 2-mercaptoethanol. It was then dialysed against 0.02M potassium phosphate (pH 7.4), 5mM 2-mercaptoethanol, 10%(w/v) glycerol (Buffer A) containing 0.2M KCl and 0.5mM EDTA (2 changes, 3h per change), (Fraction II).

Fraction II was applied to a phosphocellulose column (60cm x $24cm^2$) equilibrated with buffer A containing 0.1M KCl, and 0.5mM EDTA. After washing with about 1 liter of the same buffer, the column was eluted (120ml/h) with a 4 liter linear gradient of KCl (0.15 to 1M) in 0.02M potassium phosphate (pH 7.4), 5mM 2-mercaptoethanol, 0.1mM EDTA, 10% (w/v) glycerol. Eco RI methylase and endonuclease activities are eluted at about 0.47 and 0.61M KCl, respectively (5). Fractions were then assayed for endonuclease activity.

SDS-polyacrylamide gel electrophoresis: Some active fractions were dialysed against 0.02M potassium phosphate (pH 7.4), 5mM 2-mercaptoethanol, 0.1mM EDTA and denatured and subjected to protein electrophoresis using 0.1% sodium dodecyl sulfate and 11% polyacrylamide according to the procedure of McPherson (7).

Assay procedures: Protein content: The quantity of protein in each fraction was measured by determining the absorbance at 278nm on a Cary 219 (Varian) UV-Visible spectrophotometer. The extinction coefficient of Eco RI endonuclease was taken as 8.3 cm^{-1} for a 1% (w/v) solution of enzyme (8). The ratio of absorbance at 280nm to that at 260nm for the native protein was close to 2 which rules out significant nucleic acid contamination (8).

Additionally, protein in some fractions and dialysed samples were quantitated by Coomassie brilliant blue binding assay (9) using Coomassie blue reagent from Bio-rad. Standard protein solutions (Lysozyme or Bovine serum albumin) of known concentrations were employed to obtain calibration curves using linear least squares analysis. Concentrations of endonuclease preparations were calculated by using the standard curves. Absorbance of 1mg of Eco RI corresponds to that of 1.25mg of lysozyme or 1.5mg of bovine serum albumin. The correlation factor for lysozyme was determined by using the absorbance at 278nm and the extinction coefficient reported by Modrich and Zabel (8). The correlation factor for bovine serum albumin was used as reported by Jen-Jacobson et al (10).

Metal content: Zinc: Zinc content of samples was determined using a Varian 875 atomic absorption spectrometer equipped with a simultaneous background correction and a CRA 90 graphite furnace.

Zinc chloride solutions of known concentrations were obtained by diluting a gravimetrically prepared 50mM stock (zinc powder dissolved in concentrated HCl). All dilutions were made in assay buffer. Zinc concentrations of samples were determined by routine calibration of sample absorption at 213.9nm against zinc standard solutions (0.1μM-0.9μM) using linear least squares analysis. One microliter of each solution was subjected to

analysis as follows: 35s dry at 98°C, 15s ash at 750°C, atomize at 2000°C, 1.5s hold, ramp rate 600°C/s. Absorption of each standard was the average of at least 3 readings and for protein samples at least 8 readings were taken and averaged.

Cobalt: Cobalt solutions of known concentrations were made by gravimetric preparation of 50mM $CoCl_2$ solution in assay buffer (pH 7.0) and diluting it appropriately. The cobalt content of the samples was determined by routine calibration of the samples against standard solutions (0.1μM-0.8μM) as described for the zinc assay. The following atomization program at 240.7nm was employed for cobalt assay using 5μl aliquots: 50s dry at 98°C, 35s ash at 600°C, atomize at 2000°C, 2s hold, ramp rate 450°C/s. Absorption of each standard was the average of at least 3 readings and for protein samples the average of at least 8 readings was taken.

Endonuclease activity: Activity of endonuclease was assayed by monitoring the conversion of covalently closed circular Colicin (Col E_1) plasmid DNA having one Eco RI site, to the linear form. Plasmid pBR322 DNA substrate contains plasmid dimers and hence has two cleavage sites for Eco RI. For all assays conducted at low concentration of enzyme (pM), DNA substrate at appropriate concentrations was incubated with enzyme at 37°C. All assays were performed in assay buffer containing 5mM $MgCl_2$. The reaction was quenched with 100mM EDTA in electrophoresis buffer [0.05M Tris base(pH 7.0), 0.02M sodium

acetate, 0.018M sodium chloride] containing 50% sucrose, and 0.5% bromophenol blue with subsequent cooling to 0°C. Assays performed at high concentrations were conducted using 10^{-8}M DNA (sites) and enzyme at 37°C for 20s. To determine the site-specificity of enzyme, lambda phage DNA (26nM sites) was incubated with 150nM enzyme at 37°C for 30sec in assay buffer with 5mM magnesium chloride. The reaction was quenched as described before.

Agarose gel electrophoresis: Reaction mixtures after quenching were subjected to electrophoresis as follows. About 0.3µg of DNA was electrophoresed using 1% horizontal agarose slab gels (about 5mm thick) at 3V/cm for 3-5h in electrophoresis buffer. DNA was visualized by staining with ethidium solution for 3-18h and photographed under ultraviolet light using a Polaroid 600 camera containing a red filter and 665 positive/negative film.

Conversion of supercoiled DNA substrate to linear product was seen in gels as a decrease in intensity of the supercoiled band and an increase in the linear band. The substrate contained some nicked DNA as starting material which remained more or less constant after each assay. Plasmid pBR322 DNA contained supercoiled dimers as the substrate. Therefore the product appeared as two bands, one corresponding to DNA cleaved once and the other band corresponding to DNA cleaved twice. For

quantitative work this DNA was not used.

Quantitation of product: Photographic negatives were scanned at 500nm using a Varian Cary 219 spectrophtometer equipped with gel scanner attachment. Intensity of bands corresponded to the amount of DNA present which was proportional to the weight of each peak. The amount of product formed was given as the percentage of linear DNA formed.

RESULTS

Correlation of zinc content and activity of the restriction endonuclease Eco RI obtained from an overproducing bacterial strain: Overproduction of Eco RI by the strain M5248/PSCC2 leads to a 50-100 fold increase of enzyme over that of wild type cells. (5). Figure 1a shows the concentration of the protein as it was being eluted from the phosphocellulose column. The two peaks have about 2.5×10^{-5}M and 1×10^{-5}M protein monomer respectively. Fractions 30-80 contained Eco RI activity. Zinc content of the fractions was determined as shown in Figure 1a and the concentrations were found to be range from 1×10^{-5}-0.5×10^{-5}M in the active Eco RI fractions. A noticeable feature, however, is the absence of stoichiometric amounts of zinc in most of the active fractions.

The low zinc content associated with the protein led us to believe that the enzyme was less pure and/or it lacked the

necessary amount of zinc. Therefore, another enzyme preparation was carried out, in order to improve the purity, using phosphocellulose chromatography twice. Figure 1b shows a second enzyme preparation which was purified twice. The protein was pure as judged by SDS-gel electrophoresis. Fractions 95-110 which contain about 3-4μM protein are associated with almost stoichiometric amounts of zinc. Figure 1b further illustrates that these samples are the most active Eco RI fractions as indicated by percent activity (80-100%).

Direct exchange of intrinsic zinc with cobalt (II): The substitution of intrinsic zinc with cobalt(II) was achieved by dialysing the native enzyme with an excess of cobalt(II). In fact, it had been already carried out successfully using commercially available Eco RI (11). In this experiment, the native enzyme (1.2μM) was dialysed against assay buffer (pH 7.4 at 25°C) containing 2.5mM $CoCl_2$. Nitrogen gas was continuously bubbled through the dialysis buffer to avoid oxidizing conditions. A sample was taken out at every third change of buffer containing the cobalt salt, and dialysed against metal free buffer to remove extraneous metal (6 changes over 2 days). Each sample was examined for cobalt and zinc content and assayed for activity.

An enzyme fraction (number 95) containing stoichiometric levels of zinc, as illustrated in figure 1b, was selected for the

direct exchange. Figure 2a shows the time course of the exchange of two metals. Actually, the removal of bound zinc was relatively faster than the incorporation of cobalt. In fact, about 80% of the bound zinc was removed after 3 changes of dialysis buffer containing cobalt. This partially metal free protein which was presumably stable in solution, as shown by protein assays, incorporated sufficient amounts of metal over 6 days to gain an increase in activity corresponding to the amount of bound metal. A sample collected after one day had 20% of each metal and was the least active. The two preparations that follow showed more incorporation of cobalt and less zinc as well as an increase in catalytic activity. It is remarkable that after 4 days a sample which acquired some zinc, in addition to cobalt, to a total metal content of about 80%, was ~ 130% active. Furthermore, sample 5 which apparently incorporated only about 20% cobalt and gained 30% zinc is much less active (65%). Finally, the last sample containing 80% bound cobalt and 20% zinc has an activity (140%) much higher than that of the native protein (day 0) with 100% activity. This experiment illustrates that the intrinsic zinc ion can be exchanged with cobalt without any apparent loss of protein due to precipitation or denaturation. Furthermore, catalytic activity correlates remarkably well with tightly bound metal content and also cobalt metal incorporation renders the enzyme more efficient catalytically.

Site-specificity of the cobalt-substituted enzymes: Once the substitution with the cobalt metal was achieved it was necessary to determine whether the specificity of DNA recognition seen with the native enzyme was unaltered. Plasmids Col E_1 and pBR322 contain only one specific Eco RI site. Therefore we selected lambda phage DNA substrate containing five Eco RI recognition sites. Figure 2b illustrates the cleavage pattern of lambda DNA by the native enzyme and the cobalt substituted enzymes obtained by the direct exchange method. An identical fragmentation pattern of the phage lambda DNA occurs as a result of digestion with both native and cobalt substituted enzymes.

Metal reconstitution through apo enzyme: Using fraction 59 (Figure 1a) purified from the overproducing strain, metal reconstitution experiments at 2.5μM protein concentration were carried out. The sample, fraction 59, was dialysed against metal free buffer containing 0.1M Tris base (pH 7.5) and 0.2M NaCl (7 changes, 4 days). Concentration of protein was 2.5μM and the zinc concentration of the dialysis buffer was below 10^{-8}M. The sample which contained the least amount of zinc was assayed for activity. The apo enzyme was then reconstituted with stoichiometric amounts of metals and incubated overnight at 4°C before assaying for activity.

Figure 3 pictorially represents the results of this experiment. Plasmid pBR322 DNA was chosen here as the substrate

and gel electrophoresis showed a band pattern that was easy to observe. The active enzyme converts the supercoiled substrate (Form I) to linear (Form III) dimers and monomers. Formation of linear monomer is an indication of high activity of the enzyme. Here, metal removal made the apo enzyme less active than the native form. It is apparent that the cobalt reconstituted enzyme is more active than both the native and the zinc reconstituted enzyme as can be seen from the appearance of Form III monomer and the disappearance of Form I supercoiled DNA. Apo enzyme shows the least activity. These observations therefore suggest that the enzyme displays a requirement of a metal for maximal activity in the site-specific cleavage reaction.

Table I shows the same correlation of metal content and enzyme activity, observed in a metal reconstitution experiment performed with commercially available Eco RI. Here the microdialyser system 1200MD (BRL) was used. The protein was diluted in assay buffer to 0.8μM and 15μl samples were placed in several sample wells on to a dialysis membrane and the wells were covered on top. Dialysis buffer containing 5mM o-phenanthroline (pH 7.2 at 25°C) was passed continuously through the samples (about 4 liters total, 5-7 days). Samples containing the least amount of zinc were assayed for activity and reconstituted with zinc(II) and cobalt(II) separately. Metals (either cobalt or zinc as a solution of the chloride salt in assay buffer at pH 7.0, 25°C) were added back to the apo enzyme so that the final pH

of the solution was 7.0. Dialysis against metal free buffer was performed in order to remove residual unbound metal content.

It is clear from the activities that metal removal inactivates the enzyme resulting in its inability to cleave the substrate. This suggests a requirement of the metal for the activity of the protein. Addition of the metal reactivates the enzyme but not completely. Highest percent reactivation found is 48% observed for the cobalt reconstituted enzyme. Zinc reconstituted enzyme has 30% activity. The presence of the bound cobalt, activates the enzyme more than does the zinc ion. Also it is interesting to note here that upon storage at 4°C a sample of apo enzyme acquired adventitous zinc owing to contamination and gained some activity. Contamination is difficult to prevent as well as to detect at low concentration of protein.

Kinetics of cleavage reaction of cobalt and zinc enzymes: Kinetics of the cleavage reaction was investigated using samples obtained by direct exchange of cobalt with the native zinc ion. Three cobalt(II) substituted enzyme samples having different metal contents were compared with the native protein. Col E_1 DNA (3nM-12nM sites) was used as the substrate with 50pM enzyme. Incubation was done at 37°C for 3 minutes in assay buffer containing 5mM Mg^{+2} and the products were analysed as mentioned previously.

After determining the percentage product formed in each sample, the initial velocity of the reaction was calculated as

follows. Concentration of product in each reaction mixture was determined by using the percentage of product formed and the initial concentration of substrate. The product concentration was then converted to the rate of product formation (nM/Min). Double reciprocal plots were obtained by plotting the inverse of initial velocity against the inverse of initial substrate concentration.

Figure 4 illustrates a double reciprocal plot of initial velocity of cleavage reaction of metal substituted enzymes versus substrate concentration. The native zinc enzyme has an apparent dissociation constant (Km) of 4.3nM (data not shown) which agrees well with the reported values of 3nM by Woodhead and Malcolm (12) and 8nM by Modrich (13) for plasmid Col E_1 DNA. Activities of samples 1, 3 and 6 (see Figure 2a) having differing levels of intrinsic zinc and cobalt were compared with the native enzyme. Table II shows the values obtained for $Km_{apparent}$, defined as the dissociation constant for the binding of the enzyme to the substrate under steady state conditions. With the increase in incorporation of cobalt into the enzyme a gradual increase in the $Km_{apparent}$ and hence a decrease in affinity of enzyme is seen. As can be seen from Table II, an increase in apparent maximum velocity of reaction seems associated with bound cobalt content. Since the cleavage reaction was carried out at comparable enzyme concentrations, these values suggest that the metal may take part in catalysis and furthermore that the bound

cobalt ion appears more efficient than zinc.

DISCUSSION

Correlation of activity and zinc content of Eco RI obtained from an overproducing strain: As mentioned previously, metal reconstitution experiments contribute greatly to proving a metal requirement for the enzyme to be active. This demonstration is made difficult by the ubiquitous nature of zinc metal which leads to contamination of samples unless precautions are taken to avoid contact with external materials (contaminating levels of zinc in the environment can be in the order of 10^{-8}M). Therefore the metal substitution experiments carried out near contaminating levels of zinc and the enzyme assays which usually require concentrations as low as picomolar have to be conducted with great care. If there are methods to obtain enzyme in high enough quantities, then working at concentrations above contaminating levels of zinc is made possible. Such large quantities of Eco RI are made available through overproduction of the enzyme by E. coli strains carrying a plasmid constructed to produce this enzyme in substantial quantities.

As can be seen from Figure 1a, the Eco RI restriction enzyme was produced in large quantity. The enzyme, however, does not contain stoichiometric levels of zinc. The lack of association of zinc could be due to several different reasons. Some

concentrated fractions could have been less pure than others even though protein electrophoresis showed one band corresponding to Eco RI. Further purification led to increased zinc levels associated with the enzyme. This is a promising observation. In fact, metalloenzymes show characteristic increases in metal content upon purification (14). It is obvious, however, that the enzyme is less concentrated in this preparation and therefore external contamination is a possibility. Partial removal of metal also can take place depending on the isolation procedure. There are instances where zinc is provided in the extract, during the isolation of a metalloenzyme. For example, during the isolation of alkaline phosphatase, zinc is added to the extract before the enzyme is precipitated using ammonium sulfate. The addition of zinc prevents the enzyme from losing its intrinsic zinc atom during precipitation (14). Furthermore, the overproducton of an enzyme may not lead to a corresponding increase in the incorporation of metal into the protein from the environment. A primary reason could be the lack of adequate amounts of available metal in growth media. The problem of metal content related to overproduction will be discussed in detail in Chapter 4.

Substitution of cobalt(II) by direct exchange: Direct exchange is a convenient way of substituting the native zinc atom of a metalloenzyme by another transition metal. The advantage

here is the relative stability of the native enzyme as compared to the apo enzyme. A metal which is an integral component may impart a certain amount of structural stability to an enzyme. Therefore it may be difficult to establish the exact conditions under which the apo enzyme will be stable in solution. Direct exchange in the presence of excessive amounts of another metal usually leads to the replacement of the native zinc ion with the second metal without the loss of protein due to denaturation.

According to Figure 2, it is clear that the bound zinc ion in Eco RI can be exchanged for cobalt. The exchange of metal led to a tightly bound cobalt-enzyme complex because the removal of excessive cobalt after direct exchange did not eliminate the fraction of the metal which was apparently incorporated into the enzyme. Loosely bound metals are usually removed by dialysing the enzyme against 3-5 changes of metal free buffer. Furthermore, as can be seen from Figure 2, an increase in activity is associated with incorporation of cobalt. A change in activity brought about as a result of a change in the bound metal implies the likely involvement of the intrinsic metal ion in the catalytic reaction. Furthermore, the cobalt ion at the active center is a more efficient catalyst than the native zinc atom. A reducing atmosphere was maintained throughout the dialysis procedure. Therefore, it is very likely that the metal ion was incorporated as cobaltous ion even though air oxidation to Co(III) cannot be completely ruled out.

The native zinc enzyme used in this experiment had stoichiometric zinc levels associated with it. It is noticeable, however, that about 70% of the native metal was removed during the first few changes of dialysis. This could very likely be an indication that the protein contained only 30% intrinsically bound zinc and that during the second purification step (Figure 1b) acquired some loosely bound zinc. The incorporation of cobalt was apparently slow. It is possible that the apo protein although stable in solution had a slightly different conformation and that it incorporated cobalt slowly and in doing so returned back to its active form. Nevertheless, the metal bound tightly to the enzyme and caused an increase in activity.

Site-specificity of the cobalt substituted enzymes: Activity assays using Col E_1 DNA substrate showed that the incorporation of cobalt enhanced the activity of the enzyme. The substitution of a different metal, however, may lead to an alteration of the recognition of specific sites. In order to determine that the integrity of the site recognition was preserved, it was necessary to use phage lambda DNA as the substrate. This DNA has five Eco RI recognition sites. According to Figure 2b the fragmentation pattern remains the same when the cobalt atom is substituted for the native zinc atom. Therefore, the replacement of native zinc with cobalt does not interfere with the properties of the enzyme which make it specific for its particular six base pair recognition site.

Metal reconstitution using apo enzyme: Reconstitution of the apo enzyme with zinc to obtain active enzyme is important in demonstrating the presence of a metal site which is an integral part of a protein. According to Figure 3 which pictorially represents the activities of Eco RI samples containing different metal contents, there is a definite relationship between the activity and the metal content of the enzyme. The catalytically inactive apo enzyme was reactivated by the addition of zinc back to the enzyme. Furthermore, the apo enzyme was reactivated to different extents by two different metals i.e. zinc and cobalt. One feature of this experiment, however, requires comment. At higher concentrations of enzyme the metal was removed without the aid of a chelator. The facilitated removal of zinc was apparently a consequence of the increased salt content of the buffer. High salt enhances the solubility of the protein and in this case also helped in the removal of the metal. It is known that the dialysis of some metalloenzymes against metal free buffer having lower than 10^{-8}M zinc can result in the formation of apo enzyme (15). The addition of metal, however, back to the apo Eco RI made the enzyme catalytically active again. It was assumed that the enzyme incorporated all the metal that was added stoichiometrically. Two different metals reactivated the enzyme to different extents. The cobalt ion was a better catalyst than the zinc ion.

A more quantitative determination is shown in Table 1 which shows that the apo enzyme can also be reactivated by the addition of excess metal over protein. The unincoporated metal is removed by dialysis afterwards. Here again the cobalt incorporation made the enzyme more active than zinc incorporartion. This quantitative determination proves that the metal ion is an integral part of the enzyme. Furthermore, it suggests that the metal is required for the activity of the protein and cobalt reconstitution results in an enzyme which is more active than the zinc enzyme.

The decrease in percent reaction seen in metal reconstituted enzymes may be due to several reasons. Prolonged dialysis makes the enzyme less stable and an increase in volume which might occur results in a decrease in enzyme concentration. Sometimes addition of metal leads to partial precipitation of the protein. At low starting concentrations the enzyme tends to be less stable but the reason for this is not known. Nevertheless, binding of cobalt and zinc ions is seen here as metal/enzyme ratios and relative activities indicate the formation of catalytically active enzyme again. Residual metal ion removal was not very effective using the microdialyser system. In spite of this, results again suggest the possible requirement of a metal for the activity of protein.

Kinetics of cleavage reaction of cobalt and zinc enzymes: A metal ion which is tightly bound and necessary for catalytic activity can be further examined using kinetic methods. The kinetic parameters of the cobalt enzyme are generally distinguishable from the native zinc enzyme. For Eco RI, which is a site-specific DNA binding enzyme, the catalytic reaction involves the recognition of its cognate sequence on DNA as well as hydrolysis of phosphodiester bonds. Therefore, reaction kinetics would indicate the nature of the binding of the enzyme to its specific sequence as well as the actual hydrolysis. Comparison of the kinetics of different metal substituted enzymes is useful here because a difference in either the binding of the enzyme to the substrate or actual catalytic rate implicates the involvement of the metal in binding or catalysis of bond cleavage respectively. In fact, according to Table II, a change in both these parameters is seen. These differing kinetic parameters for the two types of enzymes reflect the participation of the metal in Eco RI activity. More specifically, the increasing apparent dissociation constants associated with more bound cobalt suggest that the metal is involved in binding of the enzyme to the substrate. In this case cobalt binds poorly to DNA. This poor binding may appear to increase the apparent maximal velocity of the reaction because of the following reason. It is reported that for Eco RI site-specific cleavage reaction the rate limiting step is the release of product (13). Therefore, a metal that

binds less tightly to substrate can be thought to release the product more readily once the hydrolysis is completed. This idea is based on the assumption that the metal site either participates or stays bound to the substrate throughout the catalysis. Hence, an enzyme that binds less tightly to the substrate may appear to enhance the reaction rate.

Besides the fact that poor binding of cobalt to the substrate can release the product easily, the metal may take part in the hydrolytic reaction itself. The increase in apparent maximal velocity observed with more bound cobalt can additionally be the result of a catalytically more efficient metal ion. The kinetic parameters observed here for the enzymes containing various amounts of zinc and cobalt vary more or less within the same order of magnitude. It is very noticeable, however, that a gradual change of both apparent binding constant and maximal velocity is associated with the content as well as the nature of the metal. Therefore, we suggest that the metal is at least involved in the binding of the enzyme to the substrate. The involvement of the metal in the hydrolytic reaction is difficult to determine by these kinetic studies alone.

Variations in kinetic parameters as a result of metal substitution are commonly observed in other metalloenzymes too. For example, a comparison of the kinetics of E. coli alkaline phosphatase show that the native enzyme binds phosphates less tightly than cobalt enzyme (Dissociation constants for the zinc

and cobalt enzymes are 1.2 x 10^6 and 0.5 x 10^6M, respectively (16). There are other examples of metalloenzymes that show different affinities for substrates depending on the substituted metal. For carboxypeptidase A, depending on the substrate used, the affinities of the zinc and cobalt substituted enzymes vary (17).

An observation more relevant to our study has been made by Speckhard et al (18) using RNA polymerase which recognizes specific promotor sequences on DNA. The apparent Km value for T7 DNA is twice as high for the native Zn-Zn as for the Co-Co enzyme. They suggested that the intrinsic metal may be involved in template binding. Furthermore, the cobalt enzyme initiated transcription less efficiently than the zinc enzyme at the A_2 promotor on the T7 DNA. The promotor A_2 is one of the three major promotors on this DNA. In addition, in vitro transcription of a restriction fragment containing lac operon, by the cobalt enzyme is less sensitive to cAMP and CRP than is the native zinc enzyme (19). These findings imply that the intrinsic zinc ion in the polymerase enzyme may be involved in binding to nucleic acids. Our observation that the Eco RI enzyme containing substituted cobalt has a higher apparent Km than the zinc enzyme therefore suggests that the metal ion in this enzyme may be necessary for substrate binding.

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Enzyme	Zinc/Enzyme	Metal/Enzyme[a]	Activity
Native	~1	-	100
Apo	0.02	-	0
Cobalt	0.02	~1.4	48
Zinc	0.02	2.2	30
Apo(3 days)	0.14	-	14

Table I: Quantitation of activity of restriction endonuclease Eco R before and after zinc removal and after metal substitution using Co (II) and Zn (II).

Microdialyser system was employed in carrying out this determination. Commercialy available native enzyme (0.4μM) was dialysed against assay buffer at 4°C (pH 7.2 at 25°C) containing 5mM o-phenanthroline followed by assay buffer lacking the chelator. After determining zinc content and activity of the apo enzyme, zinc and cobalt(II) were added back at 2.5mM concentration at pH 7.0 and incubated overnight at 4°C before determining activity. Residual metal was removed by dialysis against metal free buffer before analysis of metal content. Plasmid Col E_1 DNA (5.5nM sites) was incubated with 0.17nM enzyme and 0.1mM EDTA at 37°C for 5min in assay buffer in the presence of 5mM magnesium (II). Activity of the native protein was taken as 100%. Activity increases as ,
Apo < Zn-substituted < Co-substituted < Native protein.

It is interesting to observe that the same apo enzyme samples contaminated with small amounts of extraneous zinc regained some activity.

a: The determination of the actaul zinc content can be done to an accuracy of 1.0±0.01, but the uncertainty in the zinc to enzyme ratios could be due to the result of non-specific binding of the metal to protein.

Enzyme	(Zinc)/(Protein)	(Cobalt)/(Protein)	$Km_{apparent}$	V_{max}
Native	1.0	—	4.3	0.63
Cobalt	0.19	0.19	12.5	1.8
	0.15	0.37	16.3	1.3
	0.20	0.80	36.0	3.3

Table II: Comparison of $Km_{apparent}$ (nM) and maximal velocity of cleavage reaction (nM/Minute) catalysed by the native restriction endonuclease and the cobalt-substituted enzymes containing different amounts of metal.

The table shows that with an increase in bound cobalt content the apparent equilibrium dissociation constant ($Km_{apparent}$) increased. This suggests that the cobalt-substituted enzyme bound less tightly to DNA. On the other hand, catalytic rate increased with cobalt incorporation. Therefore cobalt in the active site may be a relatively more efficient catalyst than the native zinc metal center.

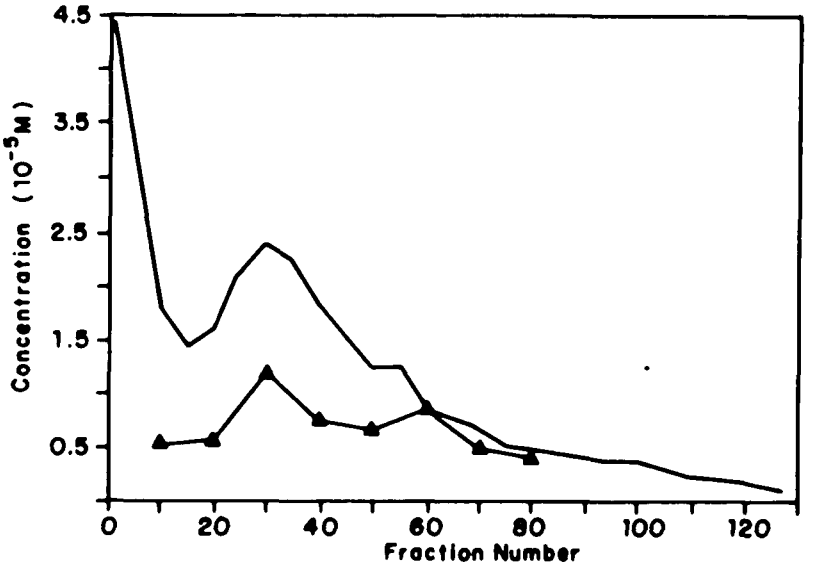


Figure 1a: Overproduction of Eco RI endonuclease using E. coli strain M5248(PSCC2) and characterization of purified enzyme by determination of protein and zinc content. ——: concentration of protein (A_{280}), ▲–▲: zinc as analysed by atomic absorption spectrometry.

Fractions 30-80 contained Eco RI activity. SDS-polyacrylamide gel electrophoresis of selected fractions indicated that they contained one band corresponding to the molecular weight of Eco RI which is 31,000d. Protein concentrations were monitored as absorption at 280nm as they were being eluted from the phosphocellulose column. Fractions were active at picomolar concentrations. However, it is obvious that most fractions lack appreciable levels of zinc, except those around 60 which contain near stoichiometric amounts of zinc. These fractions were selected for metal substitution experiments.

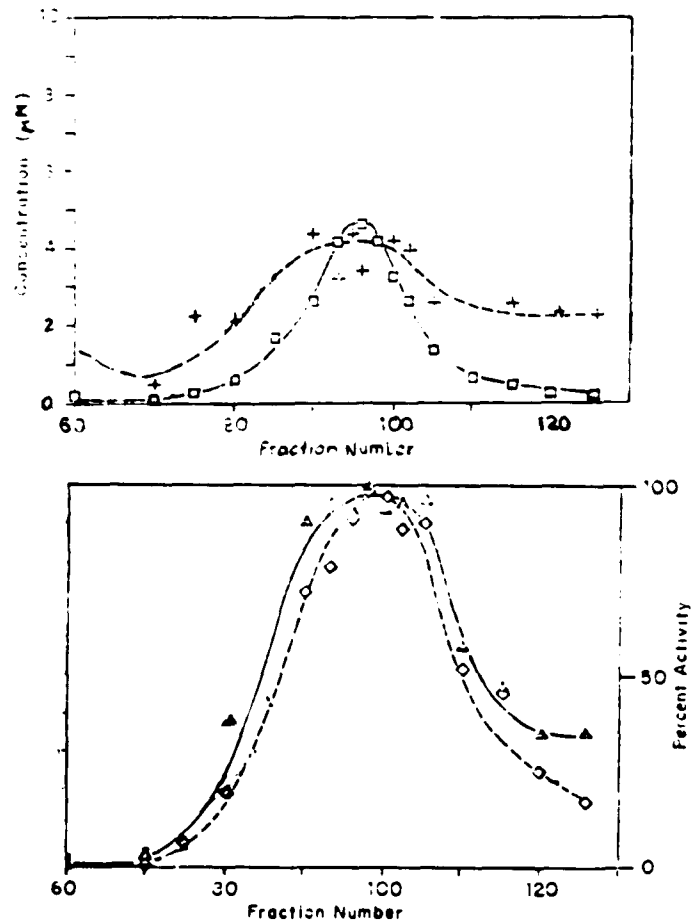


Figure 1b: Characterization of Eco RI endonuclease fractions prepared by purification using phosphocellulose chromatography twice. □—□: Protein, + —+: Zinc, and % activity at ◇-◇: high and ▲-▲: low substrate concentration.

Most active fraction was taken as 100%. Activity of the fractions was determined at two concentrations of substrate. For high concentration determinations plasmid Col E_1 DNA substrate (78 nM sites) was incubated with 40nM enzyme with 5mM magnesium (II) in assay buffer for 20s at 37°C. Low concentration incubations had 5nM sites of the same DNA with 70pM enzyme and 5mM magnesium (II) in assay buffer. The reaction was carried out at 37°C for 1min. These fractions contain lower concentrations of protein than the previous preparation (cf. Fig.1a). But it is noticeble that zinc is associated with active Eco RI fractions which range from 80-120.

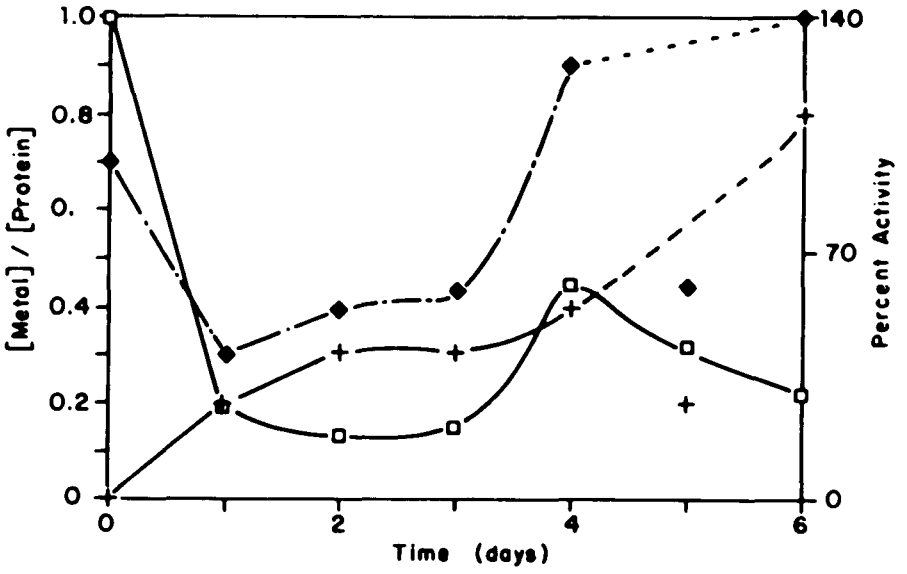


Figure 2a: Direct exchange of native zinc ion with Co(II): Time course of zinc removal and cobalt substitution and enzyme activity: □-□: bound zinc(II), +-+: bound cobalt (II), ◆-◆: activity.

Activity is given as the percentage of linear plasmid Col E_1DNA product formed. The native enzyme was taken as 100% active. Aliquots of native enzyme (1 ml) were dialysed at 4°C against zinc free assay buffer containing 2.5mM cobaltous chloride. Nitrogen gas was bubbled through the dialysate to minimize air oxidation. Samples were taken out daily at every third change of dialysis buffer and unbound cobalt was removed by further dialysis against metal free assay buffer at 4°C. All samples were examined for zinc, cobalt and protein content and activity. Plasmid Col E_1DNA(5nM sites) was reacted separately with each enzyme sample at 70pM concentration in assay buffer with 5mM magnesium chloride for 1minute. Cobalt incorporation is slow at first but with increase in bound cobalt an increase in activity is seen. Native zinc is removed relatively fast but the activity appears to correspond to the total metal content of the samples. One feature worth noticing here again is that the cobalt enzyme (day 6) is more active than the native protein (day 0) (cf. Figure 3).

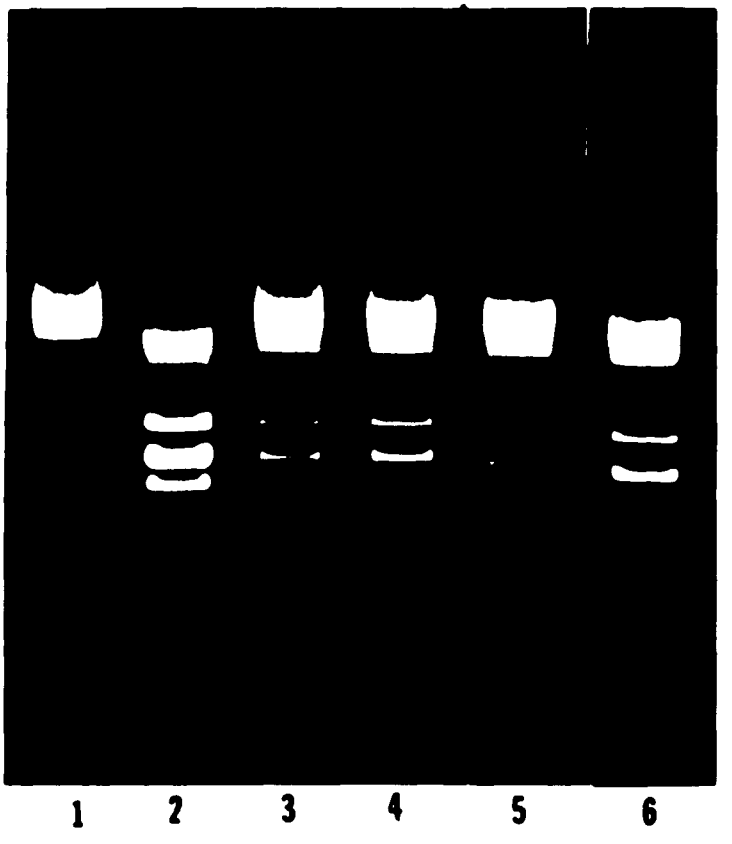


Figure 2b: Comparison of the site-specific recognition and cleavage pattern of DNA by the native Eco RI and cobalt substituted enzymes.

Lane 1 shows undigested lambda phage DNA. Lanes 2-4 show lambda phage DNA cleaved by the cobalt substituted enzymes containing 80, 30 and 20% substituted cobalt respectively. Lanes 5-6 contain lambda phage DNA cleaved using native Eco RI. The DNA (26nM sites) was incubated with 150nM enzyme at 37°C for 30sec in assay buffer with 5mM magnesium chloride and the products were analysed by agarose gel electrophoresis.

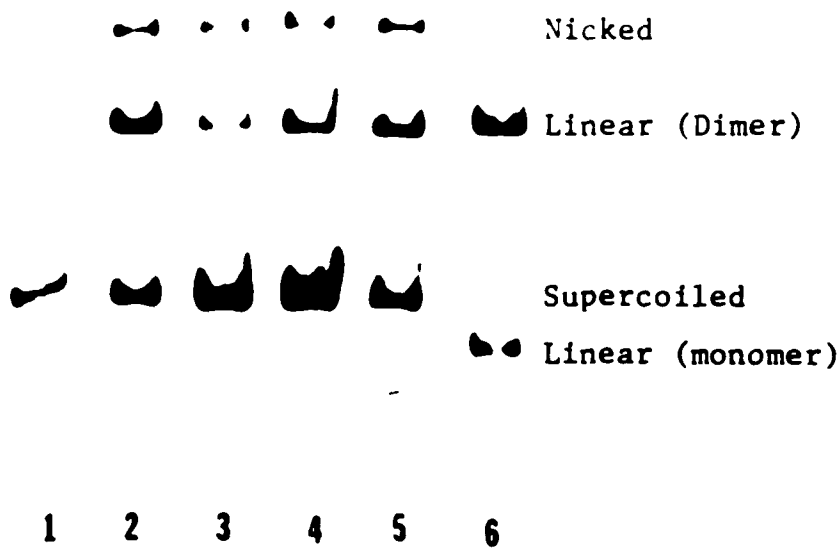


Figure 3: Site-specific cleavage reaction of plasmid pBR322 DNA by Eco RI endonuclease as seen by agarose gel electrophoresis of reaction products.

Lane 1...DNA substrate prior to cleavage, Lanes 2-6 ... activity of enzyme, 2... containing stoichiometric zinc, 3 & 4...with no detectable zinc content(apo), 5....reconstituted after removal of zinc by additon of stoichiometric zinc (II), 6 ...reconstituted after removal of zinc by addition of stoichiometric cobalt (II). This reconstitution experiment was done using fraction number 59 (see Fig. 1a) which contained stoichiometric quantities of zinc. Native zinc ion was removed by dialysing the enzyme against buffer containing 0.1M Tris base (pH 7.5 at 25°C) and 0.2M NaCl. Metals were added back as solutions of the same buffer at pH 7.0 at 25°C. A similar volume of same buffer was added to the apo enzyme but without metal as the control. Lanes 3 & 4 show the activity of apo enzyme before and after the addition of buffer respectively. Incubations were done at 37°C for 5 min with 5nM DNA (sites) and 0.7pM enzyme in assay buffer containing 5mM magnesium chloride. Note that with increase in enzyme activity formation of linear monomer product increases. Here activity appears to increase as,
Apo enzyme < Zn-reconstituted < Native protein < Co-reconstituted

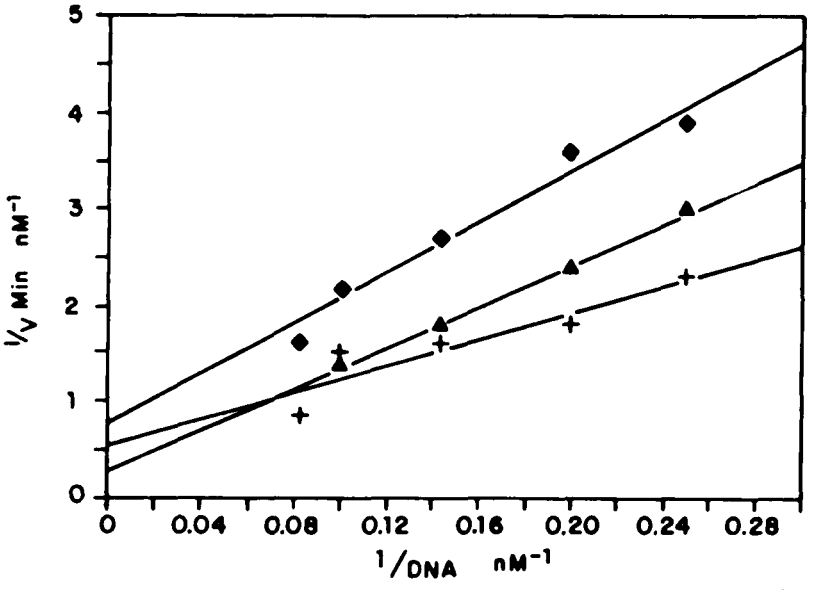


Figure 4: Double reciprocal plot of initial velocity of cleavage reaction of plasmid Col E_1 DNA by Eco RI (cobalt substituted) vs DNA substrate concentration: +—+: with 20% bound, ◆-◆: 30% bound and ▲-▲: 80% bound metal.

DNA (3nM-12nM) was incubated with enzyme (50pM) at 37°C in assay buffer with 5mM magnesium(II) for 3min. Initial velocity was taken as the concentration of linear product formed per minute of reaction. Here an increase in the apparent dissociation canstant (Km) is seen with incorporation of cobalt but maximum velocity appears to increase with bound cobalt.

CHAPTER 3

EFFECTIVENESS OF THE LIGANDS THAT COORDINATE TO ZINC AS INHIBITORS OF THE SITE-SPECIFIC CLEAVAGE REACTION OF RESTRICTION ENDONUCLEASE Eco RI.

INTRODUCTION

Ligands that coordinate to zinc with high affinity are commonly used in investigating zinc metalloenzymes and the characteristic reactions they catalyse. If an intrinsic zinc ion in an enzyme is involved in catalysis, then a ligand with high affinity for zinc may act as an inhibitor of the reaction by coordinating to the metal. The coordinated ligand could either remove the metal from the enzyme site or stay bound to the protein thus hindering any further progress of the catalytic reaction (1). Therefore, metal chelators may often be useful in investigating the native zinc enzyme in solution under physiological conditions.

Ligands that coordinate to a zinc atom of an enzyme can be employed to examine the metal center of the enzyme in a variety of ways. Metal ion can be removed by the use of ligands to obtain the apo enzyme which can be investigated for its structure and catalytic activity. In addition, metal chelating agents have been used as inhibitors of the reactions catalysed by metalloenzymes in order to study the active site structure and

the mechanism of reaction. The proteases, carboxypeptidase A and thermolysin for example, have been examined using N-phosphorylated amino acid derivatives (3). These and other similar types of investigations have led to the speculation that all zinc proteases may have similarities in their active sites and their mechanisms of action (3). Furthermore, sulfonamide derivatives have been used in examining the active site of carbonic anhydrase (4,5,6). In fact, inhibition by the sulfonamide derivative acetazolamide, has become an accepted criterion for the active-site directed nature of any process associated with the enzyme (7). Kinetic studies with anions show that the same enzyme, which catalyses a reversible hydration-dehydration reaction, is inhibited noncompetitively during CO_2 hydration (8) and competitively during the dehydration reaction (5). These investigations contributed much to the elucidation of the mechanism of carbonic anhydrase action involving its intrinsic zinc ion.

We set forth to carry out a similar type of study using restriction endonuclease Eco RI, since this enzyme contains a tightly bound zinc atom as described in Chapter 2. Ligands such as cyanide, azide, sulfanilamide and 4-(2-pyridyl azo) resorcinol (PAR) were employed in this study. These ligands are known inhibitors of other zinc metalloenzymes. The kinetics of the Eco RI endonuclease cleavage reaction was examined in the presence of these ligands in order to determine further whether the zinc ion

associated with the enzyme plays a role in catalysis.

Eco RI restriction endonuclease reaction has been previously reported to be inhibited by DNA binding agents such as distamycin A and actinomycin D (9,10). These compounds were indicated to inhibit the enzyme activity by interacting with the substrate DNA specifically at the regions flanking the Eco RI recognition site. Furthermore, the enzyme itself was observed to be competitively inhibited by polynucleotides not containing the Eco RI recognition site (11). This investigation suggests that Eco RI is competitively inhibited by nonspecific DNA. In this chapter I report the inhibitory studies carried out on the restriction endonuclease Eco RI using ligands that coordinate to zinc. Based on these observations a role for the metal ion is suggested.

EXPERIMENTAL PROCEDURES

Materials

Bacterial strains, Enzymes: These materials were either purchased or prepared as described in Chapter 2.

DNA: Plasmid Col E_1 DNA and ϕx174 DNA were purchased from Sigma Chemical Co. and BRL, respectively. These DNA samples were dialysed and stored for use as described in Chapter 2.

Chemicals: [4-(2-Pyridyl azo)] resorcinol (PAR) and potassium cyanide were obtained from Sigma Chemical Co. and

Mallinckrodt Inc., respectively. Sodium azide, ethylenediamine, 2-mercaptoethanol, acetonitrile and sulfonilamide were from Fisher. Other chemicals were obtained as described in Chapter 2. Stock solutions of all compounds used as inhibitors were prepared as 10mM solutions in assay buffer containing 0.1M Tris (pH 7.5 at 25°C), 0.05M NaCl, 1% Glycerol and 0.01% Tritonx100.

Other materials: Micro centrifuge tubes, dialysis tubing and plasticware were all cleaned as mentioned in Chapter 2.

Methods

Preparation of Eco RI endonuclease: The enzyme was prepared as described in Chapter 2.

Assay of endonuclease in the presence and absence of inhibitors: Plasmid Col E_1 DNA (5nM sites) in assay buffer was incubated with Eco RI (0.1-1nM) at 37°C for 2-10 minutes with or without 2mM inhibitor and 5mM Mg^{2+}. In some cases the enzyme was preincubated for 5-10minutes at 37°C before the addition of the DNA substrate and Mg^{2+}. The reaction was quenched by addition of 60mM EDTA and 0.1% bromophenol blue dye containing 50% sucrose in assay buffer and cooling the solution to 0°C. The concentration of 4-(2-pyridyl azo) resorcinc. (PAR) was measured using the absorbance at 415 nm with an extinction coefficient of $2x10^4M^{-1}cm^{-1}$ (12). Other inhibitors were made by dissolving the

appropriate amounts of the compounds in assay buffer to a final stock concentration of 10mM.

Agarose gel electrophoresis and quantitation of product: These procedures were carried out as described in chapter 2.

Kinetics experiments: Plasmid Col E_1 DNA (2-12nM sites) was incubated with 100pM enzyme with different fixed levels of inhibitors (0.6-1.2mM). The incubations were done for 1-10 minutes at 37°C with 5mM magnesium (II) and the reactions were quenched as mentioned in chapter 2.

Equilibrium dialysis experiments: Plasmid Col E_1 DNA (5nM sites) was dialysed against a 2mM solution of the PAR ligand in assay buffer overnight without changing the dialysate. The concentration of PAR outside and inside the dialysis bag was measured using absorption at 415 nm as described before. The concentration of DNA was measured as the absorption at 260nm using an extinction coefficient of 6600 M^{-1} cm^{-1} nucleotides.

RESULTS

Inhibitory effects of different ligands that bind to zinc on the Eco RI activity: Table 1 shows the effect of various ligands that bind zinc, on the site specific cleavage reaction of Eco RI.

Linearization of the circularly closed plasmid Col E_1 DNA by Eco RI was examined in the presence and absence of 2mM concentration of several ligands for zinc. It is clear that these ligands inhibit the reaction to varying extents. The extent of inhibition does not seem to depend entirely on the affinity of the ligand to zinc. Trends can be seen, however, based on the charge of the ligand. Those ligands that may coordinate to the metal as negatively charged species (Azide, Cyanide, 2-6-DPA, Sulfonilamide and PAR) appear to be better inhibitors of the reaction irrespective of their size. Out of the neutral ligands used, acetonitrile, which is small, is a good inhibitor. In some cases the enzyme was preincubated with the ligand at 37°C before the addition of the DNA substrate. Preincubation caused more potent inhibition by all the ligands.

The substrate used in these experiments, the plasmid Col E_1 DNA, contained about 30% form II (nicked) DNA as the starting material in addition to the supercoiled form. A significant change in the percenage of this form was not seen in the presence of inhibitors.

Kinetics of inhibition of cyanide ligand on Eco RI site-specific cleavage reaction: Two of the more effective inhibitors, cyanide and PAR, were selected in order to study the kinetics of inhibitory action. Figure 1a illustrates a double reciprocal plot of initial velocity against DNA substrate

concentrations, at different fixed levels of cyanide ligand. It is clear that these plots do not converge to any one point. On the other hand, the intercepts of these lines which correspond to the reciprocal of the apparent maximal velocity, when plotted against the reciprocal of inhibitor concentration, seem to produce a straight line as can be seen from Figure 1b. This line cuts the x axis at a value of about 1.2 which corresponds to a cyanide concentration of 830μM. This value represents the probable apparent inhibitory constant (Ki_{app}) of cyanide for the cleavage reaction.

Kinetics of inhibition of Eco RI site-specific cleavage reaction by PAR ligand : Figure 2a shows the double reciprocal plots obtained by plotting the initial velocity of reaction against substrate concentration at different fixed levels of PAR ligand. Here again, the anomalous behavior of the enzyme in the presence of the inhibitor is observed. As can be seen from Figure 2b, apparent straight line graphs were obtained by plotting the y intercepts from Figure 2a (reciprocal of the apparent maximal velocity) against the reciprocal of inhibitor concentration. The straight line cuts the x axis at a value close to 1 indicating a Ki_{app} of about 1000μM for the PAR ligand.

Equilibrium dialysis of DNA against PAR : A 2mM solution of PAR was allowed to equilibrate with DNA contained inside a

dialysis bag. This experiment was carried out at a DNA to PAR ratio of 5nM Eco RI sites and 2mM ligand. At this concentration ratio, the binding of PAR to DNA if occurred, could be easily detected by measuring the ligand concentration outside and inside the dialysis bag. This is possible because DNA provides many potential sites for the binding of PAR. The concentration of the ligand inside and outside the dialysis bag was the same indicating that PAR does not bind to DNA. Since the inhibitory experiments were carried out at a similar DNA to ligand ratio, the results suggest that the inhibitory effects observed with PAR ligand are the consequence of the ligand interacting with the enzyme rather than with the DNA substrate.

DISCUSSION

Inhibition of the site-specific cleavage reaction of Eco RI by ligands that coordinate to zinc : The site-specific cleavage reaction of Eco RI involves the recognition of a specific DNA sequence by the enzyme with subsequent hydrolysis of the phosphodiester bonds. The cleavage reaction requires the presence of magnesium(II) ion. The ligands chosen to investigate the Eco RI cleavage reaction do not have a high affinity for magnesium ion. Furthermore, the concentration of magnesium in the reaction mixture was 5mM, whereas the inhibitor concentration was at most 2mM. The fact that these ligands, which have a high affinity for zinc, inhibit the reaction, implies that a zinc ion

may be involved in the catalytic reaction. The inhibitors therefore very possibly interfere with the reaction by coordinating to zinc.

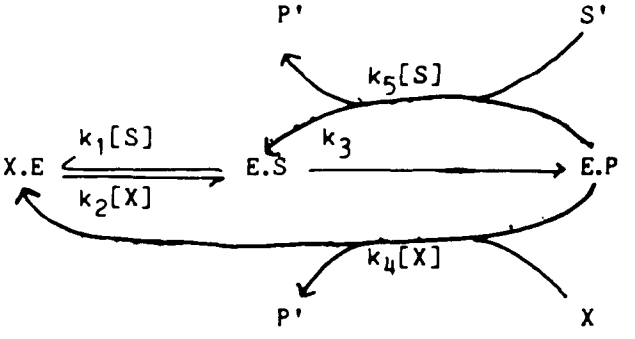
A nicked intermediate is formed if the enzyme cuts only one strand on the double helix of the supercolied substrate. No significant accumulation of a nicked intermediate is seen in the presence of the ligands. This suggests that the inhibitors do not cause inefficient cleavage thereby making the enzyme to release the product early. The enzyme is active as the dimer at catalytic concentrations (13). Therefore, the binding of an inhibitor to either subunit probably prevents the activity of the enzyme as the dimer.

The fact that the negatively charged ligands seem to be better inhibitors irrespective of their size indicate that the enzyme active site may have a positively charged environment which is fairly accessible. The ligand 2-mercaptoethanol is a poor inhibitor of Eco RI. Sulfur ligands, however, usually have a high affinity for zinc and are therefore potent inhibitors of many zinc metalloenzymes. An observation similar to ours, on the other hand, has been reported for the class of zinc metalloenzymes, neutral proteases, which are not inhibited by mercaptoethanol (14). Preincubation of the enzyme with the inhibitor alone seems to enhance the inhibitory effects of the ligands. This may be possible as a result of a slight increase in the accesibility of the metal site. On the other hand, some

of the ligands may bind at first at a site different from the metal site and then orient themselves properly to coordinate to the metal. Some derivatives of sulfonilamide have been reported to bind to carbonic anhydrase in these two modes (15,16).

Kinetics of inhibition: Involvement of the zinc ion in the cleavage reaction was examined using kinetic methods. Double reciprocal plots (Figures 1a and 2a) showed anomalous behavior in that the lines did not converge to one point. Furthermore, at higher substrate concentrations, the kinetics tend to be non-linear. This may be the result of ligands acting as both competitive inhibitors and activators of the reaction. This type of effect by an inhibitor on a reaction is usually seen on a double reciprocal plot, when the rate limiting step of the reaction is the release of the product (17). Straight line graphs, however, can be obtained by replotting the data (Slope or Intercepts vs Inhibitor concentrations) from the double reciprocal plots.

According to the following modified Briggs-Haldane scheme (17),



where X represents the inhibitor, S, the substrate, E, the enzyme and P' the product.

The following kinetic equation can be written under steady state conditions.

$$\frac{V_m}{v} = \frac{k_3.E_t}{v} = 1 + \frac{k_2[x]+k_3}{k_1[S]} + \frac{k_3(1/k_5-1/k_1)}{k_4[X]/k_5+[S]} \qquad (1)$$

At low substrate concentrations when $k_5 = 0$,

equation 1 is reduced to,

$$\frac{k_3.E_t}{v} = \frac{V_m}{v} = 1 + \frac{k_3}{k_4[X]} + \frac{k_2[X]k_3}{k_1[S]}$$

When 1/v is plotted against 1/[S], intercepts on y axis are equal to,

$$1/V_m + k_3/k_4V_m.X$$

Therefore, a plot of y intercept vs reciprocal of inhibitor concentration is a straight line which cuts the x axis at k_4/k_3. (see Fig.1b and 2b). The value for k_3/k_4 is the inhibitor concentration which reduces the maximum velocity to half of the

limiting maximum (Apparent inhibitory constant, Ki_{app}).

If for example, the zinc ion is involved in the Eco RI cleavage reaction, a ligand that can coordinate to zinc may inhibit the reaction. The same ligand may compete for the zinc ion during the release of product as well. Eco RI endonuclease activity is rate limited by the release of the product (13). Competition with the product for the zinc atom by the ligand will be seen as an activation of the reaction if the product release is the rate determining step. This step is illustrated in the modified Briggs-Haldane scheme above, as $k_4[X]$, where the inhibitor (X) competes with the enzyme-product complex (E.P) to form the enzyme-inhibitor complex (X.E). If a substrate can replace the inhibitor more easily than it replaces the product from the enzyme, then the reaction rate may be accelerated in the presence of an inhibitor. For example, succinic dehydrogenase is activated by preincubation with monovalent anions, which is caused by the displacement of the firmly bound oxaloacetate from the enzyme. The oxaloacetate is otherwise so firmly bound that it remains through the isolation procedure (18). Furthermore, the inhibitory action of glutarate on the reaction between alanine and aspartate aminotransferase could be overcome by adding chloride. There is a direct positive correlation between the amount of glutarate displaced by the chloride anions and the rate of transamination (19). In an analogous way, both the ligands used in the kinetic experiment of Eco RI seem to act as

activators. But at the same time they act as inhibitors of the cleavage reaction. Therefore it is likely that the zinc ion may be directly involved in the site-specific cleavage reaction of Eco RI.

The degree of inhibition by these ligands may be compared to the results obtained with other enzymes. The apparent inhibitory constants of cyanide and PAR for the Eco RI site-specific cleavage reaction are 830 and 880μM respectively. (Figures 1b and 2b). Cyanide ligand is a potent inhibitor of the zinc enzyme carbonic anhydrase. Carbonic anhydrase activity is reduced by 50% in the presence of 3.7μM cyanide (15). Furthermore, inhibitory studies using PAR and influenza virus reverse transcriptase show that 50% inhibition is seen at 260μM PAR concentration (20). As can be seen from these values the cyanide ligand is a more potent inhibitor of carbonic anhydrase than Eco RI endonuclease. The inhibitory effects of the PAR ligand are more or less similar for the influenza virus reverse transcriptase and Eco RI. The variable effects of these ligands on different enzyme reactions probably depend on the structure of the metal site as well as the type of reaction catalysed.

One of the inhibitors used in this investigation, namely, PAR was examined for its effect on DNA alone because it was thought that PAR could intercalate into DNA. Intercalators of DNA are known to inhibit Eco RI site-specific cleavage reaction to various extents (10,11,21 and see chapter 5). Therefore it

was important to determine whether PAR inhibition was the result of intercalation. Equilibrium dialysis experiments using Col E_1 DNA and PAR at concentration ratios comparable to those used in inhibitory experiments showed that this ligand does not bind to DNA. The inhibitory effects of PAR, therefore, are the consequence of the ligand interacting with the enzyme.

The inhibitory effects of ligands that coordinate to zinc, on a reaction catalysed by a zinc metalloenzyme suggest the participation of the metal ion in catalysis. When taken together with the metal reconstitutiton experiments, these results provide further evidence for the importance of the presence of zinc ion to the catalytic activity of the enzyme. For Eco RI site-specific DNA cleavage reaction, it is hard to determine whether the zinc ion is involved in site-specific binding to DNA and/or hydrolysis of the phosphodiester bond using the above inhibitory studies alone. Competitive inhibition by the ligands indicates that these ligands prevent the coordination of the metal to the DNA substrate. It is not clear whether the metal is required for further progress of the reaction, i.e., the hydrolysis of the phosphodiester bonds. Based on the following two observations, however, it is possible to suggest a role for the bound zinc ion. Unpublished observations in our laboratory (22) indicate that the metal may be involved in binding to DNA. It was possible to examine the binding of Eco RI in the presence of an inhibitor, since the enzyme is known to form a stable complex with its

cognate DNA (13). Nitrocellulose filter binding experiments using ^{32}P-labelled DNA in the presence of the enzyme allowed the isolation and quantitation of the stable protein bound DNA complex. The presence of the inhibitor cyanide, prevented the binding of the enzyme to DNA. Therefore it is very likely that the zinc ion may at least be involved in the binding of enzyme to DNA. Furthermore, it has been shown by Connolly et al (23) that during the site-specific cleavage reaction of Eco RI there is an inversion of configuration at the phosphorus where the hydrolytic reaction occurs. This experiment suggests a direct enzyme catalysed nucleophilic attack of H_2O at the phosphorus without the involvement of a covalent enzyme bound intermediate. It was suggested by the same authors that the phosphorus atom may be directly attacked by an enzyme bound water molecule. Our results do not rule out the possibility that the enzyme bound water molecule is coordinated to the intrinsic zinc atom. It is possible, however, that the cofactor magnesium ion which is necessary for cleavage binds to the water molecule instead of zinc. Results thus far do not allow us to describe the participation of the metal in the reaction in detail. However, we can suggest that the metal very possibly is involved in substrate binding.

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Ligand[a]	Log K[b](Ref.24)	Percent inhibition[c]	
		+preincubation	-preincubation
Azide	0.759	66	30
Cyanide	5.3	69	47
2,6-DPA	6.32	24	20
Ethylenediamine	5.75	22	12
2-Mercaptoethanol	13.71	17	6
Acetonitrile	not reported	50	23
Sulfonilamide	not reported	42	30
PAR	not reported	66	57

Table I: Effect of different ligands that coordinate to zinc on the site-specific cleavage reaction of plasmid Col E_1 DNA by Eco RI.

a: The final concentration of each inhibitor in the reaction mixture was 2mM except in the case of PAR which was 1mM.

b: Affinity constant of the ligand to zinc according to the following equilibrium,

M (Metal) + L (Ligand) M.L (24)

except in the case of 2-mercaptoethanol where the product is ML_3.

c: percent inhibition is given as the percentage of the difference between the amount of linear Col E_1 DNA (product) formed for the uninhibited and inhibited reactions. The approximate uncertainties of these values is 1±0.05.

The DNA was incubated with the enzyme (0.1-1nM) with and without the inhibitor. Reaction was started by the addition of 5mM magnesium chloride and incubated for 2-10minutes.

+preincubation: The enzyme was incubated with the inhibitor at 37°C for 5-10 minutes before the addition of DNA and magnesium.

-preincubation: The cleavage reaction was carried out in the presence of inhibitor without preincubation.

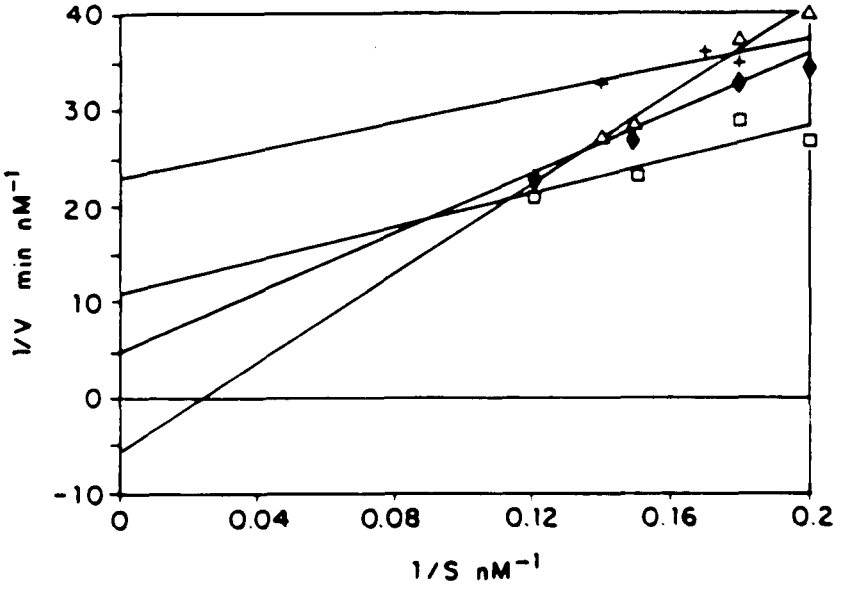

Figure 1a: A double reciprocal plot of initial velocity vs plasmid Col E_1 DNA substrate concentration in the presence of varying cyanide where: □—□—; 0mM, +—+—; 0.4mM, ◆—◆—; 0.8mM and -△-; 1.0mM inhibitor.

Plasmid Col E_1 DNA (5-10nM sites) was incubated with the enzyme at different fixed levels of cyanide (0.4-1.0mM). The incubations were done for 10minutes at 37°C with 5mM magnesium (II).

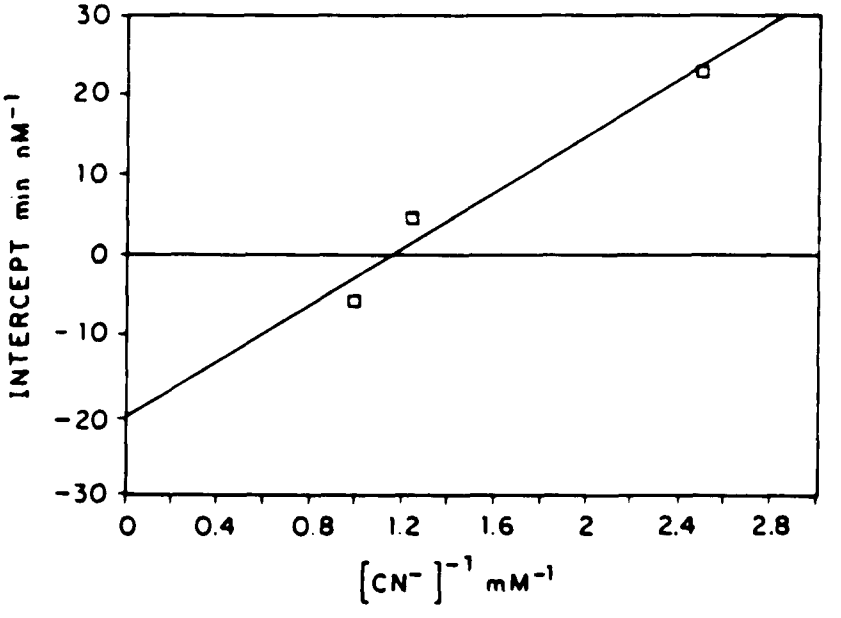

Figure 1b: Plot of intercepts from 1a vs reciprocal of cyanide concentration.

The intercepts from 1a represent the reciprocal of the apparent maximal velocity of the reaction at different concentrations of cyanide.

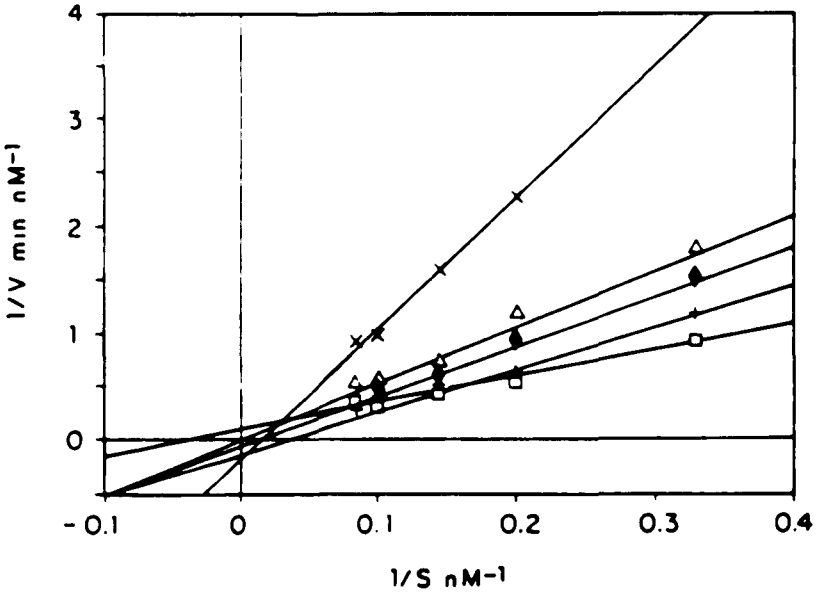

Figure 2a: A double reciprocal plot of initial velocity vs plasmid Col E_1 DNA substrate concentration in the presence of varying amounts of PAR where -□-:0mM, -+-:0.6mM, -◆-:1.0mM, -△-:1.6mM and -X-:2mM PAR.

Plasmid Col E_1 DNA (2-12nM sites) was incubated with 100pM enzyme at different fixed levels of PAR 90.6-1.2mM). The incubations were done for 1minute at 37°C with 5mM magnesium (II).

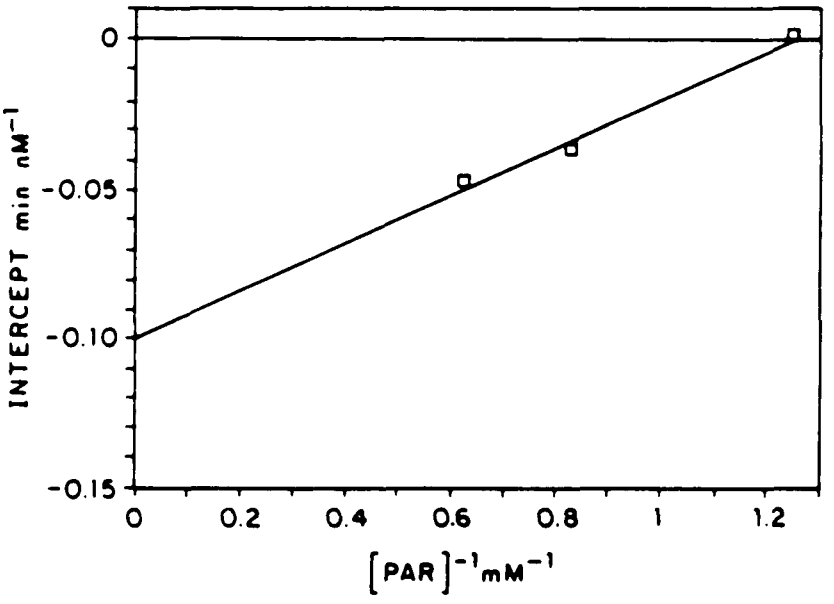

Figure 2b: Plot of intercepts from 2a vs reciprocal of PAR concentration.

Intercepts from 2a represent the reciprocal of the apparent maximal velocity of the reaction at different concentrations of PAR.

CHAPTER 4

IN VIVO COBALT SUBSTITUTION OF THE RESTRICTION ENDONUCLEASE Eco RI

INTRODUCTION

The substitution of a constitutive zinc atom in a metalloenzyme by other divalent metal ions is achieved in different ways. In vitro replacement methods include the direct exchange of the native zinc ion with another metal and the removal of zinc to obtain apo enzyme which can be reconstituted using a selected metal. In addition to these techniques, metal substitution can also be carried out in vivo. Zinc depleted growth media are enriched with another divalent metal (e.g. cobalt) and inoculated with bacteria. After sufficient amount of growth the cells are collected and the metalloenzyme is isolated. Zinc free conditions are maintained throughout the isolation and purification procedure. This technique is especially useful when the tightly bound zinc ion can not be removed by chelators. For example, in vivo cobalt substitution was essential in order to replace one tightly bound zinc atom in DNA dependent RNA polymerase from Escherichia coli (1). As mentioned in previous chapters, cobalt substitution allows the study of the enzyme in various ways such as kinetics, visible spectroscopy and electron paramagnetic resonance. Electronic spectroscopy is the commonly

used preliminary method of investigation of a cobalt substituted enzyme.

Cobalt(II) is frequently used as a substitute for zinc because of the similarity of the chemistry of the two metals. Cobaltous ion forms a large number of complexes. Many of these complexes are either octahedral or tetrahedral. Five coordinate and square complexes, however, have been observed (2). The ligand field stabilization energies favor the formation of octahedral complexes over that of tetrahedral for the d^7 cobalt(II) ion. Cobaltous ion, however, forms more tetrahedral complexes than other transition metal ions because the difference in stability between the octahedral and tetrahedral complexes is less for cobalt(II) than for other transition metals. The actual geometry obtained with given ligands depends on both electrostatic and steric factors. For example, octahedral Co(II) complexes are formed by many amine and amino acid ligands (3,4). Tetrahedral compexes are common with halide anions (5). The tendency to form complexes having varying coordination numbers is even greater for the zinc ion. Complexes having coordination numbers that vary from 2-8 have been observed for zinc. In the case of zinc complexes, however, there is no ligand field stabilization interaction. Owing to the presence of a completely filled d shell, the stereochemistry and the coordination number of the complexes are determined by ligand size, electrostatic forces and covalent bonding. Zinc complexes with coordination

numbers 4, 5 and 6 are most common (2). Complexes having a coordination number 4 can assume both tetrahedral and square planar geometries. Tetrahedral complexes can sometimes assume a distorted geometry. Trigonal bipyramid and square-pyramidal geometries are observed in five-coordinated complexes. Zinc ion forms octahedral complexes with small neutral ligands such as water or ammonia (2,6). The similarity in the stereochemical preferences of Zn(II) and Co(II) may play an important role in accepting different ligand geometries by these two ions (7,8). This property, therefore, is significant in their function and interchangeability in metalloenzymes (9).

The spectral properties of cobalt complexes have been useful in the study of cobalt substituted-zinc metalloenzymes. Unlike the d^{10} zinc ion, Co(II) ion has a d^7 configuration. Many cobalt complexes, therefore, show absorption spectra due to low energy d-d transitions. Both high and low spin complexes of Co(II) occur and the geometry around the metal can be fairly well predicted from the characteristic d-d absorption spectra. Cobaltous ion in an octahedral field usually has a molar absorbance of about 10 M^{-1} cm^{-1} (9). The pseudotetrahedral complexes, on the other hand, show more intense color with maximum molar absorbancies varying from 100-1000 M^{-1} cm^{-1} (9). Furthermore, these complexes absorb at higher wave lengths due to decreased d-d transition energies. Visible absorption spectra of cobalt enzyme can be obtained in the presence of substrate and

inhibitors as well as at different pH's. Variations of the spectrum that occur as a result of these agents are useful in studying the mechanism of enzyme catalysis.

In this chapter, I report the substitution of cobalt in vivo into the restriction endonuclease Eco RI. In vivo substitution allowed us to isolate the cobalt enzyme in relatively high concentrations to obtain a preliminary visible spectrum of the enzyme. Besides the obvious advantage of spectroscopy, in vivo cobalt substitution was carried out for another reason. As mentioned in chapter 2, the overproduction of Eco RI did not produce the enzyme containing stoichiometric zinc. This observation led us to assume that high quantities of enzyme produced may not be able to find adequate amounts of metal in the media. Therefore we decided to enrich the media with more metal. Cobalt(II) ion was selected because the enzyme can be investigated using spectroscopic methods. Additionally, any cobalt ion associated with the purified enzyme can be assumed as intrinsic because contaminating levels of cobalt in the environment are very much lower than that of zinc. As a result any cobalt associated with the pure enzyme can be assumed to have incorporated during the formation of the enzyme. Taking all these factors into consideration the enzyme was isolated from overproducing bacteria grown in cobalt containing culture media. The metal content of the purified active enzyme was determined. The enzyme was further investigated using visible spectroscopy

both in the presence and absence of DNA substrate.

EXPERIMENTAL PROCEDURES

Materials

Bacterial strains: These were received and prepared as described in chapter 2.

Preparation of the endonuclease: Native enzyme was prepared as described previously. The preparation of the cobalt enzyme is described in the methods section.

DNA: Plasmid Col E_1 DNA and lambda phage DNA were purchased from Sigma Chemical Co. and BRL, respectively.

Growth media: Bacto tryptone and yeast extract were obtained from Fisher Chemical Co.

Cobalt(II) chloride: Puratronic cobalt chloride was purchased as described in chapter 2.

Other materials: Spectral grade glycerol and 2-mercaptoethanol were obtained from Aldrich Chemical Co. Other chemicals were obtained as mentioned in chapter 2.

Methods

Growth of cells in cobalt enriched media: Transformed bacterial strain M5248/pSCC2 was grown in the presence of media containing cobalt chloride as follows. Culture media (see chapter 2 for other details) were mixed with 5μM cobaltous chloride before inoculation with an overnight culture of bacteria grown in normal L-broth not containing cobalt. The cells were then grown for 4 hours at 30°C and the temperature was raised to 42°C. Cobalt chloride (5μM) was again added just before and after the temperature was increased. The bacteria were grown at the elevated temperature for 4 hours before harvesting. The isolation and the purification of the enzyme was carried out as described in chapter 2 except this preparation was carried out on a much smaller scale (3-4 g of cell pellet). The buffers used in isolating and purifying the enzymes were made metal free by treatment with chelex 100.

Endonuclease activity and agarose gel electrophoresis: These determinations were carried out as mentioned in chapter 2.

Determination of the metal content of the endonuclease: The enzyme samples substituted with cobalt were purified using phosphocellulose chromatography. After assaying the fractions

for activity, some selected samples were examined for the cobalt/protein stoichiometry. A few of these samples were then dialysed against metal free assay buffer to remove extraneous metals and analysed to determine the protein and metal concentrations. Assay procedures used to obtain protein and metal contents are described in chapter 2.

Spectroscopy: Spectra of the cobalt enzyme were obtained using a Cary 219 (Varian) spectrophotometer with 4cm path length quartz cells. The enzymes were in 0.02M phosphate buffer (pH 7.4) containing 0.6M KCl, 5mM 2-mercaptoethnol, 0.1mM EDTA and 10% glycerol.

RESULTS

Correlation of the cobalt content with the production of the restriction enzyme Eco RI: Table I demonstrates that in vivo addition of cobalt led to the production of the cobalt derivative of Eco RI endonuclease. The data were taken from two different enzyme preparations. After the bacterial cells were grown in cobalt enriched media, the enzyme was isolated and purified as described in the experimental procedures. Fractions were then assayed for endonuclease activity and the most active fractions were selected for further experiments. Preparation 1 contained enzyme having a protein concentration of ~ 0.3μM. According to

Table I these fractions were found associated with cobalt. Dialysis against metal free assay buffer removed some of the loosely bound metal. About 60% cobalt remained tightly bound after dialysis. A second preparation produced enzyme having a higher concentration (~3 μM). Cobalt/protein ratios indicate that the enzyme is again associated with 60% cobalt. In addition to cobalt, both enzyme preparations contained some zinc (~30%) associated with it. As can be seen (cf.Figure 1a, chapter 2), the overproduced enzyme (average enzyme concentration ~ 8μM) in the absence of any added metal, produced an enzyme containing an average of ~40% zinc and no cobalt. Hence the addition of the metal to the medium clearly increases the total level of metal incorporation into the overproduced Eco RI. These results illustrate the addition of metal to growth media is a requirement in order to produce the metalloenzyme.

The cobalt substituted Eco RI made by the in vivo addition of cobalt had an activity consistent with that of its in vitro analog. Furthermore, this enzyme was also tested for its site-specificity on phage lambda DNA. In vivo cobalt substituted enzyme showed a fragmentation pattern similar to that produced by both the native and the in vitro cobalt substituted enzymes (see Figure 2b, chapter 2).

Visible spectrum of the cobalt substituted enzyme: Active fractions containing 60% cobalt were pooled to obtain spectra. The native enzyme was used as the reference sample. Both enzyme samples were in 0.02M phosphate buffer (pH 7.4) containing 0.6M KCl, 5mM 2-mercaptoethanol, 0.1mM EDTA and 10% glycerol. The enzymes were scanned from 700-370nm. The concentrations of the native as well as the cobalt enzyme were 3μM. Plasmid Col E_1 DNA (20 nM site concentration) was added to the cobalt enzyme and the scanning was repeated with the native enzyme as the reference. The change in volume of the enzyme solution upon the addition of the DNA was negligible because the latter was added as a lyophilyzed preparation.

The difference spectrum of the cobalt enzyme versus the native enzyme is given in Figure 1. Cobalt substituted enzyme shows two sharp peaks with high intensities at wavelengths 400 and 460 nm. The approximate extinction coefficient estimated is $2x10^3 M^{-1}cm^{-1}$ for both peaks. There is another band (550-625 nm) which is broad with very low intensity. It is not possible to estimate the extinction coefficient for this peak based on this spectrum. The spectrum of the zinc enzyme in the same wavelength region shows light scattering which sometimes occurred with the precipitation of the protein.

Plasmid Col E_1 DNA was added to the cobalt enzyme sample (3μM) and the difference spectrum against the zinc enzyme was taken by scanning from 700-370nm. The specific Eco RI site

concentration of the DNA was 23 nM. This indicates that, out of the total enzyme dimers, only 1.4% was bound to the specific sites and the rest was associated with non-specific sites on DNA.

The presence of bound DNA leads to a hyperchromism of the absorption. The peak at 460 nm is decreased and a distinct band at 400 nm is no longer evident. It is noteworthy that the cofactor Mg(II) ion which is necessary for cleavage was not present in the mixture. Therefore, the perturbation of the spectrum is solely due to the binding of the enzyme to DNA.

DISCUSSION

Overproduction of Eco RI in the presence of added cobalt in the medium: As mentioned in chapter 2, the metal analyses of overproduced Eco RI restriction enzyme showed that the zinc content of many active enzyme fractions were below stoichiometric proportions. In contrast, the enzyme isolated from the non-overproducing wild type strains, more reproducibly showed stoichiometric metal incorporation. We thought that the decrease in the bound zinc content of the overproduced enzyme could perhaps be the result of an unavailability of sufficient quantities of metal in growth media. The proteins, Eco RI methylase and endonuclease, when overproduced represent several percent of the total cell protein (10). Owing to the fact that

the formation of these enzymes takes place after the cell growth is arrested, the overproduction of Eco RI may face the problem of obtaining adequate amounts of metal, even in the presence of relatively high metal concentrations in the medium. Numerous other zinc metalloenzymes which are necessary for cell growth and metabolism have the first opportunity to obtain zinc from the media. As a consequence, at the stage when the production of Eco RI takes place, the available metal content may not be high enough to bind to all endonuclease molecules produced.

According to these ideas, the enzyme should be able to incorporate more metal ions, if the culture media are provided with more metal during the production of the enzyme. Therefore, we decided to enrich the growth media with cobalt. Unlike zinc, the chances of contamination with cobalt metal during isolation of a protein are lower as the latter metal is less abundant in the environment than is zinc. Furthermore, isolation of a cobalt substituted protein allows the production of the enzyme containing specifically a spectroscopic probe. Also, cobalt substitution of Eco RI produces active enzyme. As mentioned in the experimental procedures, the addition of cobalt was accomplished at three different stages. Since the growth of the cells was carried out at relatively zinc free conditions, cobalt was added to the medium before inoculation to allow normal cell growth. Cobalt was again added to the cultures just before and after the growth temperature was increased. Elevated

temperature gives the signal for the cell to overproduce Eco RI genes and polypeptides. If the medium is already depleted of cobalt, the addition of the metal at this stage could probably replenish it. These two additions were, therefore, done to make sure that there was enough metal present in the medium, when the Eco RI proteins are produced.

According to Table I, two independent enzyme preparations produced the restriction endonuclease containing bound metal 60% of which is Co(II). Therefore, the enzyme apparently incorporated the metal that was provided in the growth media. This result strongly supports the fact that the Eco RI restriction enzyme is a metalloenzyme. It is difficult at this point to determine the stage of growth of cells at which the addition of cobalt is most effective for incorporation. The enzyme preparation also contained ~30% zinc in addition to cobalt. This amounts to a total metal concentration of ~90%. Complete incorporation was not seen. This is possible, if the media did not contain optimal amounts of cobalt required for 100% incorporation. The amounts of cobalt bound, however, suggest that the absence of sufficient metal in growth media could have been the cause of the earlier production of enzyme deficient in metal.

The requirement for the presence of extra metal in the growth medium during the overproduction of Eco RI restriction enzyme brings forth a complication inherent in artificially

constructed gene systems. The construction of an artificial system is, unquestionably, the most ingenious way of obtaining large quantities of a protein which otherwise occurs in very low concentrations in the cell. The insertion of a gene into a plasmid with a strong promotor leads to the production of a protein in large amounts. This construction, on the other hand, does not necessarily mean that the protein obtained is identical in every respect to the naturally occurring polypeptide. Each metabolic process in a living system is part of an intricate network and is subjected to rigorous controls within the cell. The proteins are made in the most economical way, as and when the cell needs them. During overproduction, one or two specific proteins are made over and above the requirements of the cell. In this case, therefore, the cell may not exercise the necessary control over the production of the proteins in such a way that the most efficient product is made. Nevertheless, the living system is forced to make a protein in overwhelmingly large quantities. Therefore, the cell does not necessarily have to make an active product or it may not even have the ability to do so. In the Eco RI restriction enzyme which requires a metal for its activity, it is clear that the naturally occurring levels of zinc are sufficient for the wild type protein. The overproducer, on the other hand, requires more metal which cannot be obtained from the normal growth medium. From our results it is seen that the enzyme incorporated the cobalt metal which was added to the

growth medium.

The cobalt enzyme thus produced was compared with the native enzyme for site-specific cleavage of lambda phage DNA. As described in chapter 2 (see Figure 2b, chapter 2), a similar type of experiment was carried out using in vitro cobalt substituted enzyme. Our results show that the endonuclease which incorporated cobalt in vivo, produced an identical fragmentation pattern of lambda DNA indicating that its site-specificity was unaltered. Furthermore, the activity of the protein was comparable to that obtained by in vitro cobalt substitution. Unlike in the case of in vitro direct exchange method, no attempt was made to maintain a reducing atmosphere during in vivo addition of cobalt. The metal was added to the media as cobaltous chloride. It was assumed that the metal, once absorbed into the cell, was present as cobalt(II) ion. The possibility of oxidation of cobalt(II) to cobalt(III) may not have been prevented. On the other hand, in vivo cobalt substitution of E. coli RNA polymerase produced the enzyme which incorporated two cobaltous ions per mole of holoenzyme. Oxidation of the cobalt(II) polymerase produced the cobalt(III) derivative which had a visible spectrum different from that of the cobaltous enzyme (11).

Our next aim was to obtain the cobalt incorporated enzyme in sufficient quantities to enable spectroscopic studies. The amount of enzyme obtained permitted the examination of the

spectral properties of the protein in the presence and absence of one of its DNA substrates, plasmid Col E_1 DNA. The spectrum shows two bands of high intensities at 400nm (ε~$2x10^3 M^{-1} cm^{-1}$) and 460nm (ε~$2x10^3 M^{-1} cm^{-1}$). The peaks seen at these wavelengths for cobalt enzymes are usually attributed to charge transfer bands. The high extinction coefficients of these bands are consistent with this idea. The weaker band at 590nm is important because its low absorption indicates that the peak is the result of a d-d transition. The small degree of absorption occurred did not allow us to estimate an extinction coefficient. Such peaks are commonly observed for many cobalt substituted enzymes (see Table II).

It is interesting to note that the addition of the DNA substrate, plasmid Col E_1 DNA, reduced the intensity of the band at 460nm and the band at 400nm was no longer visible. This perturbation suggests that the metal may be involved in binding to the substrate. The amount of enzyme in the reaction mixture was very high compared to that of specific Eco RI recognition sites. Out of the total enzyme dimers, only 1.4% was bound to the specific sites. The rest bound non specifically to other sites in DNA. Therefore, non specific binding of the enzyme to the substrate caused the decrease in band intensities. It cannot be determined from the spectral data whether the metal is involved in either non specific binding only or in specific binding as well. The perturbation, then may at least be due to

the participation of the metal center in binding to the DNA. These changes in spectral properties were seen in the absence of the cofactor magnesium ion which is necessary for the hydrolysis of phosphodiester bonds. Therefore the metal center is clearly involved in binding to the substrate as the cleavage reaction was not allowed to take place.

Table II contains some spectral data of other cobalt substituted enzymes. According to this table, E. coli RNA polymerase substituted with one cobalt atom per enzyme unit has a spectrum remarkably similar to that of cobalt substituted Eco RI. (cf. Fig.1). The two peaks of the polymerase enzyme occuring at 400 and 475nm have been attributed to charge transfer transitions. These two peaks have higher molar absorptivities which are comparable to Co-Eco RI. Furthermore, the authors (12) reported that the spectrum was perturbed by the addition of ATP or UTP in the absence of magnesium and template DNA. This observation is consistent with their idea that the intrinsic metal is involved in orientation of the initiating nucleotide. Similar to this observation, the addition of DNA to Co-Eco RI perturbed its visible spectrum. The spectrum of polymerase also shows another weaker band at 580nm which was intensified after the substitution of the zinc atom with cobalt (see Table II, Co-Co enzyme). A similar type of weak band is seen in the spectrum of cobalt-substituted Eco RI having λ_{max} of 590nm. The intensity of this band is very low compared to that of the other two bands.

According to Table II the occurrence of a band near 550nm is seen commonly in cobalt substituted enzymes. In contrast to the charge transfer bands that occur near ultraviolet region, these bands usually have much lower extinction coefficients and are useful in characterizing the geometry of the metal site. We cannot identify the coordination geometry around the metal site of Eco RI, based solely on this spectrum. Since the d-d bands are not intense, higher concentrations of the enzyme are needed in order to refine the spectrum.

Possible role of the metal center in the restriction enzyme Eco RI: According to the observations described in chapters 2-4, it is possible to suggest a role for the integral metal ion in the restriction enzyme Eco RI. These chapters describe the various approaches taken to determine the role of the metal in the protein. Kinetics of the cleavage reaction of the native and cobalt substituted enzymes indicates that the variations of bound metal affects at least the association of the enzyme with the DNA substrate. A similar conclusion can be drawn from the inhibitor studies as well. The ligands having high affinity for zinc inhibit the reaction competitively. Competitive inhibition implies the direct complexation of the ligand with the metal, affecting again, the binding of the enzyme to the substrate. Additionally, the perturbations of the spectrum of Co-Eco RI endonuclease in the presence of DNA indicates that the binding of

the enzyme to the DNA may involve the metal center. All the evidence, taken together, strongly suggests that the intrinsic metal ion in the restriction endonuclease Eco RI may directly participate in the interaction of the enzyme with its DNA substrate. Whether the metal ion is involved in phosphodiester bond cleavage is not clear. Further investigations will have to be carried out to examine the hydrolytic step.

Future plans: The primary investigations described above imply the importance of the zinc ion in substrate binding. Further investigations ought to be carried out to determine the nature of the involvement of the metal in substrate binding. It is important to obtain the enzyme at a higher concentration, in order to refine the visible spectrum. Higher concentrations are necessary to observe the bands due to d-d transitions. Furthermore, the changes in the visible spectrum of the cobalt enzyme can be monitored as a function of pH. These changes could indicate metal linked ionizations which might be useful in catalysis. The variations of the ionizations in the presence of substrate and inhibitors would furnish information about the mechanism by which the metal center interacts with the DNA. Also, spectra can be obtained at lower enzyme to specific DNA site ratios to determine the changes that could occur as a result of specific binding. The addition of magnesium(II) which is a cofactor necessary for cleavage could also reveal more

information about the role of the metal. Added perturbations in the spectrum might indicate whether the metal is involved in the cleavage of the phosphodiester bond. Another important advantage of the cobalt substituted enzyme is that it enables one to study the relatively more stable enzyme-substrate or enzyme inhibitor complexes. The labile Co(II) ion can be oxidized to produce the inert Co(III) complexes which can be subjected to intensive spectroscopic investigations. The change in spectroscopic properties will also indicate the oxidation state of the enzyme preparation. Additionally, the enzyme can be substituted with other metals such as nickel, copper or cadmium. The relative activities and the spectroscopic characteristics of these enzymes would be very useful in examining the ligand environment of the metal within the enzyme.

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Endonuclease sample	Metal/protein stoichiometry[a]		
	Cobalt		Zinc
	I[b]	II[c]	I[b]
Preparation 1	0.6-4.0	0.65	~0.3
Preparation 2	0.5-0.7	0.6	~0.3
Native zinc enzyme[d]	-	-	~0.4

Table I: Correlation of protein content with the extent of in vivo incorporation of cobalt by Eco RI endonuclease.

The enzyme was prepared and purified from the overproducing E. coli strain M5248/pSSC2 as described in the experimental procedures. Cobalt was added to growth media in the form of coballtous chloride at three stages of growth, to a final concentration of 15μM. The table provides data from two independent enzyme preparations. All samples contain active restriction enzyme. As can be seen from the table, cobalt metal was incorporated into the enzyme during its formation and the metal remained undissociated throughout the isolation procedure.

a: Cobalt/protein ratios and zinc/protien ratios were determined by assaying the protein content and the metal content of each sample as mentioned in chapter 2. The uncertaintinities in these values are 1.0±.05 and 1±.1 for the cobalt and zinc enzymes, respectively.

b: Metal/protein ratios determined after initial purification of the enzyme.

c: Some of the purified enzyme fractions were dialysed extensively thereafter against metal free assay buffer and the metal/protein ratios were determined once again.

d: Metal/protein ratios of the native zinc enzyme, produced as described in chapter 2 (Figure 1a), without metal enrichment in the media.

Enzyme	λ_{max}nm (ϵ M^{-1} cm^{-1})	Reference
<u>E. coli</u> RNA polymerase		
Co-Zn enzyme	400 (3000), 475 (2700) 580 (N. E.), 700 (N. E.)	12
Co-Co enzyme	584 (200), 703 (335)	1
Alkaline phosphatase	640 (260), 605 (220) 555 (378), 510 (335) 480 (120)	13
Carboxypeptidase A	555 (250), 572 (150)	14, 15
Thermolysin	555 (90)	16
Carbonic anhydrase		
At pH 5.9	550 (200), 1220 (47), 950 (13)sh. 641 (100), 617 (135)	17
At pH 8.0	1220 (80), 900 (18), 640 (260) 617 (280), 550 (380), 520 (280)	18 19
Superoxide dismutase	530 (160), 560 (240) 595 (230)	20
Liver alcohol dehydrogenase (four cobalt atoms)	340 (15000), 655 (1000) 740 (700)	21

Table II: Electronic spectral data reported for some zinc metalloenzymes substituted with cobalt.

ϵ: Extinction coeffcient

λ_{max}: Wave length at which maximum absorption is observed.

sh: shoulder.

N. E.: Not estimated.

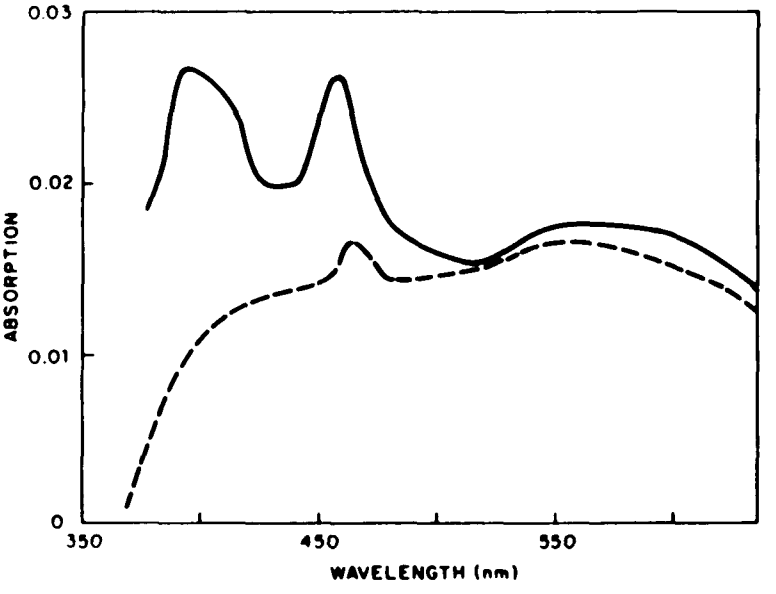

Figure 1: visible and near ultraviolet spectrum of Co-Eco RI endonuclease (—) in the absence and (---) in the presence of col E_1 DNA substrate. The spectrum was obtained by using the native enyzme as the reference. The absorption was measured using cuvettes of 4cm path length. The enzymes used were the fractions obtained after phosphocellulose chromatography and therefore were contained in 0.02M phosphate buffer (pH 7.4) containing 0.6M KCl, 5mM 2-mercaptoethanol, 0.1mM EDTA and 10% glycerol.

CHAPTER 5

The Restriction Endonuclease EcoRI Alters the Enantiomeric Preference of Chiral Metallointercalators for DNA: An Illustration of a Protein-Induced DNA Conformational Change.

Biochemistry (1986, In Press)

Abstract

A conformational change in the DNA plasmid ColE$_1$ appears to occur upon specific binding of the restriction endonuclease EcoRI. 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 delta (Δ) configuration bound preferentially by right-handed B-DNA. In the presence of EcoRI, however, this enantioselectivity is altered. The chiral intercalators, at micromolar concentrations, inhibit the reaction of EcoRI, but for each enantiomeric pair it is the lambda (Λ) enantiomer, which binds only poorly to a B-DNA helix, that inhibits EcoRI 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 EcoRI 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 EcoRI 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 EcoRI recognition site. Possible models for the recognition site are described. 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.

Introduction

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; Anderson et al., 1982; Frederick et al., 1984; McKay & Steitz, 1981; Pabo & Lewis, 1982; Steitz, et al., 1982; Tanaka et al., 1984). Based upon 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; 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 EcoRI 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; Barton, et al., 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 lambda and delta isomers of phenanthroline complexes used in this study. Complexes possessing the delta configuration may bind by intercalation easily to a right-handed helix with one ligand stacked between the base pairs and the two non-intercalating ligands disposed along the right-handed helical groove. The lambda 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 stereospecificity is apparent for the bulkier diphenylphenanthroline (DIP) 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 EcoRI restriction enzyme (Kania et al., 1976; Nath & Azzolina, 1981; Nosikov et al., 1976). EcoRI 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 non-specific 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 EcoRI to its specific DNA substrate. We report here that EcoRI 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 $[(phen)_3Ru]Cl_2$ and $[(DIP)_3Ru]Cl_2$ were prepared as described by Lin et al. (1976), and enantiomers were resolved and characterized as described elsewhere (Barton, et al., 1984a; Barton et al., 1984b; Barton & Nowick, 1984). The synthesis and resolution of $[(DIP)_3Co](tartrate)_3$ diastereomers was also conducted as previously described (Barton & Raphael, 1984). The enzyme EcoRI

was prepared in large quantity and purified to homogeneity (Modrich & Zabel, 1976) using the overproducing strain M5248/PSCC2 (S. C. Cheng, 1983) kindly provided to us by Dr. P. Modrich. The plasmid DNA $ColE_1$ (PL-Biochemicals and Sigma) and lambda 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 EcoRI (0.05 - 3 nM) in buffer containing either delta or lambda 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 3nM (sites) to 12nM were used at

different fixed values of Λ-$Ru(DIP)_3^{2+}$. The enzyme and the Mg^{2+} concentrations used were 0.1nM and 5mM respectively. Incubations were done 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 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, 0.018 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 using polaroid type 605 positive/negative film. Negatives were scanned at 500 nm 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 and linear form III DNA in each sample were determined. For kinetic studies, the weight of linear DNA formed was calculated using the percentages. The initial velocity was taken as the concentration of linear DNA formed per one minute.

In order to determine the site at which the metal complex $Co(DIP)_3^{3+}$ photolytically cleaves the DNA in the presence of EcoRI, the following procedure was used: Closed circular plasmid $ColE_1$ DNA was first linearized using the restriction enzyme, SmaI (BRL). Five units of enzyme were used per 1µg of DNA in order to achieve complete linearization. The DNA was then isolated by phenol extraction and ethanol precipitation. During ethanol precipitation, 1mM 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 using 7nM DNA sites in assay buffer containing 800µM EDTA, in the presence and absence of either Δ- or Λ-$Co(DIP)_3^{3+}$ (10µM) and in the presence of each enantiomer and EcoRI (7nM), 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 8λ electrophoresis buffer

(pH4.5), followed by the addition of $Zn(NO_3)_2$ solution to a final concentration of 5mM and 1.6 units of S_1 nuclease. After incubation for 5 min. at 37°C, the samples were quenched using 100 mM EDTA and electrophoresed as described before. Positions of the resulting bands were determined by comparison with a molecular weight marker, the authentic EcoRI fragments obtained by first digesting $ColE_1$ DNA with SmaI followed by EcoRI.

Results and Discussion

Enzyme Inhibition by the Chiral Intercalators: The tris(phenanthroline) metal complexes, like other DNA intercalating agents, inhibit the action of EcoRI. The metal complexes inhibit EcoRI cleavage of both supercoiled $ColE_1$ DNA (Figure 2A) and linear lambda 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 EcoRI 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 EcoRI in the presence of micromolar concentrations of either Λ 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.

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 build-up and early release of a nicked intermediate. The metal complexes also do not affect the recognition site specificity or even the hierarchy of recognition sites on a given DNA strand. The digestion by EcoRI of λ phage DNA, which possesses five recognition sites with a gradient of cleavage efficiencies (Rubin & Modrich, 1980; Thomas & Davis, 1975) was examined under conditions that yield partial fragmentation. Figure 2B shows this fragmentation pattern with EcoRI 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 EcoRI reaction and in fact more effectively than the delta enantiomer. Table I indicates the degree of enzyme inhibition in the presence of several chiral intercalators. Although Δ-$Ru(DIP)_3^{2+}$ does inhibit EcoRI, 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 EcoRI 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 lambda isomer.

This reversal of enantiomeric preference is found consistently with the metal complexes inhibiting the EcoRI reaction as shown in Table I. While both isomers of $Co(DIP)_3^{3+}$ inhibit EcoRI, at 6 μM cobalt more inhibition is seen with the lambda 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 ($K\Delta/K\Lambda = 1.3$) in binding to B-DNA is observed, but for the bulky $Ru(DIP)_3^{2+}$ complexes binding to B-DNA is completely stereospecific. 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 one order of magnitude lower than those of delta isomers of diphenyl phenanthroline complexes (Barton et al., 1984a). Consistent with these observations here we find that higher concentrations of $Ru(phen)_3^{2+}$ are necessary to achieve inhibition than $Ru(DIP)_3^{2+}$. 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 EcoRI and for B-DNA $k_{eth}/k(Ru(phen)_3^{2+}) = 100$. Higher concentrations of the tricationic cobalt complexes appear to be needed to achieve inhibition comparable to $Ru(DIP)_3^{2+}$ isomers, although more highly charged species bind the DNA polyanion more closely. Perhaps this finding reflects the presence of a 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 EcoRI 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 EcoRI. The double reciprocal plot of concentration of DNA sites against 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 (A. 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 nM (Woodhead and Malcolm, 1980) and 8 nM (Modrich and 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 nM (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.

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 $\geq$ -0.065); the lambda enantiomer, although undergoing 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), EcoRI binds tightly to its recognition site but does not

cleave (Greene et al., 1979; Modrich, 1979). Thus EcoRI and ColE$_1$ plasmid were incubated in assay buffer lacking magnesium but in the presence of micromolar concentrations of either Λ - or Δ-Co(DIP)$_3^{3+}$. Upon irradiation with UV light at 254 nm 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 4W 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 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 EcoRI was added in the range of stoichiometric levels. Note that for the experiment in Table II, conditions were identical to those used to measure EcoRI 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 one minute. These data show that the presence of EcoRI stimulates cleavage by Λ-Co(DIP)$_3^{3+}$.

Several aspects of this experiment require comment. Firstly 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, EcoRI cleavage of DNA is not found with either 50 μM $Co(phen)_3^{3+}$ or 50 mM $Co(NH_3)_3^{3+}$. Secondly, the quantum efficiency for photoreduction of the cobalt center in solution is clearly the same for both isomers, and if anything, the efficiency would be decreased in the presence of enzyme due to light absorption. 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 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 delta 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 EcoRI 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 EcoRI recognition site. If so, then photolysis of the SmaI linearized DNA in the presence of $Co(DIP)_3^{3+}$ and EcoRI under conditions where EcoRI does not itself cleave, should still yield EcoRI digestion fragments, i.e., a 5351 bp fragment and a 1295 bp fragment. As seen from Figure 5B, photocleavage by Λ-$Co(DIP)_3^{3+}$ in the presence of EcoRI does yield a band corresponding to the 5351 bp fragment. Therefore cobalt

cleavage and therefore 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 EcoRI. 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 EcoRI. 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(phenantholine) metal complexes bind preferentially to right-handed B-DNA.

In summary, then, EcoRI 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 EcoRI. Consistent with the enzyme inhibition results described above, EcoRI appears to change the enantiomeric preference of the chiral intercalators in binding to the DNA helix. Protein binding must induce a DNA conformational change which 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 EcoRI, the DNA site cannot maintain a regular B-DNA conformation. B-DNA binds only delta 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 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 the Z-form and without substantial chiral discrimination. Since with bound protein there is still an enantiomeric preference observed, but this preference is reversed, our results would not be inconsistent with a left-handed conformation with a deeper groove

than found in the Z-form. Actually any stacked conformation which 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 based on x-ray diffraction data (Frederick et al, 1984) for the crystalline EcoRI-DNA complex may provide a basis for some speculation. The 25° degree helical unwinding and 4 A 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. Based on 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 either to enhancements or inhibition. Furthermore there is no obvious structural explanation for the preferential binding of the lambda enantiomer, unless some protrusion into the groove, either of 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. Recently results by Kim et al. (1984) have indicated definite distortions of the DNA in solution in the specific recognition

complex of EcoRI.

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 be considered.

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Table I: Inhibition of EcoRI Activity by Chiral Metallointercalators

Complex	Concentration (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 % 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)] x 100, after reaction with EcoRI for 3 min at 37°C in assay buffer.

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

	Relative Nicking Activity[a]		
[Enzyme]/[DNA sites]	0	1	2
Enantiomer			
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 versus 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.

Table III: Fragmentation of $Col E_1$ DNA by Λ-$Co(DIP)_3^{3+}$ at EcoRI Sites

Sample	$Co(DIP)_3^{3+}$ [a]	EcoRI	Integrated Intensity		
			Intact linear 6646 bp	5351 bp fragment	Ratio 5351 bp fragment / Linear DNA
Irradiated DNA [b]	—	—	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 in Methods.

b. Samples were irradiated at 315 nm.

Table IV: Alterations in DNA-metal Interactions with EcoRI

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

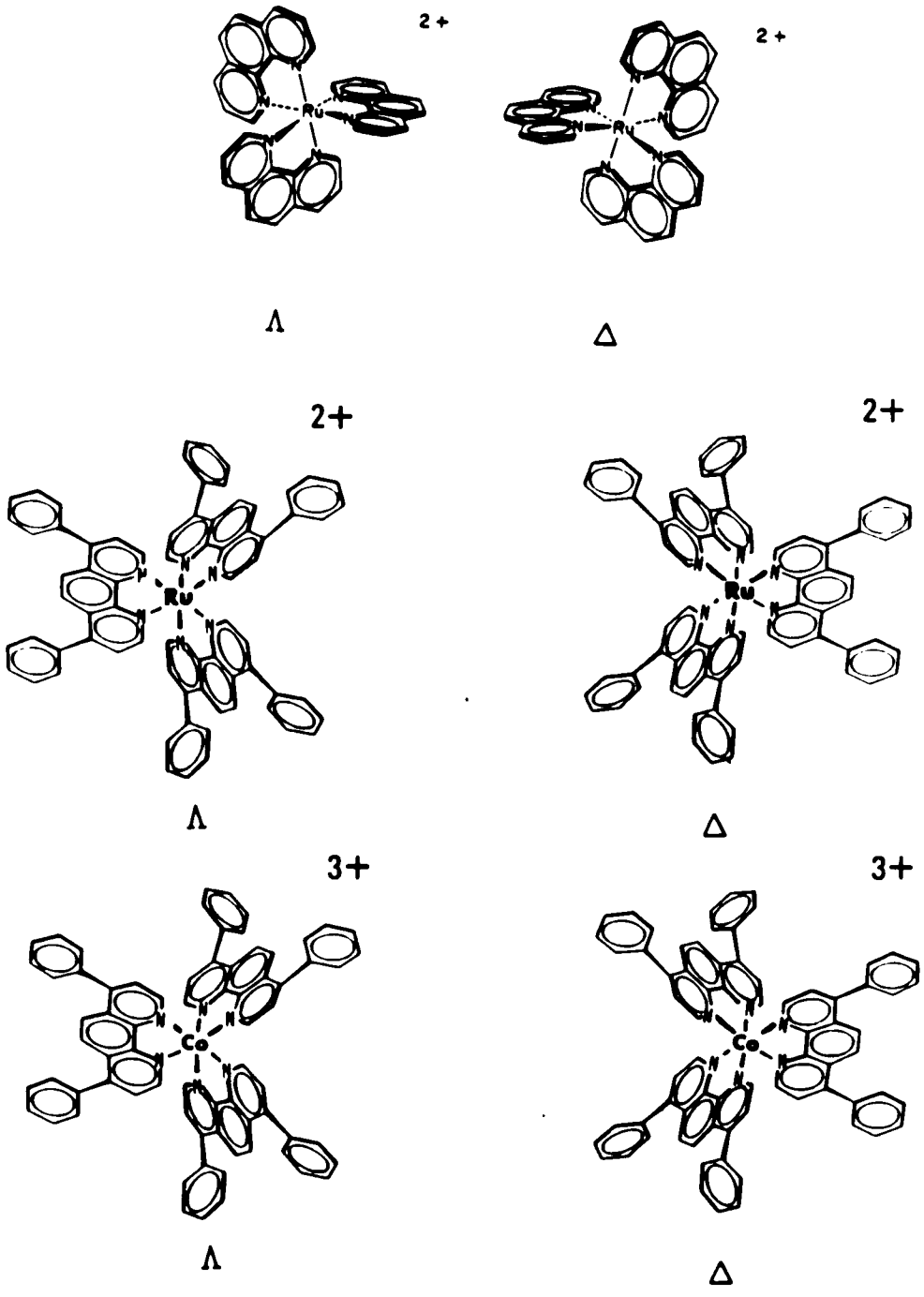
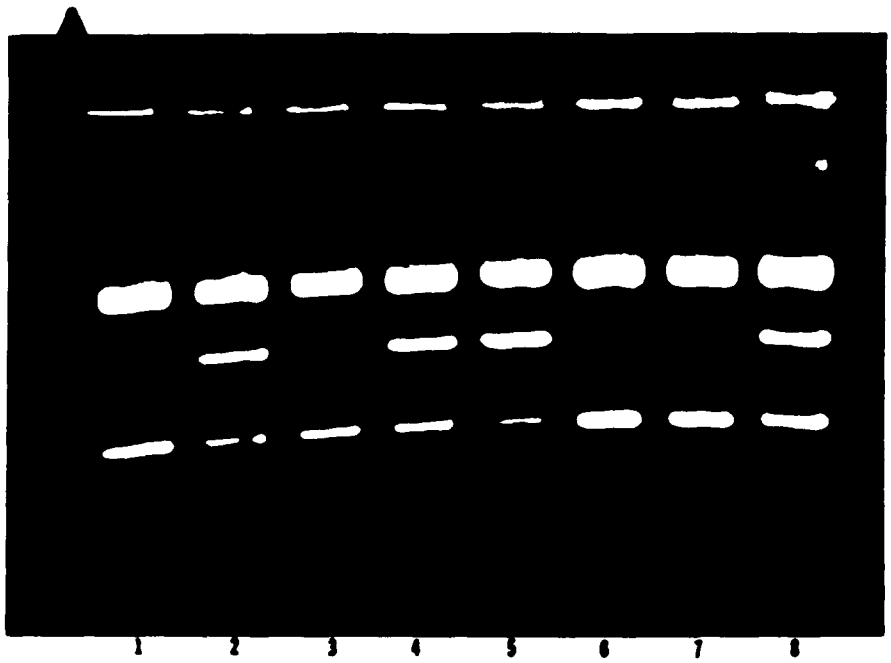


Figure 1: The chiral metallointercalators (top to bottom) $Ru(phen)_3^{2+}$, $Ru(DIP)_3^{2+}$, and $Co(DIP)_3^{3+}$.

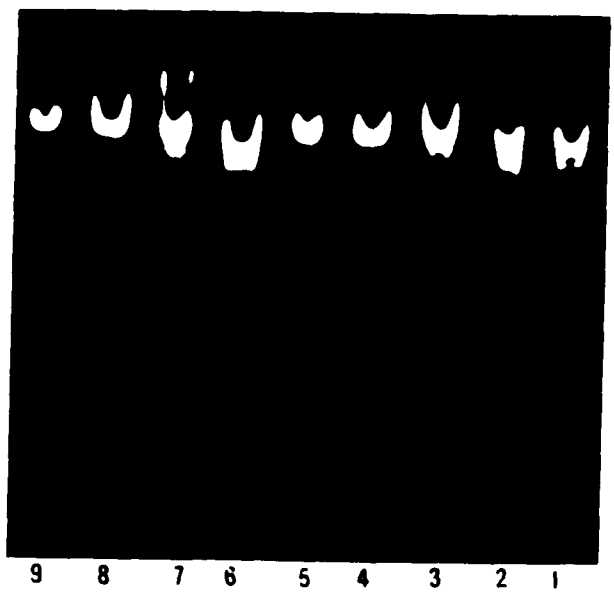
Figure 2: Inhibition of the EcoRI cleavage reaction by $Ru(DIP)_3^{2+}$ enantiomers.

(A) Agarose gel electrophoresis of $ColE_1$ plasmid DNA before (lane 1) and after reaction with EcoRI in the absence of metal (lane 2) and in the presence of various concentrations (10 μM, 5 μM, 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 4,5,8,9) and after partial digestion with EcoRI in the absence of metal (lane 1) and in the presence of 1 μM 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 using 7 nM DNA and 0.9 nM enzyme in assay buffer with and without the intercalator.



B



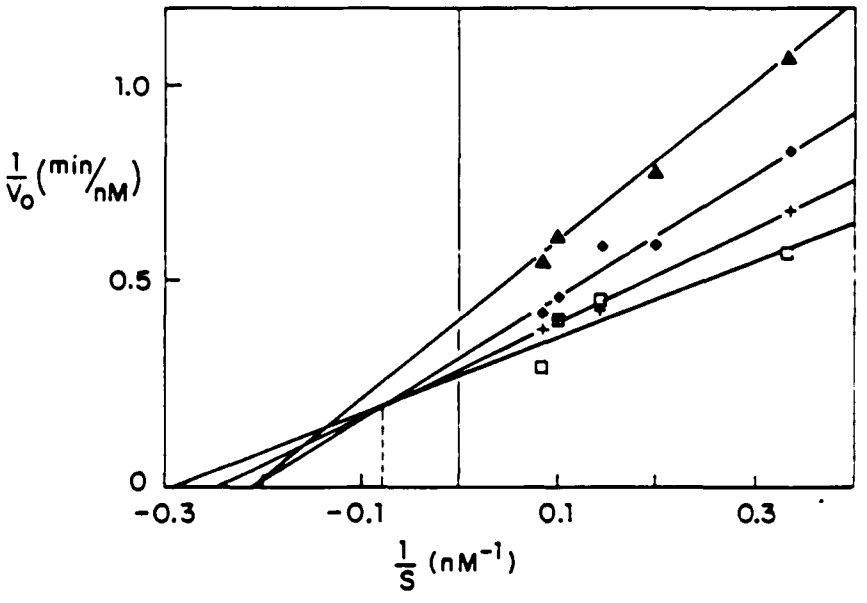


Figure 3: Kinetics of inhibition of the cleavage reaction of $ColE_1$ DNA by EcoRI by Λ-$Ru(DIP)_3^{3+}$.

Lineweaver-Burke plot of DNA site/ substrate concentration versus initial velocity (V_o) at different fixed levels [0(□), 4(+), 6(◆) and 10(♣) μM] of inhibitor. The enzyme concentration used was 0.04 μM.

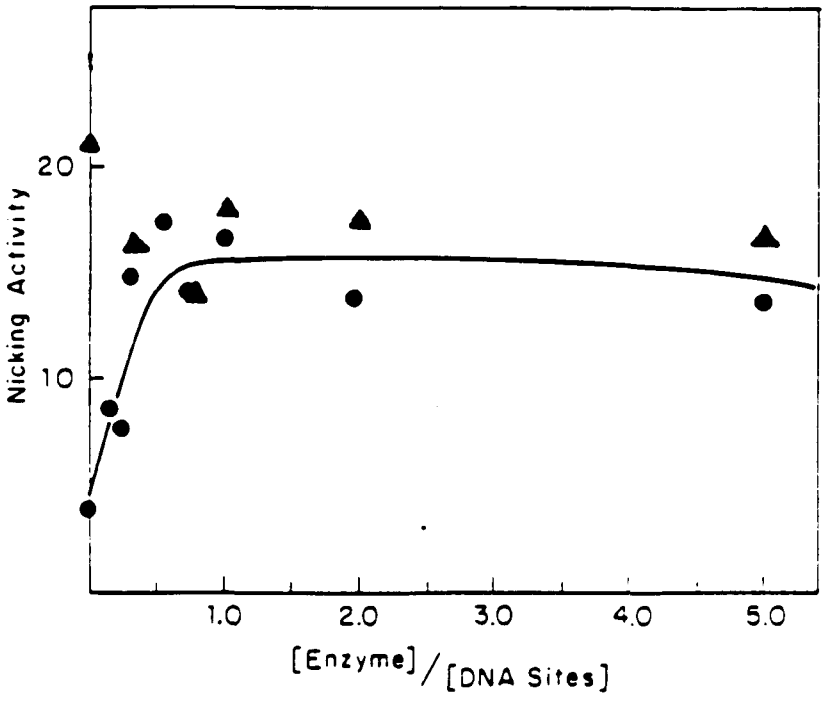


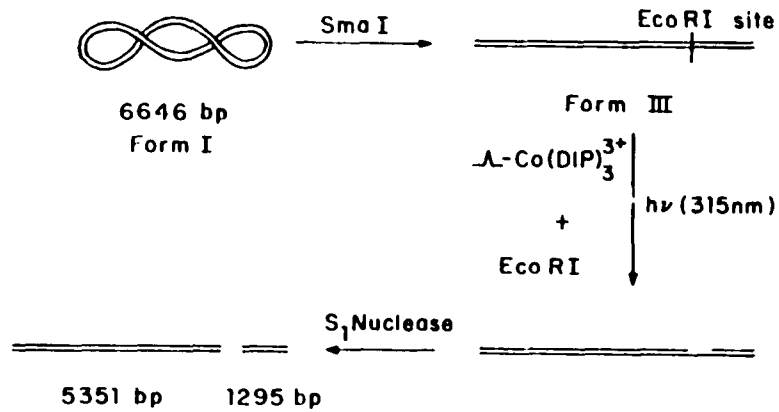
Figure 4: Photoactivated cleavage of ColE$_1$ DNA by Δ-(▲) and Λ-(●) $Co(DIP)_3^{3+}$ (6 μM) in the presence of increasing concentrations of EcoRI.

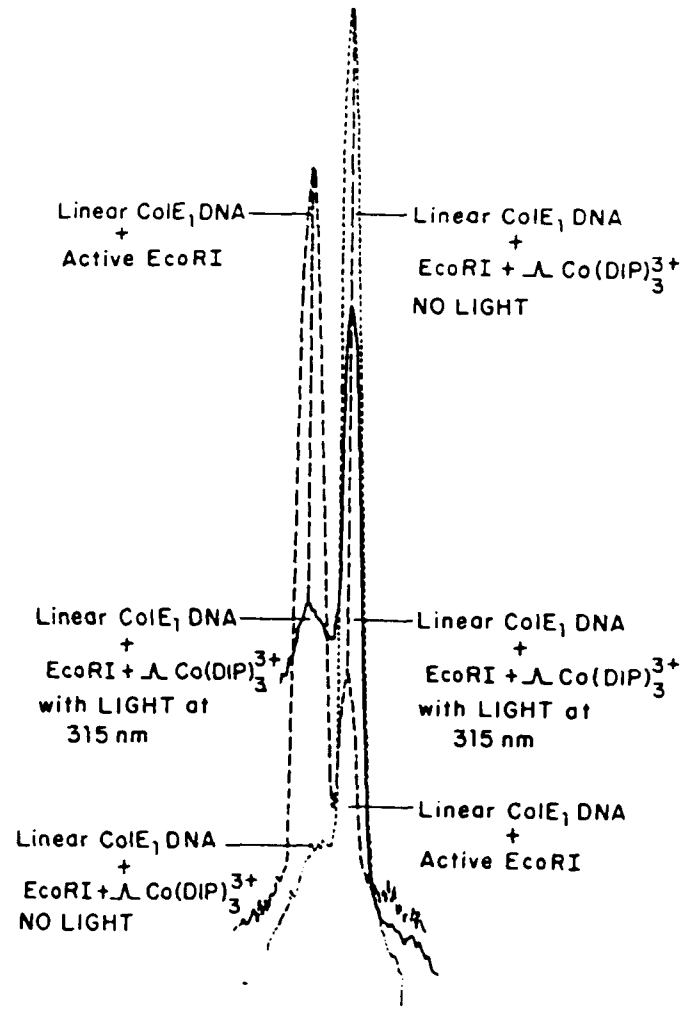
Enzyme-DNA ratios are expressed as EcoRI monomer units per DNA cleavage site. Nicking activity is given as the difference between the percentage of form II DNA of samples irradiated at 315 nm for 1 min at ambient temperature and the unirradiated control.

Figure 5:

A: A strategy for mapping site-specific cleavage of linear ColE1 DNA by Λ-$Co(DIP)_3^{3+}$ in the presence of EcoRI 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 EcoRI and 315 nm light: (----------) Linear $ColE_1$ DNA + EcoRI + Mg^{2+}; (.....) - Linear $ColE_1$ DNA + Λ-$Co(DIP)_3^{3+}$ + EcoRI without light; (_____) - Linear $ColE_1$ DNA + Λ-$Co(DIP)_3^{3+}$ + EcoRI + 315 nm light. The presence of EcoRI and light stimulates cleavage by Λ-$Co(DIP)_3^{3+}$ in the vicinity of the EcoRI recognition site, producing a 5351 bp fragment identical to that seen by EcoRI cleavage in the presence of magnesium.


Sma I
EcoRI site
6646 bp
Form I
Form III
Λ-Co(DIP)$_3^{3+}$
+
Eco RI
hν (315nm)
S$_1$Nuclease
5351 bp
1295 bp


Linear ColE$_1$ DNA
+
Active EcoRI
Linear ColE$_1$ DNA
+
EcoRI + Λ Co(DIP)$_3^{3+}$
NO LIGHT
Linear ColE$_1$ DNA
+
EcoRI + Λ Co(DIP)$_3^{3+}$
with LIGHT at
315 nm
Linear ColE$_1$ DNA
+
EcoRI + Λ Co(DIP)$_3^{3+}$
with LIGHT at
315 nm
Linear ColE$_1$ DNA
+
EcoRI + Λ Co(DIP)$_3^{3+}$
NO LIGHT
Linear ColE$_1$ DNA
+
Active EcoRI

Appendix

Communication

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The Presence of Zinc in the Restriction Enzyme *Eco* RI*

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We have determined that the restriction endonuclease *Eco* RI contains 1.0 ± 0.1 eq of zinc/monomeric enzyme. DNA cleavage by *Eco* RI is inhibited by *ortho*-phenanthroline after preincubation of the enzyme with the chelating agent. A similar inhibition by the nonchelating *meta*-phenanthroline is not seen. The sensitivity of the inhibition by the neutral ligand *ortho*-phenanthroline to preincubation is consistent with the tightly bound and inaccessible nature of the metal site. Extensive dialysis against the *ortho*-phenanthroline inhibitor leads to the release of the bound metal with the concomitant loss of enzyme activity. The tightly bound Zn^{2+} cation, then, appears to be necessary for enzyme function. The finding of zinc in *Eco* RI further illustrates the ubiquity of Zn^{2+} to DNA-protein complexes.

Zinc is essential to nucleic acid processing (1, 2). The zinc dication appears as an integral part of enzymes which bind polynucleotides either as substrate or template. DNA (3-7) and RNA (8-11) polymerases contain tightly bound zinc and, in some instances, the metal's requirement for enzyme activity has been demonstrated (4, 6, 12, 13). Other nucleic acid binding enzymes, for example, S1 nuclease, instead require the divalent zinc ion as an added cofactor (14, 15). It also appears that, in nonenzymatic reactions, Zn^{2+} is an efficient catalyst for template-directed oligonucleotide synthesis (16). Despite its prevalence, the role of Zn^{2+} in these systems is not well understood. Because of the multifunctional nature of many of these large proteins, the metal center has been difficult to examine.

In an effort to extend the characterization of DNA enzymes by their metal content and to find a system which would facilitate chemical studies of the metal center, we have examined *Eco* RI, a type II restriction enzyme from *Escherichia coli* (17-19). The type II restriction enzymes are among the simplest DNA sequence-specific enzymes known (19-21). *Eco* RI binds to the duplex palindromic sequence

5′-G↓AATTpC-3′
3′-CpTTAA↑G-5′

and cleaves both strands to form 5′-phosphoryl and 3′-hydroxyl termini as indicated (22). Crystals of *Eco* RI in the presence and absence of its recognition fragment have been isolated (23, 24). We report here that *Eco* RI contains a tightly bound zinc ion. Moreover, the zinc ion appears necessary to enzyme function.

EXPERIMENTAL PROCEDURES

Materials—Rigorously zinc-free conditions were followed throughout. Distilled deionized water was used exclusively as was plasticware, frequently presoaked in either 1% HNO_3 or EDTA. Reagents were tested before use for zinc content. Dialysis tubing was prepared to eliminate metal contaminants and free sulfhydryl sites. All protein and DNA samples were dialyzed extensively into our zinc-free media before use. Phage λ DNA was obtained from Bethesda Research Laboratory. *Ortho*-phenanthroline monohydrate was purchased from Sigma Chemical Co. and *meta*-phenanthroline was obtained from Alfa-Ventron Co., Danvers, MA.

Zinc Analyses—Metal analyses were conducted using a Varian 775 atomic absorption spectrometer equipped with simultaneous background correction and a CRA-90 graphite furnace. Zinc concentrations were determined by routine calibration of sample absorptions against standard zinc solutions in comparable buffers using linear least squares analysis. Absorbance was monitored at 213.9 nm. Reproducible values, linear with concentration, were obtained over the concentration range of 0.06–0.7 μM, using 1 μl aliquots and by averaging at least 3 injections/sample. The atomization program employed was as follows: 30 s dry at 95 °C, 15 s ash at 800 °C, atomize at 2000 °C, 1.5 s hold, ramp rate of 600 °C/s.

Protein Assays—*Eco* RI was obtained from three sources. Miles Laboratory, Bethesda Research Laboratory, and as a gift, which was prepared according to Greene, *et al.* (18), from Professor C. Cantor of Columbia Univ. Standard sodium dodecyl sulfate gels (25) showed the enzyme to be homogeneous within the limits of detection (at least 90% pure). The protein concentrations of stock solutions were determined spectrophotometrically, $E^{1\%} = 8.3$ cm (17). For routine analyses, the Coomassie blue binding assay was employed using lysozyme standards of known concentrations (26). The relative binding affinities of Coomassie blue to lysozyme *versus Eco* RI were determined in appropriate buffers using *Eco* RI standards of concentrations which were found spectrophotometrically. This indirect method allowed the routine monitoring of microgram protein samples.

Enzyme activity for all conditions described below was assayed by examining the digestion of λ phage DNA using agarose gel electrophoresis to separate fragments (18). Reactions were performed by incubation at 37 °C in assay buffer: 100 mM Tris, 50 mM NaCl, 1% glycerol, 0.01% Triton X-100, pH 7.6 (at 23 °C). Magnesium chloride, 10 mM, was added at zero time to initiate cleavage and all reactions were quenched by the addition of 100 mM EDTA at 0 °C. Following the addition of 50% sucrose, 0.5% bromphenol blue, the DNA samples were electrophoresed through 1% agarose slab gels in 90 mM Tris-borate, 2 mM EDTA, pH 8.3. Electrophoresis at 150 V was conducted for 3 h at ambient temperatures. The DNA bands were visualized after staining for 10 min with ethidium bromide (0.5 μg/ml) by illuminating from below with ultraviolet light. The gels were photographed using a Polaroid 198 camera with 1951 close-up lens through a red filter using 665 film.

For quantitation of gel bands, the absorption along the gel channels as a function of distance migrated was recorded (27) using the Varian 775 spectrometer. In order to avoid the complications of multisite fragmentation of the DNA, short time incubations were used. For the kinetic analyses, the rate of cleavage would then remain linear with the rate of loss of the intact DNA. Given constant time interval incubations (2–6 min) and constant substrate DNA concentrations (10 nM) near the reported K_m (28), the fraction of the intact phage lost and, therefore, the relative activity of the enzyme was determined. Data from one slab gel taken in duplicate using different gel negatives yielded identical results. Because *ortho*-phenanthroline quenches ethidium fluorescence, after reaction with enzyme, *ortho*-phenanthroline was added to each sample to eliminate any possible differences in fluorescent staining.

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TABLE I

Zinc content of Eco RI

Sample	Dialysis conditions	A[illegible]	A[illegible]	Concentration		Zinc: protein stoichiometry
				Zn	Protein	
					×10⁻⁵ M	
Eco RI	50 mM Tris pH 7.4, 10 mM EDTA, 10 days, 16×	0.330	0.019	4.16	4.16	1.0
	50 mM Tris pH 7.6, 50 mM NaCl, 0.01% Triton, 1% glycerol, 10 days, 18×	0.515	0.115	4.6	5.0	0.9
	5 days, 12×	0.580	0.120	7.9	8.6	0.9
	+5 mM EDTA, 8 days, 15×	0.539	0.114	4.9	4.2	1.5
	100 mM KPhos pH 7.1, 0.2 M NaCl, 0.01% Triton, 1% glycerol, 7 days, 11×	0.798	0.123	7.6	8.3	0.9
						Average 1.0 ± 0.1
Ovalbumin	100 mM Tris pH 7.6, 50 mM NaCl, 0.01% Triton, 1% glycerol, 6 days, 12×	0.165	0.120	0.7	12.5	
	+5 mM EDTA, 8 days, 15×	0.113	0.117	0	4.0	

Dialyses were conducted at 0 °C using a 10⁴ volume ratio of dialysate to sample. The frequency with which buffers were changed is indicated (×). KPhos, potassium phosphate.

[a] Atomic absorption readings were taken either on the dialyzed protein samples or on dilutions, depending on the initial protein concentrations used. The readings are given here to illustrate the relative sensitivities of the measurements to background absorptions. Concentrations were determined by daily calibration against known standards; the absolute absorptions given therefore do not correspond directly to a sample zinc concentration.

[b] In buffer as above.

RESULTS AND DISCUSSION

Table I shows the results of a series of dialysis experiments used to determine the intrinsic zinc content of *Eco* RI. We have found that an average of 1.0 ± 0.1 eq of zinc is bound per *Eco* RI monomer after extensive dialysis. As the table indicates, a range of buffers and protocols were examined. Buffers in which there is a high enzyme activity (assay buffer), high solution stability, and those containing high concentrations of chelating agent were tested to determine whether conditions existed in which the associated Zn^{++} is easily released from the native enzyme. Under all circumstances tested, at least one zinc dication appears tightly bound to *Eco* RI irrespective of the buffer system employed. The dialysis experiments were conducted using an enzyme concentration range of 10–150 μg/ml. Despite the low enzyme concentrations chosen to minimize aggregation, the atomic absorption readings, taken directly on the protein solutions or dilutions thereof, were significantly above those of the buffers which contain at most 1×10^{-8} M zinc ion. Also, protein samples were obtained from several sources to minimize the possibility of zinc contamination that is particular to an enzyme preparation. Initially, before extensive dialysis in our buffers, the zinc concentration associated with the enzyme exceeded that of the buffer and ranged from 4–8 eq of zinc/monomer. Over the course of dialysis, requiring 7–10 changes, the enzyme-bound metal concentration diminished until reaching a constant and reproducible value which was proportional to the enzyme concentration and independent of the enzyme source. Subsequent dialysis in buffers as indicated in the table did not lead to a zinc content lower than approximately 1 eq/monomeric enzyme. Moreover, we also examined ovalbumin solutions in parallel experiments to see whether our protocols in fact removed adventitious zinc contamination. Ovalbumin acts as a nonspecific metal carrier *in vivo*. Metal assays of ovalbumin solutions after extensive dialysis yielded zinc concentrations comparable to or slightly above buffer readings with a corresponding zinc content of 0.02 eq/mol of ovalbumin at most. Our results are in addition inconsistent with the specific binding of Zn^{++} to some protein contaminant in our samples. Sodium dodecyl sulfate gels indicated that the *Eco* RI samples are at least 90% homogeneous. Therefore, in order to explain our findings, the protein contaminant would necessarily contain an unreasonably high and nondialyzable concentration of zinc ion; furthermore, no contaminant was detected.

Our results therefore indicate that a zinc dication is at the very least tightly bound to *Eco* RI. The metal cannot be removed even after exhaustive dialysis with 10 mM EDTA; this chelator has a high affinity for Zn^{++}, log $K = 16.5$ (29). It is possible, therefore, that the metal is located in a protein environment that is inaccessible to solvent and the chelating anion. These results alone, however, do not rule out the possibility that the associated metal ion is a contaminant, albeit tightly bound, in our enzyme preparations. We therefore examined the effects of a metal chelating agent on the associated activity of the enzyme.

Eco RI activity in the presence and absence of the chelating agent *o*-phen[1] or its nonchelating analogue *m*-phen was determined by following the digestion of phage λ DNA using agarose gel electrophoresis as shown in Fig. 1. Digestion by *Eco* RI in its active form yields a series of low molecular weight fragments which are evident in the first channels of Fig. 1, *A* and *B*. In contrast, total enzyme inhibition results in a single high molecular weight band, that of the intact phage. Reduced activity leads to partially cleaved fragments of intermediate mobility. Fig. 1*A* shows 1% agarose gels of λ DNA after reaction with *Eco* RI (1 μg/ml) at 37 °C in assay buffer and with 2.5 mM *m*-phen or *o*-phen added. In each case, the enzyme was preincubated for 20 min at 37 °C with or without inhibitor before the addition of the DNA substrate and Mg^{++} cofactor. Under these conditions, the chelating agent *o*-phen is seen to fully inhibit *Eco* RI. This suggests that a metal ion participates in the action of the enzyme. Incubation with *m*-phen does give rise to some inhibition, but not comparable to that of *o*-phen; we estimate from the relative amounts of the primary high molecular weight fragments that there is ~10% inhibition by *m*-phen under these conditions. Fig. 1*B* shows the same experiment performed after enzyme preincubation with or without inhibitor at 25 °C. Here, while slight inhibition

[1] The abbreviations used are *o*-phen, ortho-phenanthroline; *m*-phen, *meta*-phenanthroline.

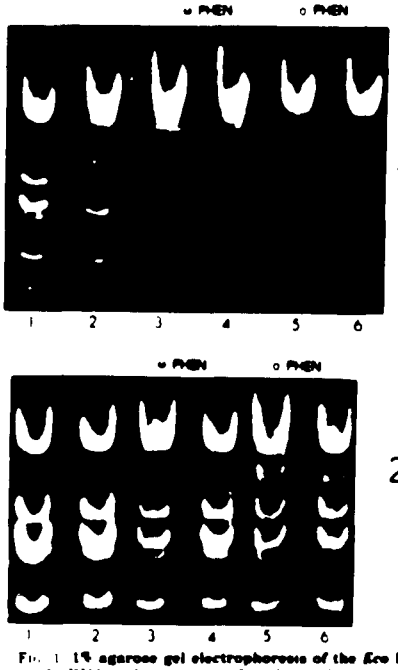

FIG. 1. 1% agarose gel electrophoresis of the *Eco* RI digestion of λ DNA in the presence of inhibitor. Effects of preincubation at 37 °C (*A*) and 25 °C (*B*). *Eco* RI samples (0.9 μg/ml) were preincubated for 20 min with (from *left* to *right* for each gel) no inhibitor (1 and 2), 2.5 mM *m*-phen (3 and 4), and 2.5 mM *o*-phen (5 and 6). Following the preincubation with inhibitor, samples were allowed to react with DNA (0.7 μg) in assay buffer upon the addition of 10 mM MgCl for either 10 min (1, 3, and 5) or 5 min (2, 4, and 6).

by *o*-phen is apparent, given the intermediate fragments formed, the striking inhibition seen after 37°C preincubation is not evident.

The dependence of the inhibition on preincubation is shown more quantitatively in Fig. 2. In these experiments, the loss of intact phage, *i.e.* cleavage at only the primary site, was measured in several trials in the presence of increasing concentrations of *o*-phen with preincubation at either 37 or 25°C. Again, dramatic inhibition by *o*-phen is apparent after preincubation with the chelator at 37°C but not at 25°C. Under these preincubation conditions, there is 50% inhibition of *Eco* RI activity with 400 μM *o*-phen. In this figure, we have plotted the inverse rates of *Eco* RI in the presence of *o*-phen relative to that of enzyme preincubated at 37°C but without inhibitor. It should be noted both from these results and in comparing *A* and *B* in Fig. 1 that there is a slight reduction in activity as a result of 37°C preincubation alone. Fig. 2, however, illustrates clearly the substantially different effects of *o*-phen on *Eco* RI activity with and without 37°C preincubation. This apparent sensitivity to preincubation is again consistent with the characterization of the zinc ion as being tightly and closely bound to the protein within a largely inaccessible region. It is likely that at physiological temperatures the native structure is somewhat unfolded and more flexible, which may facilitate access and binding of the inhibitor to the metal site. The small decrease in pH with increasing temperature may also aid access. Interestingly, a sensitivity to preincubation conditions has been observed in inhibition studies of avian myeloblastosis virus DNA polymerase by *o*-phen at comparable concentrations (6).

The fact that Mg^{++} is a necessary cofactor for cleavage (19) has precluded a similar examination of the inhibitory effects of EDTA, the affinity of this chelating agent for Mg^{++} is sufficiently high ($\log K = 8.7$) to complicate data interpretation. Inhibition by *o*-phen is not a consequence of interactions with the Mg^{++} cofactor, however. The formation constant of a phenanthroline complex of Mg(II) is negligible while with Zn(II) it is 10^6/ligand bound (30). Moreover, throughout these studies, a large excess of Mg^{++} over *o*-phen was employed. It is also noteworthy that inhibition studies of the polymerases with *o*-phen have been questioned owing to the presence of thiols and contaminating cupric ion in assay mixtures. It has been shown that *o*-phen in the presence of a reducing agent, a cupric ion salt, and molecular oxygen forms a Cu(I) complex, producing hydroxyl radicals and degrading DNA. The products of this redox reaction may be responsible for the *in vitro* inhibition of DNA and RNA synthesis (31, 32). No reducing thiol is present in our experiments, however. Moreover, no detectable levels (10^{-7} M) of copper were found in concentrated enzyme samples assayed using atomic absorption spectroscopy. A mechanism for the *o*-phen inhibition comparable to that proposed for the polymerases is therefore not likely. The inhibition studies taken alone, however, indicate strictly that chelation of a metal present in the incubation mixture leads to enzyme inactivation.

Although extensive dialysis of *Eco* RI against the polar EDTA does not release bound Zn^{++}, the metal can be removed by dialysis against 5 mM *o*-phen and the enzyme lacking Zn^{++} exhibits no activity. This finding, described in Fig. 3, indicates strongly that the neutral ligand *o*-phen does indeed inhibit *Eco* RI through chelating the intrinsically bound Zn^{++} cation. The apoenzyme has been prepared by dialysis for a minimum of 5 days at 0°C against 5 mM *o*-phen in either assay buffer or as indicated in Fig. 3. Although *o*-phen inhibition, a likely result of simple binding, requires a relatively short period of preincubation at 37°C, the complete dissociation of the metal ion from the protein requires extensive dialysis at the lower temperature. This is again consistent with the inaccessible feature of the metal site. Through this method, enzyme has been prepared having a zinc content of 0.02 eq/mol of *Eco* RI monomer. Importantly, after the parallel dialysis of *Eco* RI against buffer without *o*-phen, enzyme activity is retained. Fig. 3 shows 1% agarose gels of λ DNA after reaction with

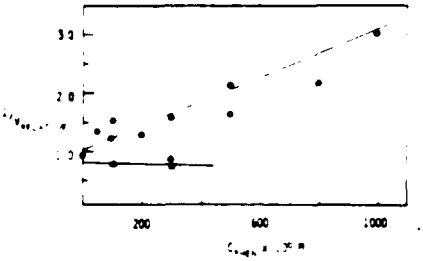

FIG. 2. The cleavage of λ DNA (10 EcoRI sites) by *Eco* RI after preincubation at 37 °C (●) and 25 °C (▲) for 30 min with increasing concentrations of *o*-phen. Relative velocities were determined as described under "Experimental Procedures" and are plotted as an inverse ratio to that of *Eco* RI preincubated at 37 °C with no inhibitor.

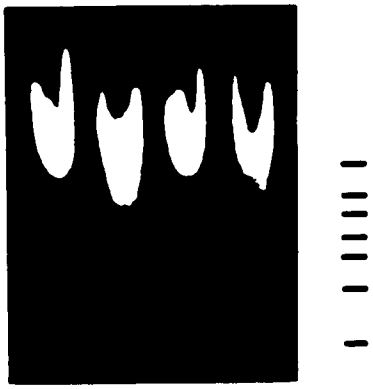

FIG. 3. **1% agarose gel electrophoresis of λ DNA after digestion by native and apoenzyme.** *Eco* RI was dialyzed for 7 days at 0 °C in buffer (10 mM K phosphate, pH 7.1, 0.2 M NaCl, 0.01% Triton) containing 5 mM *o*-phen. In parallel, native enzyme was dialyzed against buffer without the inhibitor. Shown (from *left* to *right*) are the phage DNA control and DNA after digestion (2 h) with untreated *Eco* RI, the apoenzyme, and the dialyzed native enzyme (12 μg/ml samples). The untreated *Eco* RI apo-*Eco* RI, and dialyzed native samples contained >1.5, 0.02, and 0.92 eq of zinc/monomer, respectively.

undialyzed *Eco* RI, with enzyme dialyzed against *o*-phen and enzyme dialyzed against buffer only. The zinc content is high (>1.5 eq) for the untreated sample and is 0.02 and 0.92 eq of zinc/*Eco* RI monomer for the apoenzyme and dialyzed native samples, respectively. Although 48% activity is lost after this exhaustive dialysis procedure without *o*-phen, it is clear from the figure that the loss of intrinsically bound Zn^{2+} is accompanied by a complete loss of enzyme activity; the DNA sample after treatment with the apoenzyme is identical with that of the phage DNA control. It should be noted that the apoenzyme was dialyzed twice against buffer after the *o*-phen dialysis to eliminate the inhibitory phenanthroline reagent from the enzyme incubation mixture. We have not thus far been able to restore activity by simply adding Zn^{2+} to the apoenzyme because micromolar levels of transition metal ions[1] substantially reduce *Eco* RI activity (33). Metal substitution studies are in progress.

In summary, we have demonstrated that a Zn^{2+} dication is tightly and stoichiometrically bound to *Eco* RI. Furthermore, the inhibition studies with *o*-phen and the finding that *o*-phen dialysis leads to the release of the metal from the protein with the concomitant loss of activity strongly suggest that bound zinc ion is critical to enzyme function. It is notable that Zn^{2+} appears as an integral part of this restriction endonuclease, a sample of not prototypical DNA-specific enzyme. Indeed, many DNA restriction enzymes are likely to be zinc metalloenzymes. Our findings lend support to the notion that Zn^{2+} has a general role in DNA-protein complexes. It is interesting to suggest that this metal is important to specific DNA recognition. Certainly its chemical role in this enzyme bears further scrutiny.

[1] K. Barton and L. A. Bassie, unpublished results.

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