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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.

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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 Analysis—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 0.05–0.7 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 analysis, 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 195 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.

TABLE I
Zinc content of Eco RI

Sample	Dialysis conditions[a]	A_{Zn}[b]	A_{buffer}	Concentration		Zinc/protein stoichiometry
				Zn	Protein	
					$\times 10^7$ M	
Eco RI	10 mM Tris, pH 7.4, 10 mM EDTA; 10 days, 16×	0.330	0.019	43.8	43.6	1.0
	100 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
	6 days, 12×[c]	0.580	0.120	7.9	8.6	0.9
	+5 mM EDTA[c]; 8 days, 15×	0.539	0.114	4.9	3.2	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[c]; 8 days, 15×	0.113	0.117	0	4.0	

[a] Dialyses were conducted at 0 °C using a 10^4 volume ratio of dialysate to sample. The frequency with which buffers were changed is indicated (×). KPhos, potassium phosphate.

[b] 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.

[c] 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^{2+} 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 3×10^{-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^{2+} 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^{2+}, $\log K = 16.5$ (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^{2+} 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.

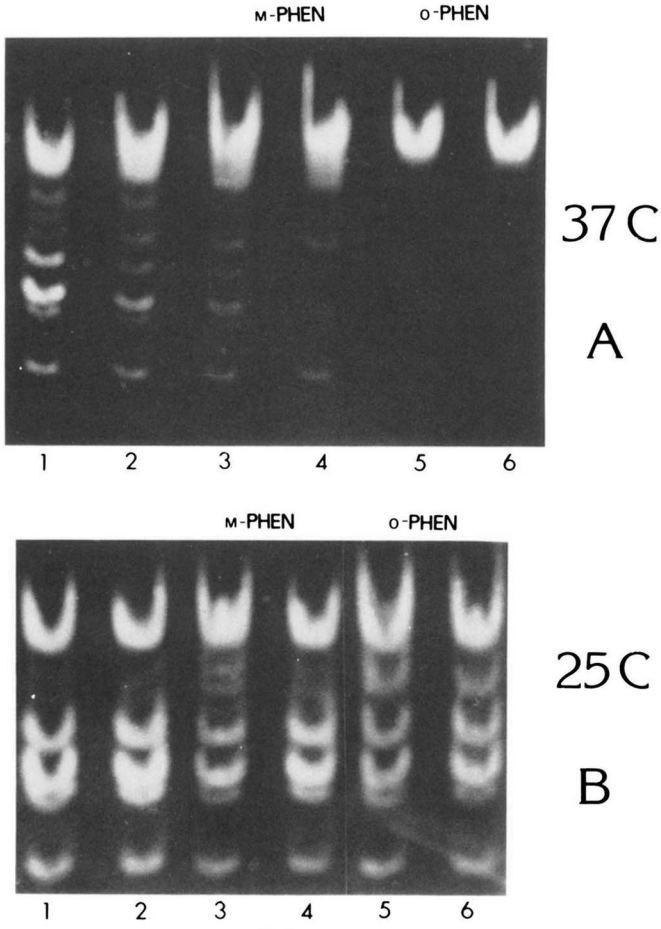

FIG. 1. **1% agarose gel electrophoresis of the *Eco* RI digestion of λ DNA in the presence of inhibitor.** Effects of preincubation at 37 °C (*A*) and 25 °C (*B*). *Eco* RI samples (0.9 μg/ml) were preincubated for 20 min with (from *left* to *right* for each gel) no inhibitor (*1* and *2*), 2.5 mM *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 $MgCl_2$ 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^{2+} 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^{2+} is sufficiently high (log K = 8.7) to complicate data interpretation. Inhibition by *o*-phen is not a consequence of interactions with the Mg^{2+} 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^{2+} 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^{2+}, the metal can be removed by dialysis against 5 mM *o*-phen and the enzyme lacking Zn^{2+} 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^{2+} 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

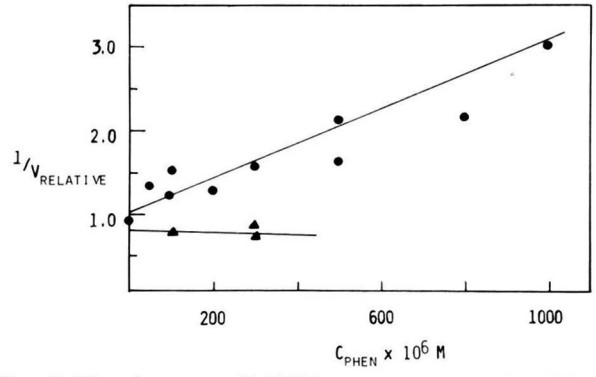

FIG. 2. **The cleavage of λ DNA (10 nM sites) by *Eco* RI after preincubation at 37 °C (●) and 25 °C (▲) for 30 min with increasing concentrations of *o*-phen.** Relative velocities were determined as described under "Experimental Procedures" and are plotted as an inverse ratio to that of *Eco* RI preincubated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

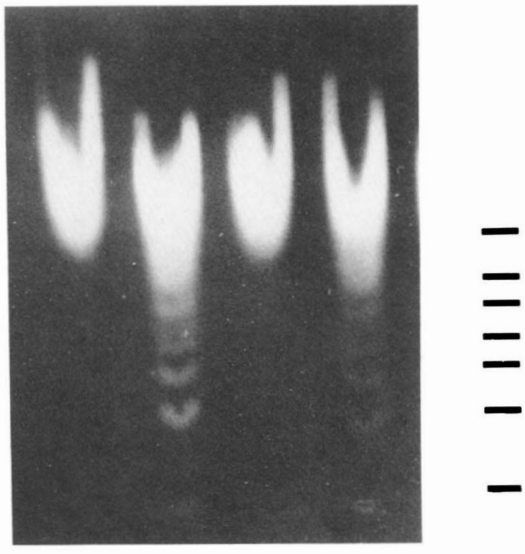

FIG. 3. **1% agarose gel electrophoresis of λ DNA after digestion by native and apoenzyme.** *Eco* RI was dialyzed for 7 days at 0 °C in buffer (10 mM K phosphate, pH 7.1, 0.2 M NaCl, 0.01% Triton) containing 5 mM *o*-phen. In parallel, native enzyme was dialyzed against buffer without the inhibitor. 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[2] 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 simple if 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.

[2] J. K. Barton and L. A. Basile, unpublished results.

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