actual binding and one corresponding to rearrangements of the anion-enzyme complex.

1 G. Mager, I. Oberbäumer, H. Ruf and E. Grell, in 'Transmembrane Ion Motions', Proc. 36e Réunion Internationale de la Société de Chimie Physique, Paris (1982), Elsevier, Amsterdam, 1983.

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A Cobalt Derivative of the Restriction Enzyme EcoRI

J. K. BARTON* and L. A. BASILE

Department of Chemistry, Columbia University, New York, N.Y. 10027, U.S.A.

Although zinc is present in a range of enzymes that bind to DNA [1], the function of the Zn(II) dication either in the DNA binding process or in subsequent enzymic action is uncertain. Recently we have shown that the type II restriction endonuclease EcoRI [2] contains one equivalent tightly bound zinc per monomer and that the zinc ion is essential for activity [3]. In order to more fully characterize the metal center and its interaction with the nucleic acid substrate, we have prepared the spectroscopically active cobalt(II) derivative. Substitution with cobaltous ion yields a particularly active restriction endonuclease which displays a substrate specificity that is comparable to the native zinc enzyme.

Cobalt-EcoRI was prepared by dialysis of the native Zn(II)-EcoRI against high concentrations (2.5 m*M*) cobalt chloride (puratronic grade). Nitrogen was bubbled through the solution continuously to avoid air oxidation to cobalt(III). The possibility that *in situ* oxidation occurred however cannot be ruled out without spectroscopic examination. The cobalt dialysis buffer was replaced a minimum of six times to achieve complete incorporation. Thereafter dialysis against buffer without cobalt was conducted to eliminate residual free cobaltous ion; again at least six changes were required.

Figure 1 shows the metal content of the enzyme over the course of one such dialysis procedure. Both the zinc and cobalt contents of the enzyme were assayed using flameless atomic absorption spectroscopy. Each time the dialysate containing $CoCl_2$ was replaced, an aliquot was removed and dialyzed in parallel against buffer lacking cobaltous ion. As can be seen in Fig. 1, over the course of the experiment a consistent rise in the bound cobalt/enzyme monomer ratio is observed with a concomitant decrease in the intrinsic zinc content. After seven days, stoichiometric cobalt incorporation is found. No subsequent increase in bound cobalt is observed. These data indicate strongly that cobalt ion specifically displaces the enzyme-bound zinc ion in view of *i*) the parallel incorporation of cobalt with the loss of zinc and *ii*) the eventual stoichiometric and tightly bound cobalt content. It is noteworthy that no significant decrease in the level of bound zinc is observed with exhaustive dialysis against buffer lacking these high concentratives of cobaltous ion. Furthermore, metal substitution cannot be practically achieved if as little as 0.1 μM zinc ion is available in solution. Quantitative values for the relative binding affinities of zinc(II) and cobalt(II) to the metal site in EcoRI are being determined presently.

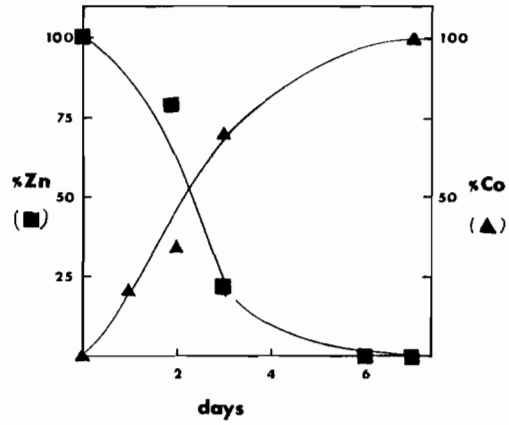

Fig. 1. Level of zinc and cobalt bound per EcoRI monomer monitored over the course of cobalt substitution into EcoRI. Zinc levels are seen to diminish with cobalt incorporation and the approximate 1:1 metal/protein stoichiometry appears to be maintained throughout. Native EcoRI (500 μg/ml) was dialyzed under nitrogen at 4 °C first against zinc-free buffer containing 10 m*M* TRIS pH 7.5, 50 m*M* NaCl, 1% glycerol, 0.01% Triton X-100, and 2.5 m*M* $CoCl_2$ with repeated changes. After each aliquot was removed from the cobalt buffer for metal and activity assays, dialysis against cobalt-free buffer was conducted to eliminate residual metal ions. All samples were dialyzed in parallel for a total of 10 days. Protein concentrations were determined by colorimetric assay and metal content by atomic absorption spectroscopy using a carbon rod furnace.

Cobalt(II)-EcoRI shows activity and a DNA substrate sequence specificity that is comparable to the native enzyme. To assay for activity we examined the fragmentation of phage λ DNA using agarose gel electrophoresis. Phage λ DNA, with a molecular weight of 30 megadaltons, has five restriction sites for native EcoRI of somewhat differing affinities, and upon digestion with EcoRI a characteristic fragmentation pattern is observed [4]. Figure 2 shows the agarose gel electrophoretic pattern of the λ DNA fragments after incubation with the various EcoRI aliquots that differ in levels of cobalt incorporation as was given in Fig. 1. It is apparent that Co-EcoRI shows the same substrate specificity as the native

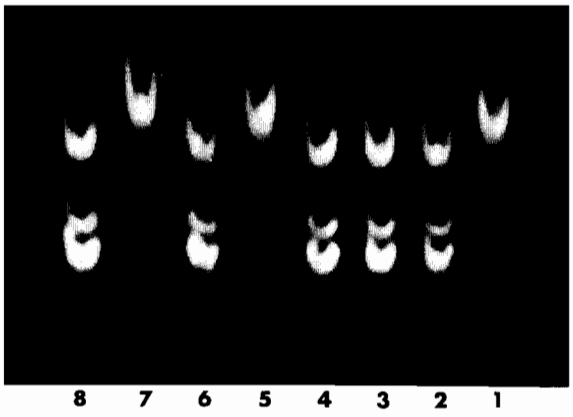

Fig. 2. The 1% agarose gel electrophoresis pattern of phage λ DNA after fragmentation by EcoRI samples containing increasing levels of cobalt substitution. Protein samples correspond to those described in Fig. 1. The DNA samples were incubated with EcoRI (2 μg/ml) for 1 h at 37 °C in 10 m*M* TRIS pH 7.5, 50 m*M* NaCl, 10 m*M* $MgCl_2$, 1% glycerol, 0.001% Triton X-100. Enzyme samples used for the incubation contain (from right to left) 0, 0.22, 0.33, 0.69, 0.9 (estimated), 0.96 bound cobalt/enzyme monomer. The enzyme aliquot used for channel 5 contained adventitous cobalt ion. Channel 7 corresponds to intact phage DNA and channel 8, to the fragmentation pattern obtained after incubation with fresh, untreated EcoRI.

enzyme; the identical fragmentation patterns are observed with complete cleavage. Interestingly, we also find identical patterns with partial digestion (not shown) which indicates a comparable hierarchy of site preferences for the cobalt and zinc metalloenzymes.

Two additional features in Fig. 2 are noteworthy. Co-EcoRI appears more active or is at least more stable than the native form of the enzyme treated similarly. Dialysis of the native Zn-EcoRI in buffer for 10 days, not surprisingly, decreases activity. This is evident in comparing channel 1, λ DNA after incubation with exhaustively dialyzed enzyme, where only intermediate high molecular weight fragments are visible and channel 8, λ DNA after incubation with untreated Zn-EcoRI, which shows complete digestion. With increasing cobalt substitution, given for channels 1–4, increasing levels of activity are found. Cobalt derivatives of EcoRI maintain high activity in dilute form for at least two months at 4 °C. Whether Co(II)-EcoRI is not only more stable to denaturation but also kinetically more efficient is being tested. Secondly the lack of cleavage in channel 5 illustrates the sensitivity of the endonuclease action to the presence of extraneous metal ions. Metal analyses of the enzyme aliquot used for this incubation revealed only partial removal of the residual cobaltous ion. The 0.5 μ*M* level of free cobalt present in the incubation mixture was sufficient to inhibit EcoRI. We find inhibition of EcoRI with various divalent metal ions at micromolar concentrations [5]. It is likely that these ions compete for the magnesium cofactor site.

Characteristics of the metal center of EcoRI are thus beginning to emerge. EcoRI contains at least one tightly bound zinc ion per monomer which is inaccessible to solvent and cannot be removed by exhaustive dialysis against anionic chelating agents. The apoenzyme, which is inactive, can however be obtained by extensive dialysis against the neutral *ortho*-phenanthroline; this perhaps indicates that the metal resides in a hydrophobic pocket. The cobalt derivative is in contrast easily prepared by direct displacement of the bound zinc ion. The small hydrated cobaltous ion gains access to the metal center and like zinc(II) binds tightly and subsequently appears non-dialyzable. Co-EcoRI exhibits high activity and the same substrate specificity as native Zn-EcoRI. Other examples of high activity in cobalt derivatives of zinc enzymes are well known; Co(II)-carboxypeptidase, for instance, displays a three-fold enhancement in peptidase activity over the native enzyme [6]. The similar substrate specificity of Co-EcoRI and Zn-EcoRI likely reflects the similar chemistry of these two metal ions. Co-EcoRi is certainly more hardy than the native enzyme, and derivatization with cobaltous ion may be generally helpful to improve enzyme lifetime for biomedical applications. Other metal derivatives of EcoRI will be important to examine in order to determine the contribution of the metal center to both the specific DNA binding and endonucleolytic steps of enzyme action.

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1 A. S. Mildvan and L. A. Loeb, *CRC Crit. Rev. Biochem., 6*, 819 (1979).
2 P. J. Modrich and D. Zabel, *J. Biol. Chem., 251*, 5866 (1976);
P. J. Greene, M. C. Betlach, H. W. Boyer and H. M. Goodman, *Methods, Mol. Biol., 7*, 87 (1974).
3 J. K. Barton, L. A. Basile and S. R. Paranawithana, *J. Biol. Chem., 257*, 7911 (1982).
4 M. Thomas and R. W. Davis, *J. Mol. Biol., 91*, 315 (1975).
5 J. K. Barton, L. A. Basile and K. Flynn, unpublished results;
J. L. Woodhead, N. Bhave and A. D. B. Malcolm, *Eur. J. Biochem., 115*, 293 (1981).
6 J. E. Coleman and B. L. Vallee, *J. Biol. Chem., 236*, 2244 (1961);
E. P. Kang, C. B. Storm and F. W. Carson, *J. Am. Chem. Soc., 97*, 6723 (1975).