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Behavior of Copper and Zinc Ions in the Binding Sites of Superoxide Dismutase, Bovine Erythrocytes

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The authors are very much interested in the selective binding of copper and zinc of bovine erythrocytes superoxide dismutase [Cu_2Zn_2SOD; superoxide oxidoreductase, EC 1.15.1.1] in the respective native binding sites without mistake. In order to examine the behavior of metals, we determined the apparent binding constants of copper ions to the apo-SOD, Cu_2E_2SOD by equilibrium dialysis against 2-picolinic acid in various pH solutions [1]. These copper binding constants were found to be pH dependent [2].

Apo-superoxide dismutase, E_2E_2SOD can also bind four zinc ions to two native copper and two native zinc binding sites. Four stepwise apparent binding constants of zinc ions to the apo-SOD were determined similarly, by means of equilibrium dialysis against 2-PA in HEPES buffer of pH 6.25.

In order to investigate the selective binding of copper and zinc ions with apo-SOD, E_2E_2SOD in the respective native binding sites, the authors carried out equilibrium dialysis in the presence of $CuSO_4$ and $ZnSO_4$ in 0.1 *M* HEPES buffer on pH 6.5. They bind selectively to their respective binding sites in the ratio of Cu:Zn = 1:1, recovering SOD activity.

As the geometry of copper ions and zinc ions is not the same, we carried out the determination of the intrinsic apparent binding constants of two zinc ions by the competitive replacement between Cu_2E_2SOD and various concentrations of 2-picolinato-Zn complex, by means of equilibrium dialysis.

The authors have established the HPLC of native-SOD, apo-SOD and metal-replaced-SOD. It is much easier to identify the purity of self-purified and marketing SOD, very rapidly. This HPLC is also helpful in the chemical study of metalloenzyme coordination.

Determination of the apparent binding constants is very useful in the investigation of the active sites of Cu_2Zn_2SOD. The authors recommend HPLC as one of the best tools in the study of bioinorganic chemistry. The mechanism of SOD activity will be discussed.

TABLE I. Apparent Binding Constants of four Cu and four Zn SOD.

	k_1	k_2	k_3	k_4	pH
Cu	13.9	13.4	11.1	10.6	6.25
Zn	10.9	11.1	7.8	6.5	6.25

1 J. Hirose, K. Iwatsuka and Y. Kidani, *Biochem. Biophys. Res. Comm., 98,* 58 (1981).
2 J. Hirose, T. Ohira, H. Hirata and Y. Kidani, *Arch. Biochem. Biophys., 218,* 179 (1982).

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Kinetic Characterization of the Active Site Region of Carbonic Anhydrase

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In native carbonic anhydrase the coordinated zinc ion, which is part of the active site region, may be replaced by Co(II), with retention of enzyme activity. The Co(II)-substituted protein has spectral properties which make it more amenable to spectroscopic studies.

The protolytic reactions in the active site region of bovine Co(II)-carbonic anhydrase B have been studied by spectrophotometric titrations employing a new computer-controlled high performance titration system [1] and by temperature jump and electric field jump relaxation spectrometry. Among the fast elementary steps involved, it has been possible to differentiate between the initial protonation/deprotonation reactions, probably occurring at the surface region, an intramolecular proton transfer to the active site region and a rearrangement within the coordination sphere of the heavy metal ion.

The binding constants of the anionic inhibitors sulphate, cyanide, cyanate and thiocyanate to bovine Co(II)-carbonic anhydrase B have been determined employing the new spectrophotometric titration system. The experiments were carried out either by pH titrations at various inhibitor concentrations or by anion titrations at various pH values.

The dynamic properties of the anionic binding site of Co(II) carbonic anhydrase have been characterized on the basis of temperature jump studies. The kinetic results obtained from the investigation of the elementary steps involved in the binding of anionic inhibitors such as cyanide, cyanate and thiocyanate provide information about the mechanism of action of this enzyme. Evidence will be presented that the overall binding of monovalent anions must be characterized by a process comprising at least two steps, one corresponding to the

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

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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.

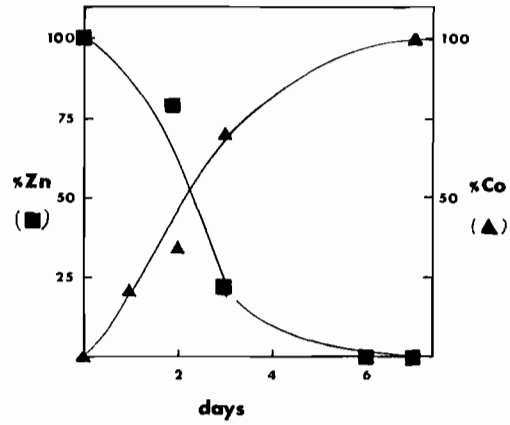

Fig. 1. Level of zinc and cobalt bound per EcoRI monomer monitored over the course of cobalt substitution into EcoRI. Zinc levels are seen to diminish with cobalt incorporation and the approximate 1:1 metal/protein stoichiometry appears to be maintained throughout. Native EcoRI (500 μg/ml) was dialyzed 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