Metallocarboxypeptidases: Stability Constants and Enzymatic Characteristics*

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In a previous communication (1), we have described some of the enzymatic and physicochemical properties of a series of metallo-carboxypeptidases prepared from the metal-free apoenzyme of bovine carboxypeptidase A. This paper presents the stability constants and enzymatic activities of a series of metallo-carboxypeptidases produced when 1 g atom of zinc in the native enzyme is replaced by 1 g atom of manganese, cobalt, nickel, copper, cadmium, or mercury. Significantly, the substitution of the various metals for zinc in carboxypeptidase induces marked changes in relative activities and specificity of the enzyme. The order and magnitude of the stability constants of this series of metallo-carboxypeptidases are in accord with the hypothesis that a sulfur and a nitrogen atom of the apoenzyme bind these metal ions to constitute the active center (2-4). The release of metal ions from the active center of metallocarboxypeptidases is in accord with the hypothesis that a sulfur and a nitrogen atom of the apoenzyme bind these metal ions to constitute the active center (2-4). The release of two H+ ions when a metal atom is bound to apocarboxypeptidase constitutes further experimental support for this hypothesis, since the apparent pK values of the ionizing groups are consistent with those to be expected of a sulfhydryl and a nitrogenous group of amino acid residues (5).

EXPERIMENTAL PROCEDURE

Beef Pancreas Carboxypeptidase [([CPD]Zn)]—Four times recrystallized zinc carboxypeptidase was prepared from beef pancreas acetone powder by the method of Allan et al.2 The solution of the final crystals in 1.0 M NaCl-0.1 M Tris buffer, pH 7.5, was homogeneous in the ultracentrifuge and by moving boundary electrophoresis when examined in 0.3 ionic strength LiCl buffers of pH 6.8 to 10.5. The proteolytic coefficient, C, of this preparation was 25 to 30 at pH 7.5, 25°, and 6 to 7 at pH 7.5, 0°. The esterase activity, expressed as a zero order rate constant, k (see below), was 1.15 × 10^9 μM H+ per minute per mg of N at pH 7.5, 25°. The zinc to protein ratio was 1950 μg per g of protein or 1.03 g atom per mole, assuming a molecular weight of 34,300 for the protein (6, 7).

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† Post-Doctoral Fellow of the National Institutes of Health of the Department of Health, Education, and Welfare.
2 The abbreviations used are (in the formulations only and when required for differentiation): [([CPD]Zn)], zinc carboxy-peptidase, with (CPD) representing the apoenzyme and the brackets indicating the firm binding of zinc or other metals substituting for it, e.g., [([CPD]Hg)]. Carboxypeptidase refers to carboxypeptidase A only.
3 Kindly supplied by the Lilly Research Laboratories.
4 Gift of Dr. H. Neurath.

Zinc-free Apocarboxypeptidase ([CPD])—Zinc-free apocarboxypeptidase was prepared by dialyzing the native enzyme against 1,10-phenanthroline. Precautions against contamination by metal ions were taken, as previously described (1, 8). The preparations used in these experiments contained between 26 and 39 μg of zinc per g of protein, or 1.3% and 2% of the original zinc content, and had 2% of the original activity.

Enzymatic Activity—Pep tidase activity was determined as described elsewhere (1) with carbobenzyoxyzglycyl-L-phenylalanine, carbobenzyoxglycyl-L-tryptophan, and benzoylglycyl-L-phenylalanine as substrates (Mann Chemical Company). Activity is expressed as an apparent proteolytic coefficient, C, as previously defined and employed (1, 9, 10). The assays were carried out at 0° in 0.02 M sodium Veronal buffer containing 0.1 M NaCl, pH 7.5; C was calculated from the linear portion of the first order reaction plots observed when hydrolysis did not exceed 15%.

Esterase Activity was determined as has been described (11) with hippuryl-dl-β-phenyllactate4 as the substrate. The activity was measured by titration with 0.1 M NaOH of the hydrogen ions released on hydrolysis with a pH Stat titrator (Radiometer, Copenhagen) and recorder (Ole Dih, Copenhagen). Assays were performed at 25° with 5 ml of 0.01 M hippuryl-dl-β-phenyllactate in 0.1 M NaCl0.005 M Tris buffer, at pH 7.5. Activities are expressed as zero order velocity constants, k, with units of μM H+ per minute per mg of N.

Metal Analyses—Zinc, cadmium, mercury, copper, manganese, nickel, cobalt, and lead were determined by chemical methods which are specific for each (12-16).

Protein concentrations were measured either by 10% trichloroacetic acid precipitations followed by drying at 104° (17), or from absorbancy at 278 mμ (18). The results of the two procedures were in excellent agreement. The Beckman model DU spectrophotometer was used throughout, and pH was measured with a Leeds and Northrup pH indicator equipped with a general purpose external glass electrode.

Preparation of Zinc, Cadmium, Mercury, Lead, Manganese, Cobalt, Nickel, and Copper Carboxypeptidase—Standard solutions of zinc, cadmium, mercuric, manganous, cobaltous, nickelous, cupric, and lead chlorides were prepared by dissolving the spectrophotographically pure metals (Johnson Matthey Company, Ltd.) in dilute, metal-free HCl. The solutions were diluted with metal-free buffer to result in the desired pH and molarity, to be 1 M NaCl and 0.1 M Tris.4

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dialyzed against the isotopically labeled enzymes for exchange experiments, or against the zinc-free apoenzyme to prepare a given metallocarboxypeptidase. The metal solutions used for the exchange and equilibrium experiments described below were labeled by the addition of tracer amounts of isotope to the standard solutions.

**RESULTS**

**Stability Constants**—Manganese, cobalt, nickel, and copper carboxypeptidase undergo significant dissociation under the experimental conditions here used for equilibrium dialysis, 1 M NaCl-0.05 M Tris, pH 8.0, 4°C. Hence, the apparent stability constants, $K_{app} = ((\text{CPD})\text{Me})/\text{CPD} (\text{Me}^{++})$, can be calculated from measurements of protein, either by trichloroacetic acid precipitation or by absorbancy at 278 mμ, and from measurement of the respective metal ion, by isotopic or microchemical means. This “direct method” has been described (1) for measurement of the stability constant of the cobalt enzyme, the prototype for this procedure.

Once the stability constant for one of these metallocarboxypeptidases is obtained in this manner, that for any other can be determined by allowing a second metal ion to compete for the enzymatic binding site, to result (at equilibrium) in a mixture of two metalloenzymes. By use of this “competition method,” the following relationship holds:

$$K_{Me} = \frac{(Me^{++})([(\text{CPD})Me])}{(Me^{++})([(\text{CPD})Me])} \cdot K_{Me}$$

in which Me represents the metal for which the stability constant is known and Me2 that for which it is unknown.7

Apo-carboxypeptidase forms a 1:1 complex with each metal, as determined by isotopic and microchemical analyses. The apparent stability constants, log $K_{app}$, for the resultant metallo-carboxypeptidases follow the order Zn > Cd > Hg > Ni > Co > Mn > Cu. (Table I, Columns A and B).

Because the solubility characteristics of the enzyme required the presence of 1 M NaCl and 0.05 M Tris, the $K_{app}$ deviates from the true stability constant, $K_{corr}$, due to the competition between these anions and the apoenzyme for the metal ions. It is, therefore, necessary to calculate the concentration of free metal ions by correcting for that moiety, bound to chloride and Tris, by which the effective concentration of free metal ions in solution is diminished. The constants for ammonia may be expected to approximate those for Tris, and the correction is significant for all the ions but Cu++. Based on the constant, the high stability constants of zinc, cadmium, and mercury carboxypeptidase had to be measured by means of the “competition method” because dissociation is so insignificant that measurements by the “direct method” fail to give acceptable results. The numerical values of the constants can be checked by variation of the species of Me, which is used to replace Me2. In most instances, Cu++ or Mn++ represented Me, the pairs of constants obtained with both ions agree closely.

### Table I

<table>
<thead>
<tr>
<th>Metal</th>
<th>Disassociation constant (apparent)</th>
<th>Log $K_{app}$</th>
<th>Log $K_{app}$ (CF, Tris)</th>
<th>Log $K_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn</td>
<td>$2.5 \times 10^{-4}$</td>
<td>5.6</td>
<td>5.6</td>
<td>$4.1^c$</td>
</tr>
<tr>
<td>Co</td>
<td>$1.5 \times 10^{-4}$</td>
<td>5.8</td>
<td>7.0</td>
<td>$7.7^c$</td>
</tr>
<tr>
<td>Ni</td>
<td>$2.0 \times 10^{-4}$</td>
<td>5.7</td>
<td>8.2</td>
<td>$9.9^d$</td>
</tr>
<tr>
<td>Zn</td>
<td>$7.4 \times 10^{-6}$</td>
<td>5.1</td>
<td>10.6</td>
<td>$a$</td>
</tr>
<tr>
<td>Cd</td>
<td>$4.7 \times 10^{-8}$</td>
<td>8.3</td>
<td>10.5</td>
<td>$10.2^d$</td>
</tr>
<tr>
<td>Hg</td>
<td>$1.2 \times 10^{-8}$</td>
<td>7.9</td>
<td>10.8</td>
<td>$11.0^c$</td>
</tr>
<tr>
<td>Cu</td>
<td>$2.0 \times 10^{-9}$</td>
<td>6.7</td>
<td>21.0</td>
<td>$22.0^c$</td>
</tr>
</tbody>
</table>

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7 The log half-life γ-emitters, Co60 and Mn54, are particularly convenient in this regard. Combining isotopic analysis of one with microchemical analysis for the other of a pair of ions represents a particularly simple and reliable procedure presenting multiple opportunities for checks of accuracy. The availability of suitable isotopes imposes some limitations. Mn64, Co60, Zn65, and Cd112 satisfy all requirements, whereas either the mode of decay or unfavorable half-lives of obtainable isotopes of Hg, Cu, Ni, and Pb made them unsuitable for this analytical method. The oxidation of Fe++ to Fe+++ during equilibrium dialysis caused precipitation interfering with precise analytical measurements.

The high stability constants of zinc, cadmium, and mercury carboxypeptidase had to be measured by means of the “competition method” because dissociation is so insignificant that measurements by the “direct method” fail to give acceptable results. The numerical values of the constants can be checked by variation of the species of Me, which is used to replace Me2. In most instances, Cu++ or Mn++ represented Me, the pairs of constants obtained with both ions agree closely.

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a Each contained 1 g atom of metal per mole of protein. The oxidation state of the copper is not known.

b Determined in 1 M NaCl-0.05 M Tris, pH 8.0, 4°C.

c These values for 2-mercaptoethylamine are not on record; those given are for cysteine (19).

d The reasons for the unavailability of constants for the cupric complexes have been detailed (20, 21).

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The long half-life γ-emitters, Co60 and Mn54, are particularly convenient in this regard. Combining isotopic analysis of one with microchemical analysis for the other of a pair of ions represents a particularly simple and reliable procedure presenting multiple opportunities for checks of accuracy. The availability of suitable isotopes imposes some limitations. Mn64, Co60, Zn65, and Cd112 satisfy all requirements, whereas either the mode of decay or unfavorable half-lives of obtainable isotopes of Hg, Cu, Ni, and Pb made them unsuitable for this analytical method. The oxidation of Fe++ to Fe+++ during equilibrium dialysis caused precipitation interfering with precise analytical measurements.

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resulted in a shift of the pK1 from 7.7 to 7.8.0.32 =I= 0.01 pmole of H+ measured. The decrease of NaCl to 0.5 values indicated by the experimental points in 1 interaction with Zn++ ions were measured by means of a pH-Stat. M values between pH 7 and 9.5, and the displacement of hydrogen titration. Zn++ ions were added to the apoenzyme at fixed pH tated a discontinuous titration for the conventional continuous below pH 5.5, but that zinc carboxypeptidase is stable at higher pH (1, 8). Hence, the dissociation of [(CPD)Zn] is negligible in the pH range from 7.0 to 9.5. We have, therefore, substituted a discontinuous titration for the conventional continuous titration. Zn++ ions were added to the apoenzyme at fixed pH values between pH 7 and 9.5, and the displacement of hydrogen ions was measured to within 0.01 μmole by keeping the pH of the mixture constant through the automatic addition of 0.01 M NaOH (22).

The enzyme and metal ion concentrations were adjusted so that the release of 0.32 μmole of H+ was equivalent to the release of one hydrogen ion from the protein. Citrate, 0.005 M, was used in the zinc stock solution, giving a final concentration of 0.00025 M in the reaction mixture. The zinc-citrate complex keeps the metal in solution between pH 7 and 9.5, yet is not sufficiently stable to compete significantly with the protein for Zn++; the pKa values of citrate are such that it does not impart buffering capacity to the solutions at these pH values. Sodium chloride, 1 M, is required for enzyme solubility, and a nitrogen atmosphere minimizes CO2 uptake at the higher pH values. Two prototropic groups of the protein are involved in zinc binding; two hydrogen ions are released when zinc combines with the apoenzyme at pH 7.0 (Fig. 1). The first hydrogen is titrated completely between pH 7 and 8; the group from which it is displaced has a pK1asp of 7.7. The pK1asp of the second ionization is 9.1.

**Substrate Specificities of Metallocarboxypeptidases** When assayed under standard conditions, the reconstituted metallo- carboxypeptidases hydrolyze carbobenzoxyglycyl-L-phenylalana nine at rates in the characteristic order Co > Ni > Zn > Mn. When carbobenzoxyglycyl-L-tryptophan or benzoylglucyl-L-phenylalanine are used as the substrates, the activities of the cobalt enzyme are approximately equal to those of the zinc enzyme in contrast to the rates observed with carbobenzoxyglycyl-L-phenylalanine (Table II). The activities of nickel carboxypeptidase are much lower, whereas those of manganese carboxypeptidase are the same with all three substrates. Copper, lead, cadmium, and mercury carboxypeptidase do not hydrolyze any of these peptides. Thus, the particular metal atom which occupies the single active site markedly and differentially affects the rates at which carboxypeptidase hydrolyzes peptide substrates.

Carboxypeptidase, like other peptidases, hydrolyzes esters, one of which is hippuryl-dl-β-phenyllactate. With the exception of copper carboxypeptidase, which hydrolyzes neither peptides nor this ester, the metalloenzyme incorporating the metals of the first transition series studied here also attack this ester substrate. However, mercury, cadmium, and lead carboxypeptidase exhibit esterase activities while being completely inactive toward peptides; cadmium and mercury carboxypeptidase, in fact, are more active than is the zinc enzyme (Table II).

The complete loss of peptidase activity observed on replacing zinc by mercury, cadmium, or lead made it important to determine whether these metals occupy the same or different sites on the enzyme as zinc and hence to discern whether the curtailment of the dual specificity for peptides and esters involves binding of these metal ions to the same or different sites of the molecule. To test this possibility, [(CPD)Zn]2+ was exposed to 1 × 10^-6 M stable cadmium ions. Bound radioactive zinc and proteolytic activity were determined simultaneously at various times (Fig. 2). The loss of peptidase activity correlates directly with the displacement of zinc, both being complete at 96 hours, when all zinc is replaced by cadmium. In a separate experiment, [(CPD)Zn], the native enzyme, was exposed to Cd2+, and esterase activity was measured (Fig. 2). As zinc is displaced, protein-bound Cd2+ and esterase activity rise concurrently to reach 1 g atom of cadmium per mole of enzyme: the

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**Table II**

<table>
<thead>
<tr>
<th>(CPD)Mo</th>
<th>Peptidase activity</th>
<th>Esterase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Carboxobenzoxyglycyl-L-phenylalanine</td>
<td>Carboxobenzoxyglycyl-L-tryptophan</td>
</tr>
<tr>
<td>[(CPD)Zn]</td>
<td>7.5</td>
<td>2.8</td>
</tr>
<tr>
<td>[(CPD)Co]</td>
<td>12.0</td>
<td>2.7</td>
</tr>
<tr>
<td>[(CPD)Ni]</td>
<td>8.0</td>
<td>2.0</td>
</tr>
<tr>
<td>[(CPD)Mn]</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>[(CPD)Cu]</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[(CPD)Hg]</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[(CPD)Cd]</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[(CPD)Pb]</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Me, 1 g atom per mole of apoenzyme.

* Assays were carried out at pH 7.5, 0°C, with 0.02 M substrate in 0.02 M sodium Veronol, containing 0.1 M NaCl.

* Assays were carried out at pH 7.5, 25°C, with 0.01 M hippuryl-dl-β-phenyllactate in 0.005 M Tris, containing 0.1 M NaCl. The coefficient of variation for these measurements is 4%.
basis of the mole fractions of [(CPD)Cd] and [(CPD)Zn] present constituted zinc enzymes interact with reagents specific for mercapto groups, whereas one -SH group is readily titrated in per mole of enzyme is incorporated (Fig. 3).

The experiments with [(CPD)Hg] were performed in similar conditions are as in Fig. 2. V, ZnB6 bound to 1 X 10e M [(CPD)Zn6], exposed to 1 X 10e M Cd++ at 0 time; , proteolytic coefficient, C, of 1 X 10e M [(CPD)Zn48], exposed to 1 X 10e M Cd++ at 0 time; , Cd155++ bound to 1 X 10e M [(CPD)Zn], exposed to 1 X 10e M Cd++ at 0 time; , esterase activity, k X 10e, of 1 X 10e M [(CPD)Zn], exposed to Cd155++ at 0 time. The dashed curves (-----) represent peptidase and esterase activities calculated on the basis of the mole fractions of [(CPD)Zn] and [(CPD)Cd] present at any given time.

Peptidase activity is lost as mercury displaces zinc, while esterase activity increases slightly, although significantly, throughout the time course of the exchange until 1 g atom of mercury is incorporated (Fig. 3). The experiments with [(CPD)Hg] were performed in similar fashion and are directly analogous to those with [(CPD)Cd]. Peptidase activity is lost as mercury displaces zinc, while esterase activity increases slightly, although significantly, throughout the time course of the exchange until 1 g atom of mercury is incorporated (Fig. 3).

The experiments with [(CPD)Hg] were performed in similar fashion and are directly analogous to those with [(CPD)Cd]. Peptidase activity is lost as mercury displaces zinc, while esterase activity increases slightly, although significantly, throughout the time course of the exchange until 1 g atom of mercury is incorporated (Fig. 3).

Discussion

These data confirm and extend past observations and hypotheses indicating the active center of native carboxypeptidase to consist of a zinc atom bound to a sulfur and nitrogen atom (2, 4). It may be recalled that neither the native nor the reconstituted zinc enzymes interact with reagents specific for mercapto groups, whereas one —SH group is readily titrated in the metal-free apocarboxypeptidase; furthermore, restoration of activity through the addition of metal ions is prevented when the —SH group is blocked. Thus, the localization of the zinc mercaptide involved in activity is limited to one particular residue, cysteine, in the amino acid sequence of the apoenzyme.

Previous evidence also indicates that cobaltous ions, like zinc, bind to a sulfur group. These two ions are bound to the same site, as proven by exchange experiments (1). Hence, the specific activity of the cobalt enzyme, higher than that of the zinc enzyme, could not be attributed to the creation of an additional, active site, contrary to other reports (23). The spectral changes accompanying the formation of cobalt carboxypeptidase show the formation of a mercapptide linkage with apocarboxypeptidase. Cobalt carboxypeptidase exhibits a distinctive red color with an absorption maximum at 530 m and an extinction coefficient of 150 (1). The shift in the absorption maximum from 512 m in the hydrated cobaltous ion to 530 m in [(CPD)Co], together with the increase in the extinction coefficient from 10 to 150, suggest binding to sulfur (24).

Similar binding of nickel, manganese, mercury, and cadmium to a sulfur atom is indicated by the relative order of the stability constants for the respective metallocarboxypeptidases which follow that expected for a sulfur containing ligand. The sulfur-nitrogen nature of the bidentate binding site is implied by the magnitude of the stability constants and by the release of two H+ ions on combination of the apoenzyme with Zn++ ions. The participation of a nitrogen atom as the second donor atom in binding zinc to apocarboxypeptidase was deduced from a number of considerations (4), which apply to this series of metallo-
carboxypeptidases.
The ranking order of stability constants for the complexes of a series of metal ions with simple ligands is found to be characteristic of its constituent donor atoms. Thus, when nitrogen and oxygen function in this capacity, the ranking order for the stability constants of metal complexes of interest here follows the sequence $\text{Hg}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Fe}^{2+} > \text{Mn}^{2+}$, analogous to that of the sulfur-containing ligands (Table I, Columns C and D). Hence, through the determination of the stability constants of such a series of metal complexes, the donor atoms of the metal-binding site of an organic ligand may be determined. If the same considerations can be shown to apply for metalloenzymes, a method for the identification of the ligand groups of the corresponding apoenzymes is at hand.

The sequence of the stability constants of the metallocarboxypeptidases studied here is in the order $\text{Hg}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Fe}^{2+} > \text{Mn}^{2+}$, copper being indeterminate because of the oxidation of the mercapto group (20, 21) (Table I, Column D). The complexes of cadmium and zinc are now distinctly stabilized over those of cobalt and nickel, the characteristic feature of the sulfur ligand series (25, 26, 28).

Thus, the metal atom known to function in substrate binding (41) also plays a role in the determination of enzymatic specificity. The coordination of the respective nitrogen or oxygen atoms of the substrates with the metal atom of the active enzymatic center will likely prove significant in detailing the mechanism of carboxypeptidase action. Many analogous aspects of the catalytic action of metals in peptidases have been discussed (42, 43).

The role of metal ions in the action of carboxypeptidase is apparent in yet another manner. Under standard conditions of assay, the relative order of catalytic efficiencies of different metallocarboxypeptidases varies as a function of the primary structure of the synthetic peptide substrate. Thus, the ranking order $\text{Co} > \text{Ni} > \text{Zn} > \text{Mn}$ observed previously for carbobenzoxyglycyl-n-phenylalanine and hippuryl-dl-β-phenylalanine, structurally differing only by the substitution of the ester for the peptide bond (Table II).
different substrates, remains to be determined, although the groups of the protein responsible for binding the metal are becoming apparent.

**SUMMARY**

Apocarboxypeptidase forms enzymatically active complexes with a series of metal ions. The stability constants are in the order $\text{Hg}^{2+} \gg \text{Cd}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Mn}^{2+}$. Both the order and the magnitudes of the constants correlate closely to those found to be characteristic of nitrogen-sulfur ligands, implying that in carboxypeptidase a nitrogen-sulfur site binds metal ions to result in enzymatic activity. This interpretation is supported by titrations showing two metal binding groups with pK values of 7.7 and 9.1, respectively, compatible with metal ions to result in enzymatic activity. This interpretation implicates that in carbosypeptidase a nitrogen-sulfur site binds to the metal-free apoenzyme before the addition of substrate occludes access of the metal to the binding site.

**REFERENCES**


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The substrate also binds to sites on the protein. If Zn$^{2+}$ ions are added to the metal-free apoenzyme before the addition of substrate, activity is completely restored. If substrate is added first and then Zn$^{2+}$ ions, activity is not restored. Apparently the substrate occludes access of the metal to the binding site (44).
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