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Binding of Transition Metal Ions by Ceruloplasmin (Ferroxidase)*

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ABSTRACT: Human ceruloplasmin (ferroxidase, EC 1.12.3.1) contains multiple binding sites for divalent transition metal ions. Equilibrium dialysis studies revealed that besides the 7 Cu ions found in the native protein, ceruloplasmin can bind an additional 10 Cu(II)'s which can be removed by Chelex. In independent and separate experiments, 3 Zn(II), 7 Ni(II), and 16 Co(II) were also bound to native ceruloplasmin. These data suggest that, in addition to the two types of copper found in the native protein, there are three other classes of binding sites in ceruloplasmin: (1) one site which is frequently occupied by Cu(II) during isolation of the enzyme; (2) two sites which serve as a substrate binding site for Fe(II) and which may bind Zn(II) and Ni(II); and (3) multiple nonspecific sites with the smallest association constants. The binding constants of these transition metal ions decrease in the order Cu(II) > Zn(II) > Fe(II) > Ni(II) > Co(II). Ferroxidase activity inhibition constants calculated for Zn(II), Ni(II), and Co(II) ions compare satisfactorily with their respective binding constants.

Ceruloplasmin (ferroxidase, EC 1.12.3.1) (Osaki *et al.*, 1966) has been known to contain copper since it was first isolated by Holmberg and Laurell (1944). The exact number of copper binding sites, however, has been in question due to the considerable variation in copper content and molecular weight reported in the past two decades (Pedersen, 1951; Kasper and Deutsch, 1963; Magdoff-Fairchild *et al.*, 1969). McDermott *et al.* (1968) defined the role of iron in ferroxidase but did not determine the binding properties of iron. This study and the investigation of the role of ferroxidase in iron mobilization (Osaki *et al.*, 1966; Ragan *et al.*, 1969; Frieden, 1971) led to the quantitative investigation of iron binding reported here.

Curzon (1960) first showed that low concentrations of Fe(II), Zn(II), Ni(II), and Co(II) activated ceruloplasmin's catalytic oxidation of *N,N*-dimethyl-*p*-phenylenediamine while higher concentrations of these ions showed marked inhibition. Huber and Frieden (1970a,b) have shown that these divalent transition metal ions similarly affected the ferroxidase activity. Both studies suggest the possibility of binding by these divalent ions to ceruloplasmin and prompted the study of the binding of these ions and a correlation with their inhibition data.

Materials and Methods

Preparation of Ceruloplasmin. Human ceruloplasmin (ferroxidase) was purified from a Cohn IV paste (Cohn *et al.*, 1946) essentially according to the method of Deutsch *et al.* (1962) using DEAE-Sephadex in the chromatography steps. The crystalline blue protein was dissolved in 0.10 M sodium acetate (pH 6.0) and showed an $A_{280}:A_{610}$ ratio of less than 23. The protein was immediately stored in acid-washed vials at −80° and rapidly thawed in water at room temperature prior to use. This treatment of the protein preparation resulted in little change in the ratio during several months storage. Upon thawing frozen ceruloplasmin, there were occasionally small amounts of a white precipitate which could be removed using a 0.45 μ Millipore filter. Only protein solutions with an $A_{280}:A_{610}$ ratio of less than 23 were used, thus indicating that the ceruloplasmin was native.

A stock solution of ceruloplasmin was prepared from frozen crystals in chelexed 0.10 M sodium acetate (pH 6.0) buffer and stored at 5°. This solution was diluted tenfold with the same buffer prior to use in equilibrium dialysis experiments. For experiments involving ^{59}Fe, all contaminating iron was removed by dialysis of the stock solution against 2% apotransferrin (Behringwerke, A.G.) for 48 hr (McDermott *et al.*, 1968). A stock solution of ceruloplasmin was routinely prepared to be 200 μM based on a molecular weight for the protein of 160,000 and $A_{610}^{1\%}$ 0.68 (Kasper and Deutsch, 1963; Kasper, 1967).

Metals, Reagents, and Glassware. All metals, acids, and other reagents used were of reagent grade purity except for ascorbic acid which was USP. Sodium acetate and ascorbate buffers were treated with Chelex-100 (200–400 mesh) (Bio-Rad) to remove any metal ion contaminants. Glassware and disposable pipets were soaked in concentrated hydrochloric acid and washed with deionized water to remove trace metals.

Equilibrium Dialysis. Plexiglas microdialysis cells (Technilab) with two 1.1-ml chambers were soaked in concentrated hydrochloric acid and washed with chelexed water to remove all traces of metal ions. Membranes for the cells were cut

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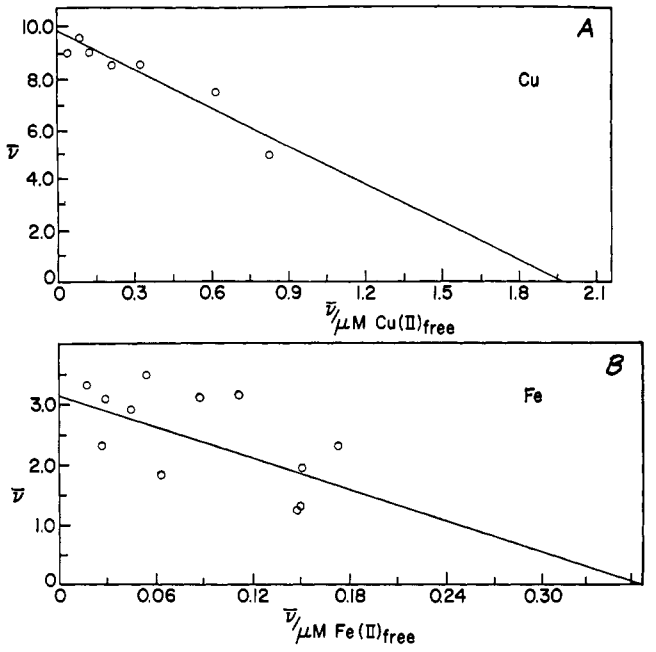

FIGURE 1: Scatchard ($\bar{\nu}$ *vs.* $\bar{\nu}/M$) plots of equilibrium dialysis data for ceruloplasmin and Cu(II) (A) or Fe(II) (B). Ceruloplasmin (20 μM) in 0.10 M sodium acetate (pH 6.0) was dialyzed against various concentrations of Cu(II) and Fe(II) for 48 hr at 5° while agitating on a Burrell wrist-action shaker. Sodium ascorbate (0.01 M) was added to maintain the iron in the Fe(II) state. Several different preparations of ceruloplasmin were used in this series of experiments.

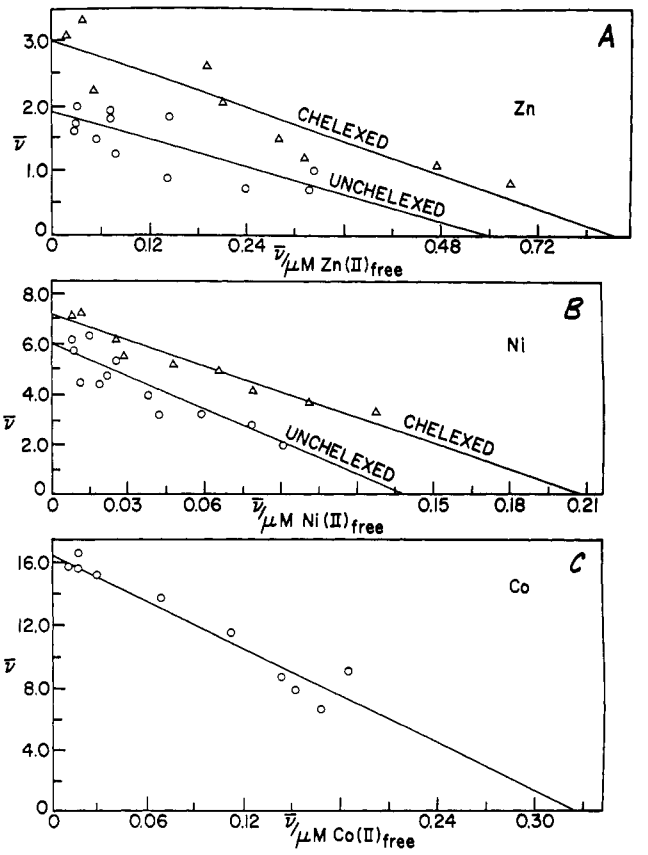

FIGURE 2: Scatchard plots of equilibrium dialysis data for ceruloplasmin and Zn(II) (A), Ni(II) (B), or Co(II) (C). Ceruloplasmin (20 μM) in 0.10 M sodium acetate (pH 6.0) was dialyzed against various concentrations of Zn(II), Ni(II), and Co(II) for 48 hr at 5° while agitating on a Burrell wrist-action shaker. For Zn(II) and Ni(II) a significant difference was found for chelexed (△) and unchelexed (○) ceruloplasmin with Zn(II) and Ni(II).

from dialysis tubing (Union Carbide) and prepared according to the method of Hughes and Klotz (1963). Assembled cells were stored at 5° until use. After removing water droplets from each cell, 1.0 ml of 20 μM ceruloplasmin was added to one chamber and 1.0 ml of chelexed buffer to the other. A divalent cation solution (1–10 μl) in 0.01 M H_2SO_4 (insufficient to noticeably change the pH) was added to each chamber of a cell. Each cell was then placed on a Burrell wrist-action shaker for 48 hr to ensure attainment of equilibrium. This procedure was followed for several different concentrations of each cation. Due to the substrate properties of Fe(II), it was necessary to use 0.01 M sodium ascorbate in the system to keep Fe(II) reduced. Cells were also kept under nitrogen atmosphere during agitation to minimize the oxidation of ascorbate and Fe(II). This problem did not arise with the other divalent ions.

Metal Analysis. A Perkin-Elmer atomic absorption spectrophotometer (Model 303) was used to determine the transition metal ion concentrations in the protein and buffer solutions of each dialysis cell. Zinc, cobalt, nickel, copper, and iron were all analyzed within the optimal limits of the instrument. Iron was also analyzed independently using ^{59}Fe (New England Nuclear) and a Nuclear-Chicago Model DSS scintillation detector with a Tracerlab Versa Matic II scaler. Stock solutions of radioactive iron were prepared and analyzed by the method of Diehl and Smith to ensure the accuracy of the analysis.

Kinetic Studies. A method developed by Johnson *et al.* (1967) was used to study the direct effect of metal ions on ferroxidase activity. Data were collected on a Cary 15 spectrophotometer after a 3-min, 30° incubation of all components except substrate, Fe(II). Reaction rates were followed at 460 nm and corrected for nonenzymic rates.

Results

Copper(II) Binding. A 20 μM solution of ceruloplasmin was analyzed for copper directly after preparation and found to contain 164 μM copper, corresponding to 8.2 atoms of copper/molecule of protein. Analysis of the same solution which had been treated with Chelex-100 showed a copper concentration of 142 μM or 7.1 copper atoms/ceruloplasmin. The latter indicates the minimum copper content in the native protein. Chelex treatment does not significantly alter the $A_{280}:A_{610}$ ratio or ferroxidase activity of ceruloplasmin (Huber and Frieden, 1970a). Except for the experiments in which excess Cu(II) was added or the ceruloplasmin was treated with Chelex, the copper content was consistently eight copper atoms per ceruloplasmin. The one copper ion removed by Chelex from typical preparations of the native protein has been labeled as the Chelexable site and represents the most tightly bound Cu(II) except for the 7 Cu ions which are an integral part of the native protein.

Equilibrium dialysis data suggest that ceruloplasmin has the potential to bind a total of seventeen copper atoms, including the seven usually isolated in the native enzyme (Kasper and Deutsch, 1963). Considering only those copper ions which can be removed by Chelex treatment, a Scatchard (1949) plot (Figure 1A) shows the number of Cu(II) binding sites to be 9.8 ± 0.3. The data indicated that these ions had an average binding constant of 2.0×10^5 M^{-1} using a least-squares plot.

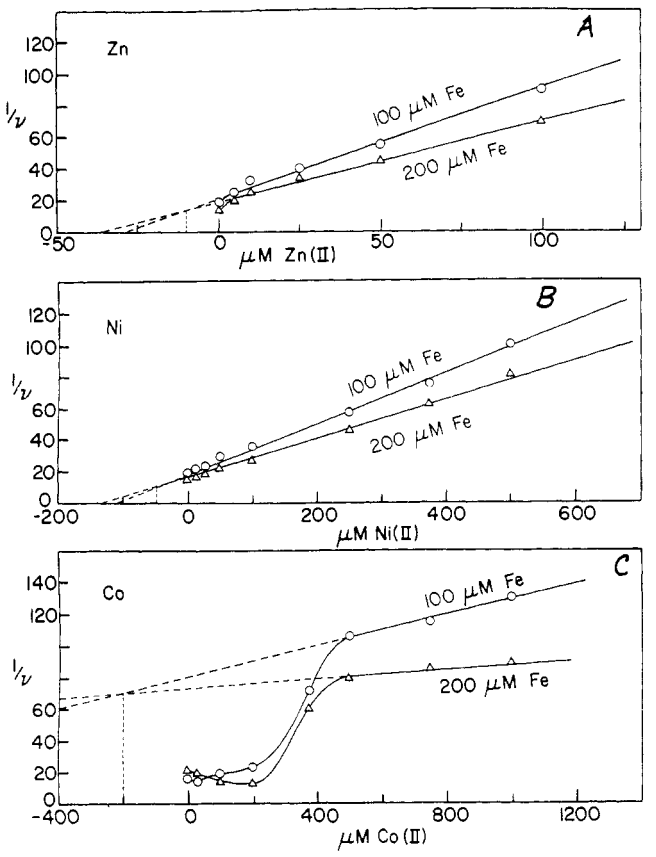

FIGURE 3: Dixon ($1/v$ *vs.* (Ir)) plots of kinetic data for ceruloplasmin using 100 μM Fe(II) (○) or 200 mM Fe(II) (△) as substrate with various concentrations of Zn(II) (A), Ni(II) (B), or Co(II) (C) as inhibitor. Final concentrations were 0.10 or 200 μM Fe(II) and inhibitor.

TABLE I: Values of n, K_a, and $1/K_i$ for Ceruloplasmin (Ferroxidase) and Transition Metal Ions.

Metal Ion	Ceruloplasmin Chelexed	n^a (Ceruloplasmin Mol Wt 1.60×10^5)	$K_a \times 10^4$ M	$(1/K_i) \times 10^4$ M
Copper(II)[b]	+, 0	9.8 ± 0.3	20 ± 3	
Iron(II)	+, 0	3.1 ± 0.3	11 ± 3	2.0, 170[c]
Zinc(II)	0	1.9 ± 0.1	29 ± 7	
Zinc(II)	+	3.0 ± 0.3	23 ± 5	10
Nickel(II)	0	6.0 ± 0.3	2.3 ± 0.3	
Nickel(II)	+	7.1 ± 0.3	3.0 ± 0.3	2.0
Cobalt(II)	+, 0	16.2 ± 0.5	2.0 ± 0.2	*d*

[a] n is the number of binding sites and K_a the protein–metal ion dissociation constant obtained from Scatchard plots in Figures 1 and 2. Values for n would become 82.5% of the values shown if based on a molecular weight for ferroxidase of 1.32×10^5 as reported by Magdoff-Fairchild *et al.* (1970). [b] The values for n and K_a do not include the copper ions which can be removed by Chelex treatment. [c] These values are the reciprocal of the K_m found for Fe(II) as substrate by Osaki (1966). [d] Cobalt(II) activates at low concentrations and inhibits at high concentrations.

Iron(II) Binding. Though there is invariably a measurable concentration of iron bound to ceruloplasmin prior to Chelex treatment, the amount is not consistent from one preparation to the next. Chelex treatment of the protein reduces the iron content below a level measurable on the atomic absorption spectrophotometer, but there remains a residual iron content which is involved in catalytic activity (McDermott *et al.*, 1968). Equilibrium dialysis of both chelexed and nonchelexed ceruloplasmin provided the data plotted in Figure 1B. Least-mean-squares treatment of the data showed the number of binding sites to be 3.1 ± 0.3 with an average binding constant of 1.1×10^5 M^{-1}. Data at low iron concentrations were scattered. However, these data suggest the possibility of a difference in the types of binding sites.

Zinc(II) Binding. Nonstoichiometric but measurable quantities of zinc were found in the various preparations of ceruloplasmin, although zinc plays no known role in the function of the protein. Data for ceruloplasmin which had not been chelexed prior to dialysis against zinc(II) indicated an average number of binding sites to be 1.9 ± 0.1 with a binding constant of 2.9×10^5 M^{-1}. After Chelex treatment with the number of binding sites increased to 3.0 ± 0.3 with an average binding constant of 2.3×10^5 M^{-1} (see Figure 2A).

Nickel(II) Binding. Nickel could not be detected in any ceruloplasmin preparation within the limits of the sensitivity of atomic absorption. Figure 2B shows data for nickel(II) binding before and after Chelex treatment of ceruloplasmin. Unchelexed ceruloplasmin contains 6.0 ± 0.3 binding sites for Ni(II) while the chelexed protein has a total of 7.1 ± 0.3. The former were calculated to have an average binding constant of 2.3×10^4 M^{-1} while the latter had a value of 3.0×10^4 M^{-1}.

Cobalt(II) Binding. No cobalt was detected in our native ceruloplasmin preparations. No significant difference could be found in data for chelexed or unchelexed ceruloplasmin; therefore, Figure 2C includes data for both. The number of binding sites found for Co(II) was calculated to be 16.2 ± 0.5 with an average binding constant of 2.0×10^4 M^{-1}.

Inhibition Studies. The inhibition of ferroxidase activity by Zn(II), Ni(II), and Co(II) was studied at two substrate concentrations, 100 and 200 μM Fe(II). This permitted analysis of the data by the method of Dixon (1964). By plotting $1/v$ *vs.* (I) (Figure 3A–C), extrapolation of the lines beyond zero inhibitor concentration yields intersection points above the (I) axis in each case. For Zn(II) and Ni(II), this indicated competitive inhibition. For Co(II) there is more uncertainty because only very high concentrations of the metal were considered in the extrapolation due to the activation at low concentrations. Inhibition constants, as taken from the value of (I) at the point of intersection, were $K_i = 10$ μM for Zn(II), $K_i = 50$ μM for Ni(II), and $K_i = 200$ μM for Co(II).

Discussion

The results of the binding studies indicate that various divalent transition metal ions can bind to ceruloplasmin. Values of n, K_a, and $1/K_i$ are summarized in Table I. From these values a pattern developed which suggested the possibility of as many as three distinct types of metal binding sites in the native protein. These exclude the two types of Cu(II) sites within the native protein (Malmstrom *et al.*, 1968). (It is possible, but not necessary that the Cu(I) sites might constitute an additional category of sites.) The first site might be labeled the "Chelexable site" to which one Cu(II) is nor-

mally bound in the isolation process but which loses this ion after Chelex treatment of the enzyme. The fact that this treatment increases the potential number of Zn(II) and Ni(II) ions bound by approximately one suggests that these ions also can bind to this site. It has been shown that, depending on conditions, there may be one accessible SH group in ceruloplasmin (Witwicki and Zakrzewski, 1969; Erickson *et al.*, 1970). Since there is one "Chelexable site" per molecule, this might reflect the reactivity of the metal ions with this SH site, an idea that might be confirmed by the preparation of specific SH derivatives. The number of Co(II) ions bound did not differ significantly with or without Chelex treatment, but this may have been due to the difficulty in distinguishing a change of one metal ion in the presence of a total of sixteen ions bound.

A second type might be referred to as the "substrate site(s)," those which bind Fe(II). It has been previously shown (Osaki, 1966) that ferroxidase has two K_m values for Fe(II) as substrate. Binding data at very low Fe(II) concentrations suggested the presence of one Fe(II) site with a much higher binding constant.

A final set of sites would be the nonspecific sites to which large numbers of divalent cobalt, copper, and nickel ions will bind. The binding constants follow the same general trend (Cu(II) > Ni(II) > Co(II)) as that predicted for complexes of the same ions with nitrogen and oxygen as donor atoms (Irving, 1959). Table II summarizes the types of metal binding sites observed for the native protein.

TABLE II: Number of Metal Ions Bound to the Various Binding Sites of Ceruloplasmin.

Metal Ion	Chelex Site	Substrate Site	Nonspecific Site	Ferroxidase Inhibition
Cu(II)	1		9	
Fe(II)	0	2 (1)[a]	1 (2)[a]	
Zn(II)	1	2	0	Complete
Ni(II)	1	2 (0)	4 (6)	Complete
Co(II)	0	2 (0)	14 (16)	

[a] The parentheses indicate a possible alternative number representing the distribution of binding sites.

The reciprocal of the inhibition constants of Zn(II), Ni(II), and Co(II) agree satisfactorily with their respective binding constants considering the differences in temperature at which these two sets of experiments were conducted and the much faster equilibration time in the kinetic experiments. Thus the kinetic data and the binding data provide independent support for the interaction of these metal ions and native ceruloplasmin. Preliminary studies of divalent transition metal ion binding by apoceruloplasmin indicate that the apoprotein does significantly bind these ions, but further work will be necessary to avoid denaturation of the unstable apoprotein.

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