Ferritin
BINDING OF BERYLLIUM AND OTHER DIVALENT METAL IONS*

Daniel J. Price and Jayant G. Joshi
From the Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37996-0840

Rat liver homogenates in 0.1 M Tris, pH 7.5, were heated to 80 °C, cooled immediately, and centrifuged at 24,000 × g, and ²⁷⁰B was added to the supernatant. Twenty-five per cent of the radioactivity was bound to a single protein. It was purified to homogeneity and identified to be ferritin as judged by different criteria. These were sucrose density gradient centrifugation, electrophoresis in polyacrylamide gel of the native or sodium dodecyl sulfate-treated protein, reactivity to antibodies, isoelectric focusing, and total amino acid composition. Comparative study of the ability of ferritin or apoferritin to bind Cd⁡⁺⁺, Zn⁡⁺⁺, Cu⁡⁺⁺, and Be⁡⁺⁺ was conducted by using a gel equilibrium technique, Centrifree micropartition technique, and microcentrifuge desalting technique. Ferritin could be saturated with Cd⁡⁺⁺ or Zn⁡⁺⁺ or Cu⁡⁺⁺ but not with Be⁡⁺⁺ even after 800 g atoms of Be⁡⁺⁺ were bound. None of the bound Be⁡⁺⁺ was dialyzable at 4 °C in 0.05 Tris acetate buffer, pH 6.5, but at pH 6.5 over 80% of the bound metal ion was dialyzed after 72 h. By contrast, apoferritin bound similar amounts of all four metal ions, some of which were dialyzable. By spectrophotometric titrations at pH 6.5 of Be⁡⁺⁺ with sulfosalicylic acid (SSA), Be⁺⁺SSA was calculated to be 5.0 × 10⁻⁶ M and by competition of sulfosalicylic acid and ferritin for Be⁡⁺⁺ the BeKderritinas calculated to be 6.8 × 10⁻⁶ M.

Excessive intake of any cation is toxic. The toxic level and the expression of toxicity vary with the cation. For example in experimental animals, exposure to Pb causes, among other ill effects, inhibition of certain specific enzymes involved in the synthesis of heme (1). In addition Pb causes replacement of Fe in heme by Zn thus generating a nonfunctional Zn-protoporphyrin (1). In some instances, living systems respond to the toxic metal ions such as Cd⁡⁺⁺, Cu⁡⁺⁺, or Zn⁡⁺⁺ by synthesizing metallothionein to sequester the detoxicant. The resulting protein binds a maximum of about 8 g atoms of the metal ions/mol (2).

One of the less commonly occurring metal ions in the environment is beryllium. This metal ion, atomic weight of 9.0122, is the lightest of the divalent metal ions. It is also one of the most toxic elements known. All forms of Be⁡⁺⁺, even at very low concentrations, adversely affect living systems (3).

The molecular basis for the toxicity of Be⁡⁺⁺ is as yet unknown. However, it is well established that some enzymes are inhibited by micromolar concentrations of Be⁡⁺⁺. Thus, out of great many enzymes tested, only three were inhibited at low concentrations of Be⁡⁺⁺ (4, 5). These were alkaline phosphatase (6, 7), phosphoglucomutase (6, 9), and (Na⁺K⁺)-ATPase (10).

Our interest in Be⁡⁺⁺ toxicity originated during our earlier investigations on the structure-function relationship of phosphoglucomutase from diverse origin (8). One of the parameters chosen for such a study was the effect of Be⁡⁺⁺ on the activity of pure phosphoglucomutase from different species. The results showed that the rabbit muscle phosphoglucomutase binds a maximum of 1 g atom of Be⁡⁺⁺ and such an enzyme metal complex is inactive. The binding of Be⁡⁺⁺ to the enzyme is facilitated by chelating agents such as EDTA or cysteine because they do not chelate with Be⁡⁺⁺ but remove other metal ions already bound to the enzymes (8). Subsequent studies showed that although partially purified phosphoglucomutase from rat liver was completely inactivated by micromolar concentrations of Be⁡⁺⁺, the enzyme activity in crude homogenates could be inhibited only partially (11). Indeed, at comparable concentrations of protein and Be⁡⁺⁺, the susceptibility to the inhibition of different preparations of liver phosphoglucomutase were: pure phosphoglucomutase > dialyzed extract > crude extract (11). This suggested that in a normal rat liver this enzyme is protected by at least two factors of different molecular weights; pure phosphoglucomutase contain neither, crude extracts contain both, and the dialyzed extracts contain only one, the large molecular weight protector(s).

Our search for the nondialyzable component led us to the isolation of a protein with a high molecular weight and capable of binding large quantities of Be⁡⁺⁺. In this paper it is identified as the iron storage protein, ferritin. Further, binding of Be⁡⁺⁺ to ferritin is compared to that of Zn⁡⁺⁺, Cd⁡⁺⁺, and Cu⁡⁺⁺. Binding of Be⁡⁺⁺ is in significantly greater amount than of other divalent metal ions. The majority of this Be⁡⁺⁺ appears to bind to the iron core of ferritin. However, binding of Be⁡⁺⁺ to the protein shell is also likely because apoferritin bound up to 160 g atoms of Be⁡⁺⁺. Speculation is made as to how the physical and chemical characteristics of beryllium would give rise to the observed binding.

EXPERIMENTAL PROCEDURES

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1 The "Experimental Procedures" are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-561, cite the authors, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Ferritin and Beryllium Binding

Materials
- Phenylmethylsulfonylfluoride. The homogenate was centrifuged at 70,000 x g at 4°C and the pellet was washed three times with phosphate-buffered saline. Horse spleen ferritin was purchased from Sigma and further purified as described below. Apoferritin was purchased from Boehringer Mannheim (Irvine). Sephadex 4B and 100 were purchased from Pharmacia (Uppsala, Sweden). Sephadex CL-65 was purchased from Pharmacia (UK). The molecular weight protein standards were purchased from Pharmacia.
- BeSO₄ was purchased from Aldrich.

Method
- Effluents from columns were monitored at 280 nm. Whenever necessary protein samples were concentrated by either the method of Lowry et al. (12) or Bradford (11) using bovine serum albumin as a standard and multiplied by appropriate correction factors. For the Lowry assay, protein values for ferritin and apoferritin were multiplied by 0.7 to correct for the assay.
- The procedure of Undeen and Garvey (24) was used to determine the concentration of ferritin and apoferritin in the horse spleen ferritin samples.

Iron in ferritin or apoferritin was determined by atomic absorption spectrophotometry on an L 157 Instrumentation Laboratories spectrophotometer. Radioactivity was measured with a Beckman Instruments liquid scintillation counter. Ferritin and apoferritin were measured by the ferritin assay, except that for the beryllium binding protein, the assay was carried out using the method of Pietruszka et al. (23). For each column run approximately 0.6 mg of protein was used and radioactivity was measured in 0.3 ml of a 10% trichloroacetic acid solution.

Results
- Ferritin from homogenates was purified by the procedure of Linder et al. (25) with slight modifications. Purification procedure was the same as that for the beryllium binding protein up to and including the steps leading to the heat stable supernatant. The heat stable supernatant, pipetted with 0.15 M acetic acid. After 1 hour at 50°C the centrifuged precipitate was washed three times with 0.15 M acetic acid. At 50°C the centrifuged precipitate was washed three times with 0.15 M acetic acid.
- The homogenate was centrifuged at 70,000 x g at 4°C and the pellet was discarded. The supernatant was heated to 80°C in a boiling water bath, cooled immediately and centrifuged at 27,000 g for 10 minutes. To the supernatant was added 0.5 M Tris-HCl (pH 7.4) containing 0.1 M sodium dodecyl sulfate (SDS) to a final concentration of 5%. After centrifugation at 27,000 g for 10 minutes more cold acetone was added to the supernatant to yield a final concentration of 50%.
- The solution was then centrifuged at 27,000 g for 20 minutes for 1 hour. The supernatant was removed by centrifugation at 70,000 x g for 9 hours in an SW50.1 rotor.
- Ferritin was quantitated by ultraviolet absorption spectroscopy, using bovine serum albumin as a standard and multiplied by 0.7 for ferritin and 0.82 for apoferritin to get the best correspondence of absorbance to protein concentration.

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Ferritin was purified by the procedure of Linder and Muñoz (12) with slight modifications. Purification procedure was the same as that for the beryllium binding protein up to and including the steps leading to the heat stable supernatant. The heat stable supernatant, pipetted with 0.15 M acetic acid. After 1 hour at 50°C the centrifuged precipitate was washed three times with 0.15 M acetic acid. At 50°C the centrifuged precipitate was washed three times with 0.15 M acetic acid.
Horse spleen ferritins containing various amounts of bound Be$^{2+}$ were prepared by using the microcentrifuge desalting technique of Hermonrth and Stokes (24). A small amount of glass wool was placed in a 10 ml plastic syringes body and then packed with G-75 sephadex (medium) pH 6.5 and 0.005% azide to produce a bed volume of 5 ml. Columns were pre-centrifuged for 3 minutes at 1960 rpm in a swinging bucket type clinical centrifuge. Samples containing horse spleen ferritin were incubated with 0.05 M Tris- HCl pH 6.5 and various amounts of BeSO$_4$ (as prepared above). Incubation mixtures were concentrated approximately 10 fold by ultrafiltration (PM 30 filter) and the concentrated protein was applied to the pre-centrifuged column. The column was then centrifuged as before, but for 3 minutes. The eluted Be-ferritin was then quantified for the amount of Be$^{2+}$ bound. Protein was measured by absorbance at 280nm, utilizing extinction coefficients of 16.90 A$^{280}$/mg ferritin and 1.23 A$^{280}$/mg apoferritin.

The affinity of Be$^{2+}$ for SSA was measured by spectrophotometric titrations. Varying amounts of BeSO$_4$ were added to SSA in 0.05 M Tris pH 6.5 buffer in a final volume of 3.0 ml and the increase in absorbance at 310 nm was measured with a Beckman DU-7 spectrophotometer.

The beryllium chelator, sulfosalicylic acid (SSA), was used to remove Be$^{2+}$ from Be-ferritins. Be-ferritin, prepared as described above, was incubated with varying amounts of SSA and then the Be-ferritin was separated from Be$^{2+}$ which remained bound to ferritin by centrifugation in Centrifree filters for 15 minutes at 3100 rpm. Measurements of the radioactivity in the filtrate quantified the amount of Be$^{2+}$ removed from ferritin by SSA.

RESULTS

Isolation of a High Molecular Weight "Be$^{2+}$-binding Protein"—As observed earlier (11), in crude liver extracts phosphoglucomutase is partially protected by dialyzable and nondialyzable factors. We first tested to see whether metallothionein could be one of the dialyzable factors, because it binds divalent metal ions such as Zn$^{2+}$, Cd$^{2+}$, and Cu$^{2+}$ and its synthesis is induced by these metal ions. Accordingly, groups of rats were injected with Cd$^{2+}$ or Be$^{2+}$ and the isolation of liver metallothionein was attempted. Although our procedure for the induction and purification of Cd-thionein yielded the expected protein, injection of beryllium failed to induce metallothionein synthesis. When Be$^{2+}$ was added to the heat-stable supernatant of the liver homogenates of rats, 25% of the soluble Be$^{2+}$ appeared in the 0.4 ammonium sulfate precipitable fraction, a fraction in which metallothionein does not precipitate (17). The remainder of the Be$^{2+}$ was either free or bound elsewhere. A procedure developed for the purification of this Be$^{2+}$-binding protein is described under "Methods." The purified beryllium red protein eluted in the void volume of a Sephacryl S200 column, suggesting a molecular weight of at least 150,000. In polyacrylamide gel electrophoresis the native protein migrated as a single band containing the bound beryllium (Fig. 1).

Identification of the Be$^{2+}$-binding Protein as Ferritin—In 20-50% sucrose gradients the Be$^{2+}$-binding protein sedimented very closely to the horse spleen ferritin which was used as a standard. To test the identity of ferritin with the Be$^{2+}$-binding protein, rat liver ferritin was purified by the established procedure (18). In sucrose density centrifugation, rat liver ferritin and the Be$^{2+}$-binding protein sedimented as single proteins at 64.8 and 61.4 S, respectively. Visible and UV absorption spectroscopy indicated that both proteins had a broad absorbance below 400 nm, although the rat liver ferritin had a greater absorbance per milligram of protein. However, these differences disappeared when the proteins were treated with thioglycolate and dialyzed to remove iron. Both proteins had a subunit molecular weight of about 20,000 (Fig. 2) which is expected for ferritin. The two proteins had similar amino acid composition as well as electrophoretic patterns on isoelectric focusing gels (data not shown).

The identification of Be$^{2+}$-binding protein as ferritin was further established by their identical antigenic properties. Fig. 3 shows that the rat liver ferritin and Be$^{2+}$-binding protein formed continuous precipitin lines and formed a single spur when adjacent to horse spleen ferritin.

Thus, the two proteins were indistinguishable as judged by sucrose density sedimentation, sodium dodecyl sulfate-poly-
acrylamide gel electrophoresis, total amino acid composition, isoelectric focusing, and reaction with antibodies.

Quantitation of the Binding of Zn²⁺, Cd²⁺, Cu²⁺, and Be²⁺ to ferritin and apoferritin—Once the Be²⁺-binding protein was identified as ferritin, horse spleen ferritin was used for further studies. First, the equilibrium gel filtration method of Pierson et al. (23) was employed to determine equilibrium binding of metals to ferritin or apoferritin at a 0.2 mM concentration of Zn²⁺, Cd²⁺, or Cu²⁺. Fig. 4 shows a typical elution profile for ferritin run on the Zn²⁺ equilibrated column. Similar elution profiles were obtained for Cd²⁺ and Cu²⁺. Fractions containing protein-bound metal ions were pooled and dialyzed against Tris-HCl buffer or Tris-HCl containing EDTA to distinguish the loosely and tightly bound metal ions (Table I). As seen, under equilibrium conditions ferritin bound 175 g atoms of Zn²⁺ and of Cd²⁺ but only 58 g atoms of Cu²⁺. After dialysis against Tris-HCl, only 64 g atoms of Zn²⁺, 83 g atoms of Cd²⁺, and 34 g atoms of Cu²⁺ remained bound to ferritin. Dialysis in the presence of EDTA removed all but a small percentage of the bound metal, and different ferritin metal complexes were not distinguished on this basis.

In contrast to ferritin, apoferritin bound much less of Zn²⁺ and Cd²⁺. The amount of Cu²⁺ bound to apoferritin (50 g atoms) was similar to that bound to ferritin (58 g atoms). Dialysis of metal-apoferritin complexes against Tris-HCl reduced all metals bound by about 50% and further dialysis against EDTA reduced Cd²⁺ and Cu²⁺ to 3 and 11 g atoms, respectively, similar to the levels remaining in ferritin. Zn²⁺, however, was not significantly removed by EDTA.

In another series of experiments the equilibrium binding of Cd²⁺, Cu²⁺, and Zn²⁺ to ferritin or apoferritin was studied by Centrifree separation. This method is less time-consuming and allowed the variation in the concentration of metal ion incubated with the protein. Fig. 5 shows the Scatchard plots (25) for Cd²⁺ and Zn²⁺. From these the dissociation constants, $K_D$ (−1/slope), and the binding capacities, $n$ ($x$-intercept/protein concentration) for Cd²⁺, Cu²⁺, and Zn²⁺ were calculated.

**Table I**

<table>
<thead>
<tr>
<th>Gram atoms of divalent metals bound to ferritin and apoferritin in equilibrium gel filtration</th>
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<tr>
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<tr>
<td></td>
</tr>
<tr>
<td>Zn</td>
</tr>
<tr>
<td>Cd</td>
</tr>
<tr>
<td>Cu</td>
</tr>
</tbody>
</table>

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Zn-equilibrium gel chromatography of horse spleen ferritin. The protein sample was dissolved in Zn-containing buffer, applied to a Sephadex G-75 (coarse) column, and eluted as described under “Experimental Procedures.” Protein was monitored by absorbance at 280 nm and Zn concentration was measured by a $^{60}$Zn tracer in a gamma counter.

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** Scatchard analysis of divalent metal binding to ferritin (○) or apoferritin (●). Metal ions tested for binding by the Centrifree method were Zn²⁺ (A) and Cd²⁺ (B). These plots were then analyzed for gram atoms of metal bound ($n$) and dissociation constant ($K_D$) as shown in Table I.
dissociation constant of Be$^{2+}$ for SSA at pH 6.5. Spectropho-
tometric titration of SSA with Be$^{2+}$ (Fig. 7) revealed that binding of Be$^{2+}$ decreases the absorbance at 300 nm and increases the absorbance at 310 nm.

The concentration of Be-SSA complex was determined by the equation:

$$[\text{Be-SSA}] = \frac{(\text{Abs}_{310} - \text{Abs}_{588})}{E_{588}}$$

(1)

where $E_{588}$ is the molar extinction coefficient for Be-SSA = 4.088 x 10$^4$. The term $\text{Abs}_{588}$ is equal to the absorbance of free SSA at 310 nm expressed as a fraction of a total of 0.05 mM SSA. This is calculated as follows:

$$\text{Abs}_{588} = \frac{5 \times 10^{-3} - [\text{Be-SSA}]}{5 \times 10^{-8}}$$

(2)

where $\text{Abs}_{588}^{310}$ is absorbance of 0.05 mM SSA alone at 310 nm = 0.842. Combining Equations 1 and 2 and solving for [Be-SSA],

$$[\text{Be-SSA}] = \frac{(\text{Abs}_{310}^{588} - \text{Abs}_{310}^{588})}{(E_{310}^{588} - \text{Abs}_{588}^{310})}$$

(3)

Using Equation 3 and the absorbance values at 310 nm, the amount of Be-SSA complex and amount of free SSA are calculated.

From the Scatchard plot of the data (Fig. 8) the SSA$\text{K}_{d}$ at pH 6.5 was calculated to be 5.04 x 10$^{-6}$ M.

Affinity of Be$^{2+}$ for Ferritin—The competitive removal of Be$^{2+}$ by SSA was determined by adding increasing amounts of SSA to Be$^{2+}$-ferritin followed by the separation of the Be$^{2+}$-SSA from ferritin by Centrifree method. Fig. 9 shows the increase in Be$^{2+}$ removal from ferritin at increasing concentrations of SSA. Three Be$^{2+}$-ferritins were utilized. They contained 83, 335, and 698 g atoms of Be$^{2+}$, respectively. Horizontal lines represent the maximum amount of Be$^{2+}$ that could be removed from each Be$^{2+}$-ferritin. As can be seen in the three curves, SSA removed all but 20–40 g atoms of Be$^{2+}$.

The affinity of Be$^{2+}$ for ferritin was calculated by using the

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**Table II**

Binding parameter of divalent metals for ferritin and apoferritin as determined by micropartition followed by Scatchard plot

<table>
<thead>
<tr>
<th>Metal</th>
<th>Ferritin</th>
<th>Apoferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>$K_D$ ($\times 10^{-4}$ M)</td>
</tr>
<tr>
<td>Zn</td>
<td>135.2</td>
<td>5.25</td>
</tr>
<tr>
<td>Cd</td>
<td>76.7</td>
<td>1.56</td>
</tr>
<tr>
<td>Cu</td>
<td>61.6</td>
<td>6.11</td>
</tr>
</tbody>
</table>

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The abbreviation used is: SSA, sulfosalicylic acid.
Following relationships:

\[
\text{Be-ferritin} \rightleftharpoons \text{Be} + \text{ferritin}, \quad \text{Be}_{\text{K}} = \frac{[\text{Be}][\text{ferritin}]}{[\text{Be-ferritin}]} \tag{5}
\]

\[
\text{SSA} + \text{Be} \rightleftharpoons \text{Be-SSA}, \quad \text{Be}_{\text{K}_{\text{SSA}}} = \frac{[\text{Be-SSA}]}{[\text{Be}][\text{SSA}]} \tag{6}
\]

SUM: Be-ferritin + SSA \rightleftharpoons ferritin + Be-SSA,

\[
K_{\text{eq}} = \frac{[\text{ferritin}][\text{Be-SSA}]}{[\text{SSA}][\text{Be-ferritin}]} \tag{7}
\]

Thus, the equilibrium expression (7) of the reaction carried out is the sum of two individual components (5 and 6). From these three reactions,

\[
\frac{[\text{ferritin}][\text{Be-SSA}]}{[\text{SSA}][\text{Be-ferritin}]} = \text{Be}_{\text{K}_{\text{ferritin}}} \cdot \text{Be}_{\text{K}_{\text{SSA}}} \tag{8}
\]

When half of the total Be\(^{2+}\) is removed from ferritin, the concentrations of ferritin and Be-ferritin are equal. Therefore, Equation 8 simplifies to:

\[
\frac{[\text{Be-SSA}]}{[\text{SSA}]} = \text{Be}_{\text{K}_{\text{ferritin}}} \cdot \text{Be}_{\text{K}_{\text{SSA}}} \tag{9}
\]

From the data for Be-ferritin containing 699 g atoms of Be\(^{2+}\) (Fig. 8) half-maximal removal of Be\(^{2+}\) was achieved at 3.7 \(\times\) 10\(^{-5}\) M SSA. We now simplify the left half of Equation 9 and substitute the known values:

\[
\frac{[\text{Be-SSA}]}{([\text{SSA}]_{\text{total}} - [\text{Be-SSA}])} = \frac{(1.55 \times 10^{-3} \text{ M})}{(2.7 \times 10^{-5} \text{ M} - 1.55 \times 10^{-3} \text{ M})} = 1.35
\]

The value of Be\(_{\text{K}_{\text{SSA}}} \) in Equation 9 is calculated as the inverse of the dissociation constant of Be for SSA as determined in the previous section:

\[
\text{Be}_{\text{K}_{\text{SSA}}} = \frac{1}{1.98 \times 10^{6} \text{ M}^{-1}}
\]

We then substitute the values of (Be-SSA/SSA) and Be\(_{\text{K}_{\text{SSA}}} \) into Equation 9 and solve for the dissociation constant of Be\(^{2+}\) for ferritin (Be\(_{\text{K}_{\text{ferritin}}} \)):

\[
\text{Be}_{\text{K}_{\text{ferritin}}} = \frac{1.35}{198 \times 10^{6}} = 6.80 \times 10^{-4} \text{ M}
\]

Thus, by an indirect method the affinity of Be\(^{2+}\) for ferritin is calculated.
The data presented here clearly establish ferritin as one of the major beryllium binding proteins in liver cytoplasm.

Consistent with the observations of Piotrowski and Szymanska (26), in our hands, injections of Be\(^{2+}\) did not induce the synthesis of metallothionein. In addition, apothionein did not bind Be\(^{2+}\) in vitro.\(^3\) This was expected because divalent metal ions form mercaptides with metallothionein (2) and Be\(^{2+}\) does not form such bonds (27). Be\(^{2+}\), however, does complex with phosphates and carboxylates (28, 29). Ferritin contains such groups. Indeed, carboxyl residues in ferritin bind Zn\(^{2+}\) and Tb\(^{3+}\) (30). Chasteen and Theil (31) have shown that vanadyl ions, VO\(^{2+}\), compete with Zn\(^{2+}\), Fe\(^{3+}\), Fe\(^{2+}\), and Tb\(^{3+}\) for the binding to apoferritin. Similar EPR signals of VO\(^{2+}\) complexed with malonate or apoferritin further underscore the importance of carboxylate residues for metal binding (31). Beryllium also complexes with various nuclear non-histone phosphoproteins (3) and other phosphate compounds (27). The well documented presence of phosphate in the iron core of ferritin (32) makes this protein well suited as a Be\(^{2+}\) chelator. We have observed in one of our preparations that horse spleen ferritin containing 1500 g atoms of iron had 322 g atoms of phosphate whereas its apoprotein containing 29 g atoms of iron had no detectable phosphate. Thus, most if not all of the phosphate in ferritin seem to reside in the iron core.

Beryllium salts in aqueous solution and at neutral pH form hydroxides. Unlike other group IIA elements, beryllium tends to form bonds of substantially covalent rather than ionic character. This has been attributed to the fact that beryllium has a relatively high nuclear charge coupled with a small atomic radius (33). Beryllium hydroxides are quite insoluble in water. Kosel and Neuman (27) have shown that a 50 mM aqueous solution of BeSO\(_4\) titrated with NaOH formed insoluble Be(OH)\(_2\) at pH 5.5 and above. This presents a problem when the binding of Be\(^{2+}\) to biological substrates is measured. Therefore, Parker and Stevens (34) used the SSA-Be\(^{2+}\) complex as a Be\(^{2+}\) donor to determine the affinity of Be\(^{2+}\) for nuclear acidic non-histone proteins. In calculating these affinities, however, the affinity of SSA for Be\(^{2+}\) appeared to have been ignored. It was therefore not surprising that SSA-Be and citrate-Be complexes gave very different results for the affinity of Be\(^{2+}\) to non-histone proteins. The data presented here show SSA\(_{Kd,Be}\) to be 5.04 \times 10^{-6} \text{ M}. In these calculations, a 1:1 binding of SSA to Be\(^{2+}\) is assumed. Das and Aditya (35) have shown 1:1 binding of SSA to Be\(^{2+}\) at pH 4.5 and 4.0. They have reported that at higher pH (between 9 and 11), SSA binds to Be\(^{2+}\) at a 1:1 ratio. Since our pH (6.5) was much closer to 4.5, we concluded that the binding of our experiments was primarily in a 1:1 ratio. The fact that our calculated SSA\(_{Kd,Be}\) (5.04 \times 10^{-6} \text{ M}) was much smaller than that determined by Das and Aditya (5.4 \times 10^{-5} \text{ M} at 0.05 ionic strength and 29.5 °C) may be due to increased ionization of SSA at the higher pH. Using our value for SSA\(_{Kd,Be}\), the affinity of Be\(^{2+}\) for ferritin was determined.

The large amount of Be\(^{2+}\) bound to ferritin is especially noteworthy. Ferritin bound greater than 800 g atoms of Be\(^{2+}\) under both equilibrium and dialyzed conditions. This large amount of tightly bound Be\(^{2+}\) bound is probably associated with phosphate (322 g atoms) which is present in the iron core. We were also able to get as high as 1200 g atoms of Be\(^{2+}\) bound to ferritin at a higher total Be\(^{2+}\) concentration. The high levels of Be\(^{2+}\) bound to ferritin under these conditions may be an artifact if a loss of subunits from the ferritin shell has occurred. If the calculation of molar quantity of ferritin molecules is erroneously low due to this damage, then it would appear that the gram atoms of Be\(^{2+}\) were greater than were actually bound to each ferritin molecule. Perhaps, Be\(^{2+}\) tightly associated with the core disrupts the iron oxohydroxide-phosphate lattice and results in the partial breakdown of the ferritin molecule.

Apoferritin binds substantially less Be\(^{2+}\) than did ferritin and of the 160 g atoms bound only 50–60 are tightly bound. Since the percentage of tight binding Be\(^{2+}\) is so much lower for apoferritin than for ferritin, one would suspect a different mode of binding. Thus rather than binding to the residual iron (29 g atoms) or phosphate (none detected) of apoferritin, we suspect that Be\(^{2+}\) is bound to the carboxyl residues of aspartic or glutamic acid or the hydroxyls of tyrosine on the protein shell.

Binding of Zn\(^{2+}\), Cd\(^{2+}\), and Cu\(^{2+}\) to ferritin or apoferritin is compared to that of Be\(^{2+}\). In some instances Scatchard plots are employed despite the limitations of this method (36) because it permitted better comparison of our data with those of Macara et al. (37) who studied the binding of various divalent metal ions such as Zn\(^{2+}\), Cd\(^{2+}\), Cu\(^{2+}\), Mn\(^{2+}\), and Tb\(^{3+}\) to apoferritin. It was found for all these metal ions except Mn\(^{2+}\), that 2–3 metal ions/subunit were tightly bound and 3–4 metal ions/subunit were loosely bound. For Mn\(^{2+}\) there is only 0.5 metal ion tightly bound and 2 metal ions loosely bound per subunit. Our results for the binding of Zn\(^{2+}\), Cd\(^{2+}\), and Cu\(^{2+}\) to ferritin were very similar to the results of Macara et al. (37). Although we obtained a hyperbolic Scatchard plot for these metals as did Macara et al., we have only determined values for what are the most tightly bound metals. Our results for the binding of Zn\(^{2+}\), Cd\(^{2+}\), and Cu\(^{2+}\) to ferritin show some different trends. Zn\(^{2+}\) and Cd\(^{2+}\) binding to ferritin showed an increased number of low affinity sites, but there was also about a 50% increase in the high affinity sites compared to apoferritin. Cu\(^{2+}\) binding is not increased for ferritin as was the case for the other metals. Harrison et al. (42) has reported that ferritin and apoferritin have the same

\(^2\) D. J. Price and J. G. Joshi, unpublished observations.
number of high affinity sites for Zn\(^{2+}\). Our results suggest that during the formation of ferritin from apoferritin some new high affinity Zn\(^{2+}\) sites are created.

The binding of Be\(^{2+}\) to apoferritin was of particular interest when compared to the binding of other metals. We show that apoferritin binds a total of 6.7 g atoms/subunit and of these only 2.1–2.5 are tightly bound. Thus, our findings of loosely and tightly bound Be\(^{2+}\) to apoferritin are in close agreement with those observed for other metals by Macara et al. (37). It is therefore likely that in the ferritin molecule the carbohydrate residues are required for Be\(^{2+}\) binding just as they have been shown for the chelation of Zn\(^{2+}\) (38).

The binding of Be\(^{2+}\) to ferritin appears to be different from that of other metals. Although all the metal ions except Cu\(^{2+}\) showed binding which could be attributed to the iron core, beryllium binding was by far the highest in amount and affinity. This binding could be to either phosphate or ferric hydroxide components of the core. However, since phosphate is known to bind Be\(^{2+}\), the binding of Be\(^{2+}\) to the phosphates of the core is the most likely possibility. Experiments are now in progress to test this possibility. To quantify the tightness of the binding of Be\(^{2+}\) to ferritin, we have measured the removal of the metal ion from ferritin by a known chelator, SSA. This technique is analogous to that of Vallee and Coombs (39) who estimated the binding of Zn\(^{2+}\) to alcohol dehydrogenase.

The sequestering of Be\(^{2+}\) by ferritin is significant not only because of the large amount bound and the tightness of binding, but also because the bound metal would have only limited accessibility to other proteins. The x-ray data of Banyard et al. (40) has shown that the only access to the ferritin core is through pores of 10 Å diameter of the 4-fold axis of the protein shell. Thus, formation of beryllium complexes in the iron core of ferritin would make Be nonaccessible to other proteins which might have a higher affinity for beryllium. In particular, enzymes which are susceptible to inhibition by beryllium could be protected by this sequestration.

The tissues shown to accumulate highest amounts of injected beryllium, liver and spleen (41), are also rich sources of ferritin (42). We have reported previously that Be\(^{2+}\) injected into rats is sequestered at least in part by ferritin (43) and that in vitro ferritin can reactivate alkaline phosphatase, phosphoglucomutase, and (Na\(^{+}\)) - ATPase which are inhibited by micromolar concentrations of Be\(^{2+}\) (44). This is so despite the fact that, as shown in this paper, Be\(^{2+}\) in ferritin is 6.8 \times 10^{-6} M. A partial explanation for this apparent discrepancy is that these studies were done at pH 7.4 and at alkaline pH the Be\(^{2+}\) bound to ferritin is nondialyzable. Presumably, like the ferric hydroxy phosphate sequestered by ferritin, Be\(^{2+}\) is also internalized although this remains to be established. In addition, under reactivating conditions used for enzyme studies the concentration of ferritin is greater than that of the enzymes, and compared to the enzymes ferritin binds far more Be\(^{2+}\). It is not claimed that ferritin is the only Be\(^{2+}\) binding protein capable of a protective effect. Nevertheless, based on the properties of beryllium binding shown here, ferritin may function as a natural detoxicant for beryllium.

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D J Price and J G Joshi


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