Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 3116–3119, May 1982
Biochemistry

Ferritin: A zinc detoxicant and a zinc ion donor

(cadmium/metallothionein)

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Communicated by Irwin Fridovich, February 8, 1982

ABSTRACT **Rats were injected with 1 mg of Zn^{2+} as zinc sulfate or 2 mg of Cd^{2+} as cadmium sulfate per kg of body weight on a daily basis. After seven injections, ferritin and metallothionein were isolated from the livers of the rats. Significant amounts of zinc were associated with ferritin. Incubation of such ferritin with apoenzymes of calf intestinal alkaline phosphatase, yeast phosphoglucomutase, and yeast aldolase restored their enzymic activity. The amount of zinc injected was insufficient to stimulate significant synthesis of metallothionein, but similar experiments with injection of cadmium did stimulate the synthesis of metallothionein. The amount of Zn^{2+} in ferritin of Cd-injected rats was greater than that in ferritin in Zn-injected rats, which was greater than that in ferritin of normal rats. Thus at comparable protein concentration ferritin from Cd-injected rats was a better Zn^{2+} donor than was ferritin from Zn-injected or normal animals. Ferritin is a normal constituent of several tissues, whereas metallothionein is synthesized under metabolic stress. Thus ferritin may function as a "metal storage and transferring agent" for iron and for zinc. It is suggested that ferritin probably serves as the initial chelator for Zn^{2+} and perhaps other metal ions as well and that under very high toxic levels of metal ions the synthesis of metallothionein is initiated as the second line of defense.**

Ferritin, molecular weight 480,000, is composed of 24 subunits. The molecular weight of subunits varies between 19,000 and 22,000. Ferritin from heart contains predominantly heavy subunits, whereas ferritin from liver contains predominantly light subunits. Other tissues contain ferritin composed of a mixture of these subunits. Fully saturated ferritin contains as much as 38% by weight of Fe(III), which can be converted to Fe(II) as needed. Injection of Fe(III) induces the synthesis of apoferritin, which sequesters the iron (1). The major physiological role of ferritin is considered to be that of storage, transport, and detoxification of iron. In addition to iron, at least *in vitro*, ferritin binds several other metal ions, albeit in smaller quantities. We have observed that it also binds beryllium (2, 3) the smallest and the most toxic of all divalent metal ions known. Indeed, holoferritin bound up to 50 mol of Be^{2+} per mol of protein. The bound Be^{2+} was nondialyzable. In contrast, apoferritin bound only 7–12 mol of Be^{2+}, all nondialyzable. The three enzymes known to be inhibited by micromolar Be^{2+} are Na^+,K^+-ATPase (4), phosphoglucomutase (5), and alkaline phosphatase (6). *In vitro*, holoferritin but not apoferritin protected all three against the inhibition by Be^{2+} and reactivated Be-inhibited alkaline phosphatase and Na^+,K^+-ATPase. We therefore suggested a possible expanded role for ferritin not only in iron storage and transport but also in control of toxicity of Be^{2+} and perhaps of other divalent metal ions (3).

In this report we show that (*i*) Zn^{2+} when injected to rats is sequestered by ferritin in the liver, (*ii*) the bound zinc is readily available to some zinc-requiring apoenzymes, and (*iii*) injection of Cd^{2+} into rats mobilized Zn^{2+} from other tissue and at least some of this zinc is deposited in liver ferritin.

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MATERIALS AND METHODS

Male Sprague–Dawley rats weighing between 250 and 275 g were injected subcutaneously with 1 mg of Zn^{2+} or 2 mg of Cd^{2+} (as $ZnSO_4$ or $CdSO_4$) per kg of body weight per day. Each group contained six rats. After seven injections and 7 days the animals were sacrificed, the livers were pooled and homogenized, and ferritin was isolated from liver as described by Linder and Munro (7). It was homogenous as judged by sucrose density gradient centrifugation, polyacrylamide gel electrophoresis, and reaction with antibodies. The latter were a generous gift of I. Listowsky of Albert Einstein School of Medicine, New York. Ferritins isolated from normal and Cd^{2+}- or Zn^{2+}-injected animals are abbreviated as ferritin-control, ferritin-Cd, and ferritin-Zn, respectively. Yeast phosphoglucomutase was isolated from Fleischmann's yeast as described (8) and aldolase was isolated as described by Rutter and Hunsley (9). Calf intestinal alkaline phosphatase was purchased from Sigma. Electrophoretically pure metallothionein I and II were isolated from Cd^{2+}-injected rats as described by VanderMallie and Garvey (10). Protein concentrations were determined by the method of Lowry *et al.* (11), using bovine serum albumin as the standard, with a 29% upward correction for ferritin as shown by Linder and Munro (7).

Apoenzymes were prepared and used on the same day. A Sephacryl S-200 column (1 × 18 cm) was washed with 50 ml of 0.1 M EDTA brought to pH 7.6 by addition of Tris base, and EDTA was removed by washing the column with 0.01 M Tris·HCl, pH 7.6, which was made metal-free by treatment with Chelex-100 (5 g of Chelex was suspended in 1,000 ml of buffer and stirred overnight at 4°C). Aliquots of alkaline phosphatase and aldolase were incubated for 24 hr and 4 hr, respectively, with 15 mM EDTA, pH 7.6, in a final volume of 0.3 ml, passed through the Sephacryl S-200 column, and eluted with metal-free Tris buffer as above. After such a treatment the enzymes had lost 98% of their activity.

Yeast phosphoglucomutase was incubated with 0.5 mM EDTA and 40 mM Tris·HCl, pH 7.6, for 30 min prior to the passage through the column. The eluted enzyme was less than 5% active without any added metal, 100% active with 5 mM Mg^{2+}, and 85% active with 0.4 mM Zn^{2+}. Activity of phosphoglucomutase was measured by coupled assay as described by Joshi *et al.* (12).

Yeast aldolase was assayed according to the method of Rutter and Hunsley (9). Alkaline phosphatase was assayed according to the method of Garen and Levinthal (13). Metal analysis of different samples were performed by Stewart Laboratories

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(Knoxville, TN). All reagents were of analytical grade and were purchased from Sigma or Baker. Chelex-100 was purchased from Bio-Rad. Only glass-distilled water passed through a deionizer was used.

RESULTS AND DISCUSSION

Ferritin is known to bind a variety of divalent metal ions (Cu^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}) *in vivo* and *in vitro* (14–16). Our earlier observations have shown that Be^{2+} also binds to ferritin, and that ferritin can prevent Be^{2+} inhibition of rabbit muscle phosphoglucomutase. Thus, an expanded role for ferritin as a metal detoxicant was suggested (3). An extension of this idea led to the investigations of a possible dual role for ferritin as a metal detoxicant as well as a metal donor for metal ions other than iron.

The three enzymes chosen for the study of zinc transfer from ferritin were yeast phosphoglucomutase, yeast aldolase, and calf intestine alkaline phosphatase. Of these alkaline phosphatase and aldolase contain tightly bound Zn^{2+} and the holoenzymes do not require added metal; however, their apoenzymes are inactive. In contrast, pure yeast phosphoglucomutase does not contain any bound metal ion but requires the presence of added divalent metal ion for activity. It is most active with 5.0 mM Mg^{2+}. The relative maximal activities with other metal ions were Zn^{2+} (at 0.5 mM) 84%, Co^{2+} (at 2.0 mM) 95%, and Mn^{2+} (at 0.5 mM) 62% (8).

Reactivation of Yeast Phosphoglucomutase. Results in Fig. 1 confirm and extend our earlier observations. As seen, the demetalloenzyme was less than 1% active without any added metal ion. Although 2 μM Cd^{2+} activated the metal-free enzyme, the activity elicited was only 10% of that obtained with 0.4 mM Zn^{2+} and 8% of that obtained with 5mM Mg^{2+}. Simultaneous presence of 0.4 mM Zn^{2+} and 2 μM Cd^{2+} was as effective as 0.4 mM Zn^{2+} alone. Furthermore, activity elicited by Mg^{2+} or Zn^{2+} was unaffected by 0.156 nM ferritin-control, ferritin-Zn, or ferritin-Cd. Thus under these conditions the metal ions were available to the enzyme. However, as seen in Fig. 1, the presence of ferritin from metal-injected rats during assay activated the apoenzyme in a concentration-dependent manner. The metal transfer was complete within 1 min because preincubation of metal-free yeast phosphoglucomutase with ferritin-Zn or ferritin-Cd for longer periods did not yield any additional phosphoglucomutase activity. As expected, ferritin-control was a poor activator and ferritin-Cd was a better activator than was ferritin-Zn.

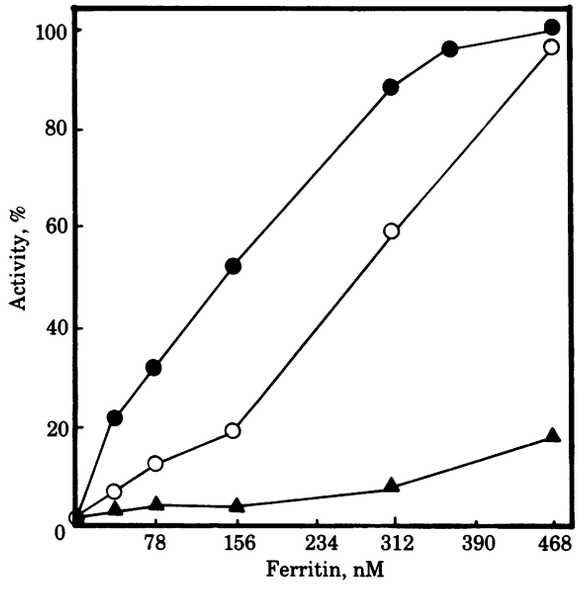

FIG. 1. Effect of various preparations of ferritin on phosphoglucomutase from yeast. The activity was measured by coupled assay (8) in the presence of various concentrations of ferritin-control (▲), ferritin-Zn (○), or ferritin-Cd (●). The concentration of enzyme was 18.7 nM. Activity observed in the presence of 0.5 mM Zn is equal to 100%.

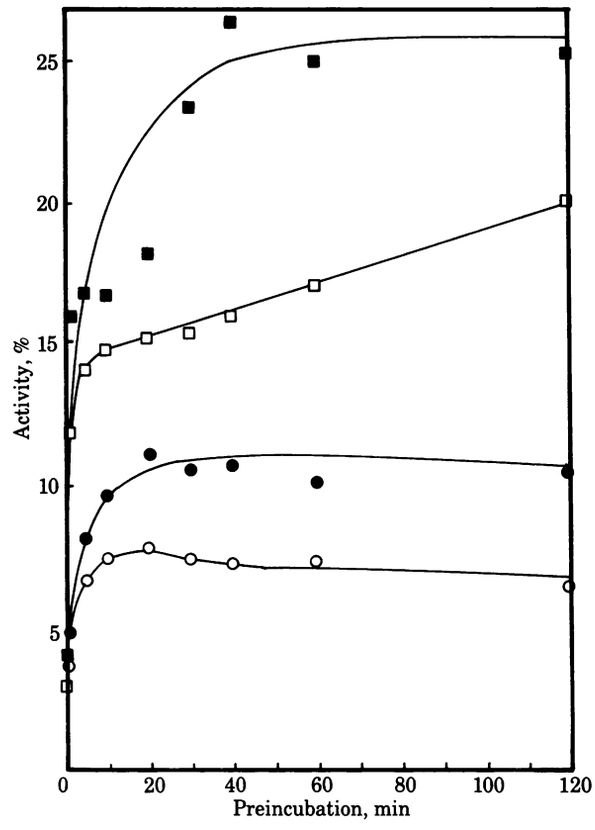

FIG. 2. Reactivation of alkaline phosphatase. Apo-alkaline phosphatase (calf intestine) was incubated at 0.97 μM in a final volume of 0.5 ml of 40 mM Tris·HCl, pH 7.4, with various activators. ○, Ferritin-control at final 0.78 μM protein, 1.05 μM bound zinc; ●, ferritin-Zn at final 0.78 μM protein, 2.44 μM bound zinc; □, ferritin-Cd at final 0.78 μM protein, 7.31 μM bound zinc; ■, $ZnSO_4$ at final concentration of 7.3 μM. Aliquots (5 μl) were assayed at various time intervals in 1 mM *p*-nitrophenylphosphate and 1.0 M Tris·HCl, pH 8.0. Under such conditions 100% alkaline phosphatase activity represented a ΔA_{414}/min of 1.34.

Activation of Apo-alkaline Phosphatase from Calf Intestine. Alkaline phosphatase was also activated by ferritin (Fig. 2). As with yeast phosphoglucomutase, ferritin-Cd and ferritin-Zn were better sources of Zn^{2+} than was ferritin-control. However, the rate of activation was slower than for yeast phosphoglucomutase, and a maximum of only 25% of the expected activity was recoverable. Measurement of the amount of metals bound to various ferritins (Table 1) showed that reactivation of alkaline phosphatase was proportional to the amount of zinc in ferritin. As can be seen, ferritin-control contained the least amount of

Table 1. Metal analysis of different preparations of ferritin

Ferritin source	Metal content, mol/mol of ferritin			Protein recovered, mg ferritin per g liver
	Zn	Cd	Fe	
Zn-injected rat	3.88	0	1,045.7	0.255
Cd-injected rat	9.32	0.68	851.7	0.203
Noninjected rat	1.33	0	840.9	0.258

zinc. Significantly, ferritin-Cd had 8 times more zinc and ferritin-Zn had 3 times more zinc than ferritin-control. This excess of bound zinc was thus available for enzyme reactivation. When $ZnSO_4$ was used as an activator at a metal concentration equivalent to that bound to ferritin-Cd, the course of reactivation was similar, although reactivation occurred at a faster rate and to a slightly higher final level. Similar results were obtained with $ZnSO_4$ levels equivalent to other ferritins.

Activation of Apoaldolase from Yeast. The results of these experiments are seen in Table 2. This enzyme, like yeast phosphoglucomutase, was completely reactivated by ferritin-Zn or ferritin-Cd. The activation was instantaneous and dependent upon the concentration of ferritin. In response to ferritin-control, 23% of aldolase activity returned, whereas only 14% of yeast phosphoglucomutase activity returned under similar conditions. Rate of reactivation by ferritin-Cd was only slightly greater than with ferritin-Zn.

Zinc, a biologically essential trace element, serves as a structural stabilizer for certain proteins such as bovine superoxide dismutase and as an essential cofactor for several enzymes (17). In some proteins such as alkaline phosphatase it serves a dual role of a cofactor and a stabilizer. Yet high doses of zinc are toxic. As a defense against these toxic levels, animals synthesize metallothionein, which sequesters Zn^{2+} (18). Indeed, the same protein is synthesized in response to other metal ions such as Cd^{2+} or Ag^{2+}; the amount of protein synthesized varies with the metal ion. Thus under comparable conditions Cd^{2+} is a far better inducer than is Ag^{2+} or Zn^{2+}. Until recently, the physiological role of metallothionein was considered to be only that of a metal detoxicant. However, Udom and Brady (19) have recently observed that metallothionein can serve as a zinc donor *in vitro* to several zinc-requiring apoenzymes. The amount of activity restored due to metal transfer varied with the apoenzyme. Thus yeast apoaldolase was fully and instantaneously reactivated, yeast apo-alcohol dehydrogenase was not activable at all, and apothermolysin and apo-alkaline phosphatase were partially reactivated to submaximal levels (19). Thus it appeared essential to examine whether yeast phosphoglucomutase is activated by metallothionein and whether metallothionein I and II were equally effective in this respect. Accordingly, metallothionein I and II were isolated from the Cd-injected rats and 15.6 nM yeast apophosphoglucomutase was assayed in the presence of a 4-fold molar excess of either of the metallothioneins. In each instance full and instantaneous reactivation was observed.

The data presented here show that, in addition to metallothionein, ferritin can function as a metal chelator and donor for other proteins. Although the synthesis of metallothionein is induced to various degrees by several metal ions, that of ferritin is known to be specifically induced by iron salt (20). The degree of induction is dependent upon the age of the animal, amount of injected metal ion, and duration of the dosage (21). The fact that ferritin-Cd was a better Zn donor than was ferritin-Zn led to measurement of zinc and cadmium in these preparations. As shown in Table 1, the amounts of ferritin isolated from normal or Cd- or Zn-injected rats were very similar, yet the ferritins varied in metal content. This suggests that the existing ferritin absorbed, at least in part, the injected Zn^{2+} and spared the animal the biologically expensive procedure of making metallothionein. As stated earlier, zinc, though toxic at high concentrations, is indispensable for several biological processes. In contrast, Cd^{2+} is nonessential and toxic. Upon its entry into the animal it induced the synthesis of metallothionein, as observed by Winge *et al.* (22), and it was also deposited into ferritin (Table 1). Of particular interest is the fact that cadmium injections mobilized zinc from other locations and caused its deposition into ferritin (Table 1). Although the source of this zinc remains to be determined, it is clear that once bound to ferritin it is available to the apoenzymes as evidenced by increased reactivation of apoenzymes by ferritin-Cd compared to ferritin-Zn. Ferritin-control, which contained the least amount of zinc, was a poor zinc donor.

Table 2. Effect of various preparations of ferritin on the activity of aldolase and apoaldolase from yeast

Conditions	Activity, %
Holoenzyme	100.0
Holoenzyme + ferritin-control	98.6
Holoenzyme + ferritin-Zn	100.0
Holoenzyme + ferritin-Cd	97.2
Apoenzyme	4.2
Apoenzyme + ferritin-control	22.9
Apoenzyme + ferritin-Zn	91.9
Apoenzyme + ferritin-Cd	97.2

The activity was measured by coupled assay (9). Concentration of ferritin was 0.147 μM. This represented final Zn^{2+} concentrations of 0.15 μM (ferritin-control), 0.35 μM (ferritin-Zn), and 1.06 μM (ferritin-Cd).

The interrelationships between different divalent metal ions and metallothionein are well recognized (23) and continue to be a subject of active interest. For example, Brown *et al.* (24) recently studied the metabolism of Zn^{2+}, Cu^{2+}, and Cd^{2+} in pretumorous livers from mice exposed to diethylnitrosamine. They observed that diethylnitrosamine-exposed mice had lower concentrations of Cu^{2+} and Zn^{2+} in cytosol of tumorous as well as pretumorous tissue and that this reduction was from a "heat stable, high molecular weight" protein pool. Exposure to Cd^{2+} alone resulted in its accumulation in metallothionein, whereas simultaneous exposure to Cd^{2+} and diethylnitrosamine resulted in the accumulation of the metal ion in a "heat stable, high molecular weight protein pool." We have found that high molecular weight protein pools of comparable heat stabilities contain predominantly ferritin (2, 3). Thus, carcinogens such as diethylnitrosamine seem to cause redistribution of metal ions amongst metallothionein and high molecular weight protein(s), such as ferritin. Both ferritin and metallothionein represent potential metal chelator–donors. Ferritin is known to be present at concentrations of at least 0.25 mg/g of liver (Table 1), and it can bind at least 50 mol of Zn^{2+} per mol (14). In contrast, metallothionein binds a maximum of only 8 mol of zinc per mol and is present only in nanogram quantities in either control or zinc-treated rats at the levels used here. Stimulation of the synthesis of Zn-thionein by zinc injections required at least 5 mg of zinc per kg of body weight, and, even at this level, the amount of metallothionein synthesis stimulated was only a fraction of that stimulated in response to cadmium (data not shown). Thus, ferritin, because it is present at high constitutive levels, is probably more suited to handle transient fluctuations in levels of divalent ions. In turn, binding of divalent ions to ferritin may also interfere with normal iron storage and mobilization. Indeed, deposition of iron into cellular ferritin was inhibited by Zn^{2+} in a dose-dependent manner (14), and Pb^{2+} bound to ferritin or ferritin-like substances in liver, spleen, and kidney of Pb-poisoned cattle suggesting an alteration in the storage of iron (15). In the case of such extreme toxicity, synthesis of metallothionein might serve to handle excess divalent metals and allow ferritin to continue to function as an iron-storage protein.

The *in vitro* data presented here suggest an analogous role for ferritin as a zinc donor *in vivo*. Because ferritin is known to bind Cu^{2+} (16), it is tempting to speculate that it may function

as a donor of Cu^{2+} to the corresponding apoenzymes. Plants contain phytoferritin, yeasts contain a ferritin-like protein (25). These are high molecular weight proteins. In addition to these, rat liver and plants are known to contain lower molecular weight proteins or even peptides capable of binding metal ions (26, 27). Perhaps such proteins may also function as divalent metal donors in a manner similar to that described for ferritin in this paper.

Technical assistance of Ms. Sara Porter is acknowledged. This work was supported in part by grants from the American Heart Association to J.G.J. and the National Institutes of Health to the University of Tennessee. This work is submitted as a partial fulfillment of the requirements for the Ph.D. degree of D.P.

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