Mechanism of Renal Lead-Binding Protein Reversal of δ-Aminolevulinic Acid Dehydratase Inhibition by Lead

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ABSTRACT

The bioavailability of lead in kidney is mediated in part by binding to endogenous high-affinity cytosolic lead-binding proteins (PbBP), which are not detectable in liver. Addition of semipurified 11,500 dalton PbBP to liver δ-aminolevulinic acid dehydratase (ALAD) reaction mixtures reverses inhibition of this enzyme by lead and thus provides an explanation for the relative insensitivity of renal ALAD to lead inhibition in vivo and in vitro. This effect results in part from a marked increase in binding of 209Pb to the PbBP relative to control liver cytosol (no PbBP) as demonstrated by Sephadex G-150 gel filtration chromatography. Zinc is known to activate ALAD and is an endogenous component of the PbBP fraction (6 μM in reaction mixtures). Zinc activated hepatic and renal ALAD over a range of 1.5 to 50 μM and also reversed the IC50 lead-inhibited activity. Studies of zinc release and/or displacement from PbBP under ALAD assay conditions (37°C, + glutathione, pH 6.8) were conducted utilizing Sephadex G-25 chromatography. Fifteen to twenty-five percent of the zinc in the PbBP fraction was released, and this value was not markedly influenced by addition of IC50 lead, temperature (4°C) or absence of glutathione; however, zinc release was primarily dependent upon the pH of the reaction mixture. These data indicate that the PbBP fraction attenuates lead inhibition of ALAD in vitro both by chelating lead and apparently serving as a zinc donor for this enzyme under optimal conditions of the ALAD assay.

The intracellular bioavailability/homeostasis of many essential metals is regulated by a number of metal-binding proteins. For example, the storage and transport of iron are facilitated by the proteins ferritin and transferrin, respectively (West et al., 1966). Copper in serum is primarily associated with ceruloplasmin (Holmberg and Laurell, 1948), a protein which has been proposed as the primary donor of copper to extrahepatic tissues (Hsieh and Frieden, 1975). Intestinal calcium absorption appears to be regulated by a vitamin D-dependent calcium-binding protein (Wasserman and Taylor, 1966). Calmodulin represents the major intracellular calcium receptor in all non-muscle and smooth muscle eukaryotic cells (Means and O'Malley, 1983). Metallothionein, a low molecular weight, cysteine-rich protein with a high affinity for copper and zinc, is postulated to function as a reservoir for the storage of these two metals and/or as a donor through which copper and zinc can be made readily available for synthesis of copper- and zinc-requiring apoenzymes (Kojima and Kagi, 1978).

The bioavailability/activity of toxic heavy metals such as cadmium and lead also appears to be regulated by binding to high-affinity proteins. Metallothionein, which has a high affinity for mercury and cadmium, appears to play an important role in regulating the acute intracellular toxicity of these metals (Foulkes, 1982). After induction of metallothionein synthesis by prior exposure to these cations, the protein acts as an integral part of a detoxification system by sequestering a high percentage of these toxic metals, making them less available to interact with sensitive organelles or enzyme systems (Probst et al., 1977; Roberts and Schnell, 1982; Squibb et al., 1984; Goering and Klaassen, 1984).

The inhibition of ALAD (EC 4.2.1.24), a cytosolic enzyme that catalyzes the second step in heme biosynthesis, is a sensitive and specific indicator of intracellular lead toxicity, including exposure to low levels (Granick et al., 1973; Finelli et al., 1975; Buchet et al., 1976). ALAD activity is inhibited markedly by lead in a number of tissues, including liver and blood (Gibson and Goldberg, 1970; Doyle and Schimke, 1969), but in kidney, inhibition of ALAD occurs only after high-level lead exposure (Hubernont et al., 1976; Roels et al., 1977; Fowler et al., 1980; Goering and Fowler, 1984). These data suggest that tissue-specific factors may be involved in regulating this effect because the enzyme itself is identical in all mammalian tissues examined (Coleman, 1966; Weissberg and Voytek, 1974; Anderson and Deenick, 1979; Chang et al., 1984).

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ABBREVIATIONS: ALAD, δ-aminolevulinic acid; PbBP, lead-binding protein(s); GSH, reduced glutathione; V/V0, retention coefficient; HMW, high molecular weight; VLMW, very low molecular weight; IC50, inhibitor concentration at which control enzyme activity is half-maximal.
Recently, an explanation for the relative insensitivity of renal ALAD to inhibition by lead was provided by Goering and Fowler (1984), who demonstrated that a low molecular weight, high-affinity renal PbBP component (Oskarsson et al., 1982), which appeared to possess properties distinct from metallothionein, reversed the in vitro inhibition of hepatic ALAD activity by lead. It was postulated that the PbBP was acting to chelate lead away from ALAD in a manner similar to the capacity of metallothionein to sequester cadmium and mercury away from sensitive organelles and metabolic processes (Roberts and Schnell, 1982; Squibb et al., 1984; Goering and Klaassen, 1984). The present study was undertaken to determine the mechanism of PbBP reversal of lead inhibition of hepatic ALAD activity in vitro.

Materials and Methods

Animals. Adult, male random-bred CD rats (Charles River/Kingston, Stone Ridge, NY), 300 to 350 g, were housed in plastic cages in groups of four and were exposed to a 12-h light/dark cycle in an environmentally controlled room at 22–25°C. Food (NIH 31 Rat Chow; National Institutes of Health, Bethesda, MD) and water were provided ad libitum.

Metals. Lead solutions were prepared by dissolving lead acetate (certified American Chemical Society grade; Fisher Chemical Co., Fairlawn, NJ) in deionized water with several drops of concentrated nitric acid. Zinc solutions were prepared by dissolving ZnCl2 (ultrapure; Morton Thiokol, Inc., Alfa Products, Danvers, MA) in deionized water. The homogenate and subsequent fractions were analyzed for Pb and Zn by atomic absorption spectrophotometry.

Isolation of PbBP. The 11,500 PbBP in kidney was isolated as described by Goering and Fowler (1984). Kidneys were removed from untreated rats after decapitation and subsequently homogenized on ice with a Teflon pestle and Potter-Elvehjem glass homogenizer after addition of 1 ml cold 10 mM Tris-acetate buffer (containing 10% glycerol, 0.5 mM monothioglycerol, and 0.1 mM phenylmethylsulfonyl fluoride), pH 7.4, per g wet weight tissue. The homogenate was centrifuged at 105,000 × g for 50 min at 4°C to obtain the cytosolic fraction. For gel filtration column calibration, kidney cytosol was incubated with 0.5 μCi (10-11 M Pb) 203Pb acetate per ml cytosol (10 mM/Ci/ml, 30 mM/Ci/mg; New England Nuclear, Boston, MA) for 4 h at 4°C. Cytosol was applied to a 60 × 2.2 cm Sephadex G-75 column previously equilibrated with buffer (containing no phenylmethylsulfonyl fluoride) to determine the retention coefficient (Ve/Vo) of the 11,500 PbBP. Elution was performed with the same buffer using a gravity flow system (25 ml/h) and 150 sixty-drop (3 ml) fractions were collected and analyzed for 203Pb by gamma spectrometry (Packard Auto-Gamma Collector 500). After column calibration, isolation of the 11,500 PbBP fraction was conducted in subsequent studies without 203Pb. Fractions containing PbBP were pooled and concentrated at 4°C using an Amicon cell containing a YM5 membrane (Amicon Corp., Danvers, MA). After two washes with 1 volume of 10 mM Tris-acetate buffer, pH 6.8, the fractions were standardized by concentrating the PbBP to 3 mg/ml of total protein, as determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. The specific high-affinity binding capacity was approximately 105 pmol of Pb per mg of protein as determined by Scatchard analysis (Mistry et al., 1988). The PbBP could be stored at −20°C for up to 2 months with no loss of activity with respect to reversal of lead inhibition of ALAD activity.

ALAD assay. The activity of ALAD was determined by measuring the rate of formation of product, porphobilinogen, in the 13,000 × g supernatant fractions of liver and kidney homogenates (1/9, w/v) using a modification of the methods of Gibson et al. (1955) and Baron and Tephly (1969). The reaction mixture contained 1 ml of 13,000 × g tissue supernatant (10–15 mg of protein per ml and 5–10 mg of protein per ml for liver and kidney, respectively) prepared in 0.25 M sucrose/10 mM Tris-acetate, pH 6.8, and 3.3 mM ALA plus 3.3 mM GSH (Sigma Chemical Company, St. Louis, MO) in 2 ml of 0.1 M sodium acetate, pH 6.8. The pH optimum for the assay is 6.8 (Coleman, 1966). Reaction mixtures were incubated at 37°C for 1 h. Formation of product was linear with respect to incubation time, substrate and protein. The apparent extinction coefficient used at 553 nm was 61 mM−1 cm−1.

Chromatography of ALAD reaction mixtures. To determine if the 11,500 PbBP acts to chelate lead during the ALAD reaction in vitro, gel filtration chromatography of hepatic ALAD reaction mixtures with and without exogenous PbBP was performed. A Sephadex G-150 column (37°C) was calibrated as described above by incubating kidney cytosol (100,000 × g supernatant) with 0.5 μCi/ml for 4 h at 4°C. Reaction mixtures were prepared as described above and contained 1 ml of 13,000 × g liver supernatant, 2 ml of substrate (+GSH), 0.8 ml PbBP or 10 mM Tris-acetate buffer (pH 6.8) and an aliquot (<10 μl) of 203Pb equivalent to 0.4 μM Pb in the reaction mixture. After a 1-h incubation at 37°C under N2 atmosphere, the reaction mixtures were placed on ice for 5 min and subsequently loaded on a Sephadex G-150 column (60 × 2.2 cm) equilibrated with 10 mM Tris-acetate, pH 6.8. Elution was performed with the same buffer using a gravity flow system (25 ml/h) and 150 sixty-drop (3 ml) fractions were collected and analyzed for 203Pb.

Zinc release/displacement experiments. Because zinc is an endogenous component of the 11,500 PbBP fraction and zinc is known to activate ALAD in vitro (Davis and Avram, 1978), the possible release or displacement of zinc from PbBP by lead under ALAD assay conditions was assessed by Sephadex G-25 chromatography. ALAD reaction mixtures without tissue supernatant were prepared as described above and contained 2 ml of ALA and GSH, 1 ml of buffer (0.25 M sucrose/10 mM Tris-acetate, pH 6.8) instead of tissue, 0.6 ml PbBP (189 high-affinity lead-binding equivalents) or 10 mM Tris-acetate buffer, pH 6.8. Lead (0.4 μM) was added to some reaction mixtures containing PbBP and assay conditions were varied (4°C or 37°C, +GSH) to study possible effects on zinc release/displacement. After a 1-h incubation, reaction mixtures were placed on ice for 5 min and subsequently loaded on a Sephadex G-25 column (fine) (25 × 1 cm) equilibrated with 10 mM Tris-acetate, pH 6.8 or 7.4. Elution was performed with the same buffer using a gravity flow system (40 ml/h) and 40 thirty-drop (1.5 ml) fractions were collected and analyzed for zinc by atomic absorption spectrophotometry (305B; Perkin-Elmer Corp., Norwalk, CT). All buffers were free of zinc contamination as assayed by atomic absorption spectrophotometry.

Statistical analysis. Data were compared by Student's t test or by an analysis of variance followed by Tukey's multiple comparison test (Steel and Torrie, 1960; Winer, 1971).

Results

To isolate the renal 11,500 PbBP for use in subsequent in vitro ALAD experiments described below, kidney cytosol (105,000 × g supernatant) was fractionated using gel filtration chromatography. The PbBP present in kidney eluted from the Sephadex G-75 column with a retention coefficient (Ve/Vo) of 2.0 to 2.5 (fig. 1A). This fraction was pooled and concentrated as described in the "Materials and Methods" section. By comparison, fractionation of liver cytosol confirmed that the PbBP is not detectable in this tissue.

Calibration of a Sephadex G-150 column for use in studies described below disclosed that the renal 11,500 PbBP eluted at V/Vo = 2.7 to 3.1 and that this protein component is not detectable in the liver (fig. 1B). In addition, gel filtration of kidney cytosol using Sephadex G-150 fractionated the 63,000 PbBP (Oskarsson et al., 1982; Mistry et al., 1985), which elutes at V/Vo = 1.5 to 1.7. The 63,000 PbBP does not reverse the inhibition of ALAD by lead as shown by Goering and Fowler (1984) and was not studied further in these experiments.
reaction different from each other, PbBP reveal that this protein contains a single class of binding equivalents (see table 1 for details) added was 105 pmol/mg of protein (Mistry et al., 1985).

The effect of renal 11,500 PbBP on lead-induced inhibition of hepatic ALAD activity is shown in table 1. In the presence of 0.4 μM Pb, ALAD activity was inhibited to 61% of control. Addition of PbBP (1.8 mg of protein; 189 pmol of binding equivalents) partially reversed this inhibition; activity was inhibited to only 84% of control. Addition of PbBP alone to control reaction mixtures resulted in a 19% elevation of ALAD activity. It should be noted that this same increase in activity was observed after addition of fresh PbBP to control reaction mixtures (P. L. Goering, unpublished data).

Results of studies concerning the order of PbBP and Pb addition to hepatic ALAD reaction mixtures with respect to Pb inhibition of enzyme activity are shown in table 2. The order of PbBP and Pb addition did not alter the protective effect of PbBP on ALAD activity at a given concentration of PbBP. A concentration-dependent effect of PbBP on reversing lead inhibition of ALAD activity was observed; higher concentrations of PbBP reversed the inhibition to a higher degree.

To determine if the mechanism of reversal of lead inhibition of ALAD was related to an altered distribution of lead within the reaction mixture resulting from PbBP chelation of lead into a metal-protein complex, ALAD reaction mixtures plus or minus PbBP were fractionated by gel filtration chromatography.

### TABLE 1

**Effect of 11,500 dalton renal PbBP on inhibition of hepatic ALAD activity**

Values are expressed as nanomoles of porphobilinogen formed per hour per milligram of protein and represent mean ± S.E.M. (n = 3). All values are significantly different from each other, P < .05. Saturation and Scatchard analysis of the 11,500 PbBP reveal that this protein contains a single class of high-affinity, low-capacity binding sites with an apparent K_d of 40 nM and capacity of approximately 105 pmol/mg of protein (Mistry et al., 1985). The PbBP was added to hepatic ALAD reaction mixtures in a 600-μl volume that contained 1.8 mg of protein. Thus, 189 pmol of 11,500 PbBP high-affinity binding equivalents were added.

<table>
<thead>
<tr>
<th></th>
<th>Hepatic ALAD Activity</th>
<th>Mean Percentage of Control</th>
</tr>
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<tbody>
<tr>
<td>Control + buffer</td>
<td>8.97 ± 0.15</td>
<td>100</td>
</tr>
<tr>
<td>Pb (0.4 μM) + buffer</td>
<td>5.46 ± 0.12</td>
<td>61</td>
</tr>
<tr>
<td>Pb (0.4 μM) + PbBP (189 pmol)</td>
<td>7.54 ± 0.20</td>
<td>84</td>
</tr>
<tr>
<td>Control + PbBP (189 pmol)</td>
<td>10.7 ± 0.53</td>
<td>119</td>
</tr>
</tbody>
</table>

### TABLE 2

**Effect of addition order of PbBP and Pb on hepatic ALAD activity**

Values expressed as nanomoles of porphobilinogen formed per hour per milligram of protein and represent mean ± S.E.M. Numbers in parentheses, n. No significant differences for a given amount of high-affinity binding equivalents added were evident as determined by Student’s t test, P < .05. Total specific 11,500 PbBP high-affinity binding equivalents (see table 1 for details) added was 105 pmol/mg of protein (Mistry et al., 1985).

<table>
<thead>
<tr>
<th></th>
<th>Hepatic ALAD Activity</th>
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<tbody>
<tr>
<td></td>
<td>Pb + PbBP</td>
</tr>
<tr>
<td>pmol</td>
<td></td>
</tr>
<tr>
<td>42.3</td>
<td>4.42 ± 0.43 (2)</td>
</tr>
<tr>
<td>94.5</td>
<td>7.49 ± 0.52 (2)</td>
</tr>
<tr>
<td>189</td>
<td>7.55 ± 0.45 (3)</td>
</tr>
</tbody>
</table>

![Fig. 1. Representative Sephadex G-75 (A) and Sephadex G-150 (B) gel filtration elution profiles of renal and hepatic cytosol incubated 4 h at 4°C with 200 Pb (0.5 μCi/ml). In A, the 11,500 PbBP in kidney (solid line) elutes at V/V_0 = 2.0 to 2.5 (arrow), whereas this protein is not detectable in liver (broken line). The fractions containing the 11,500 PbBP were pooled and concentrated as described in "Materials and Methods." The peaks beyond V/V_0 = 3 represent free lead or lead bound to VLMW peptides or amino acids. In B, calibration of the Sephadex G-150 column reveals that the 11,500 PbBP (solid line) elutes at V/V_0 = 2.7 to 3.1. As in A, the protein is not detectable in liver. The other PbBP (63,000) in kidney elutes at V/V_0 = 1.4 to 1.8, whereas this protein is not detectable in liver. The peak eluting at V/V_0 = 2.1 in the liver profile is primarily hemoglobin. Average recovery of cytosolic lead was 92%.](image)

![Fig. 2. Representative Sephadex G-150 elution profiles of lead in hepatic ALAD reaction mixtures containing renal 11,500 PbBP (solid line) or buffer (broken line). Total high-affinity lead-binding equivalents added in a 600-μl aliquot was 189 pmol based on Scatchard analysis (see table 1 for details) of the PbBP by Mistry et al. (1985). Lead eluting with retention coefficients (V/V_0) of 1.0 to 1.4, 2.7 to 3.1 and 3.3 to 3.7 was considered bound to HMW proteins, 11,500 PbBP and VLMW species, respectively.](image)
phy on a Sephadex G-150 column. Sephadex G-150 was utilized to determine if the PbBP aggregates into the 63,000 PbBP. Aggregation did not appear to occur as no increases in lead binding to the 63,000 PbBP peak were observed. The distribution of lead in control reaction mixtures and reaction mixtures to which renal PbBP had been added was different as indicated by representative elution profiles (fig. 2). In control reaction mixtures, the majority of lead was associated with HMW proteins (Ve/Vo = 1.0–1.3) and a fraction which eluted as free lead or lead bound to VLMW species (Ve/Vo = 3.3–3.7).

In contrast, in reaction mixtures to which PbBP had been added, a high percentage of lead eluted was bound to the PbBP (Ve/Vo = 2.7 to 3.1) while binding to HMW proteins and VLMW species was reduced compared to controls.

A compilation of gel filtration data is illustrated in figure 3. The concentration of lead associated with HMW proteins was less in PbBP-containing reaction mixtures compared to controls; however, the difference was not statistically significant. The most striking effect observed was the marked increase in binding of lead to PbBP when it was present. This increase was accompanied by a substantial reduction in lead binding to VLMW species. When expressed as a proportion of total lead within the reaction mixture, the same trends were observed. In controls, 44% of the lead was associated with HMW proteins and 54% with VLMW species. In contrast, in reaction mixtures containing PbBP, 39% of the lead was associated with HMW proteins and 36% with VLMW species; however, 25% of the lead was bound to the PbBP.

The effect of various concentrations of zinc on control and IIC lead-inhibited hepatic (A) and renal (B) ALAD activity in vitro. Values are expressed as mean ± S.E.M.; n = 3 to 11 for liver, except at the highest zinc concentrations where n = 1; n = 4 for kidney. †Significantly different (P < .05) from control (no zinc); ‡significantly different (P < .05) from IC20 lead (no zinc) group. Asterisks indicate a significant difference (P < .05) between values obtained in presence of zinc only and corresponding values in presence of zinc and IIC lead. PBG, porphobilinogen.

Fig. 3. Distribution of lead in hepatic ALAD reaction mixtures containing renal 11,500 PbBP (shaded bars) or buffer (open bars). Total high-affinity lead-binding equivalents added was 189 pmol based on Scatchard analysis (see table 1 for details) of the PbBP by Mistry et al. (1985). Results in the lower panel are expressed as percentage of total PbBP recovered in the three peaks. Average recovery of total PbBP was 95%. Values represent mean ± S.E.M. (n = 3). Values with no letters in common are significantly different (P < .05).

Fig. 4. Effect of various concentrations of zinc on control and IC20 lead-inhibited hepatic and renal ALAD activity in vitro. Values are expressed as mean ± S.E.M.; n = 3 to 11 for liver, except at the highest zinc concentrations where n = 1; n = 4 for kidney. †Significantly different (P < .05) from control (no zinc); ‡significantly different (P < .05) from IC20 lead (no zinc) group. Asterisks indicate a significant difference (P < .05) between values obtained in presence of zinc only and corresponding values in presence of zinc and IC20 lead. PBG, porphobilinogen.

The effect of various concentrations of zinc on control and lead-inhibited hepatic and renal ALAD activity in vitro is shown in figure 4. Addition of zinc alone to liver and kidney reaction mixtures resulted in a biphasic effect on ALAD activity with activation at low doses and inhibition at higher doses. In liver, zinc alone (solid circles, solid line) activated ALAD 20 to 40% over a range of 1.5 to 25 μM (fig. 4A). Significant inhibition of ALAD activity by zinc occurred at concentrations in excess of 100 μM. The presence of zinc also reversed the inhibition of ALAD by 0.4 μM Pb (approximate IC20) over a range of 1.5 to 50 μM (open circles, broken line), with the effect diminishing at zinc concentrations in excess of 100 μM. In kidney, zinc alone activated ALAD activity 40 to 60% above control values over a range of 3 to 50 μM with maximal activation occurring
at 12.5 μM (fig. 4B). As in liver, significant inhibition of ALAD activity by zinc occurred at concentrations higher than 100 μM. Zinc also reversed the inhibition of ALAD by 2.5 μM Pb (approximate IC50) over a range of 1.5 to 100 μM, with the effect no longer evident at 200 μM Zn.

Because zinc is an endogenous component of the 11,500 PbBP fraction (6 μM in reaction mixtures) and ALAD in liver is activated by zinc concentrations as low as 1.5 μM (fig. 4A), the effect of lead binding to PbBP with potential displacement of zinc from PbBP was assessed by Sephadex G-25 chromatography. Incubation of PbBP alone in ALAD reaction mixtures (no tissue) resulted in release of zinc from the PbBP fraction as illustrated by a representative elution profile (fig. 5). Addition of lead (0.4 μM) appears to have only minimally increased the amount of zinc released from the PbBP. It should be noted that zinc in the PbBP fraction before addition to ALAD reaction mixtures appears to be fully bound with negligible amounts of zinc existing as free ion. This observation is supported by our evidence, which revealed that the zinc concentration in the concentrated PbBP fraction did not change after washing (2×) with 1 volume of buffer and subsequent reconcentration (P. L. Goering, unpublished data). In addition, storage of the PbBP at −20°C in small aliquots and thawing for use did not alter zinc release (P. L. Goering, unpublished data).

Compilation of Sephadex G-25 elution data is shown in table 3. Incubation of PbBP alone under typical assay conditions (37°C, +GSH) resulted in release of approximately 15% of the zinc from the PbBP whereas 24% was released from the PbBP in the presence of lead; however, the contribution of lead to zinc release was not highly significant (P = .09). The presence of GSH did not markedly affect zinc release from PbBP in reaction mixtures containing lead (data not shown). In an effort to explain the release of zinc from the PbBP, this protein was incubated under a variety of conditions. The major factor influencing release of zinc from PbBP was the pH (P = .01) of the reaction mixture; zinc was readily released at pH 6.8 but not pH 7.4. GSH appeared to play a moderate role in the release of zinc (P = .03) whereas temperature had no effect. These results are most readily observed in the table column where the data are expressed as percentage of free zinc based on total amount of zinc eluting from the G-25 column (table 3).

**Discussion**

The present data provide a mechanism of action for the reversal of lead-induced inhibition of hepatic ALAD in vitro by the high-affinity 11,500 renal PbBP. This effect appears to be mediated by two properties of the PbBP: 1) its ability to chelate lead and sequester it in a relatively inert metal-protein complex, and 2) its ability to release zinc with potential donation to ALAD. The chelation hypothesis is an especially attractive one for the PbBP, which has an apparent dissociation constant (Kd) of 10−4 M for lead (Mistry et al., 1985). Metallothionein, which also has a high affinity for toxic metals, e.g., cadmium and mercury, serves as an integral part of a detoxication mechanism in acute exposure situations by sequestering these metals in an inert complex in the cytosol, making them less available to interact with critical organelles and cytosolic enzymes (Probst et al., 1977; Roberts and Schnell, 1982; Squibb et al., 1984; Goering and Klaassen, 1984). In the present study, addition of renal PbBP resulted in an altered distribution of lead within the hepatic postmitochondrial supernatant fraction (figs. 2 and 3). In the presence of renal PbBP, a higher percentage of lead binds to the PbBP compared to controls (25%, vs. 2%). This 23% increase in binding is due to decreases in binding to the other lead-binding ligands; a 5 and 18% decrease in binding to HMW proteins and VLMW species, respectively, accounts for the increase in binding to the PbBP. Because ALAD is included in the HMW peak, one would expect a much greater decrease in binding to HMW proteins if the mechanism of reversal of lead inhibition of ALAD was solely due to sequestration of lead by PbBP. However, it should be noted that not all HMW proteins may be equally affected by the chelation effect. Thus, some proteins such as ALAD may have greatly reduced lead binding whereas other proteins, which exist in much higher cellular concentrations and with more stable binding sites, would retain lead to a higher degree. Ferritin would

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**Fig. 5.** Representative Sephadex G-25 elution profiles of ALAD reaction mixtures (no tissue) illustrating the release of zinc from the 11,500 PbBP fraction determined by atomic absorption spectrophotometry. Zinc bound to the PbBP elutes in fractions 7 to 10 and free zinc in fractions 13 to 16.

**TABLE 3**

Release of zinc from 11,500 dalton PbBP under various ALAD assay conditions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Bound±</th>
<th>Free±</th>
<th>Total±</th>
<th>% Free±</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbBP (37°C, +GSH)</td>
<td>13.2 ± 1.0</td>
<td>2.3 ± 0.6</td>
<td>15.5 ± 1.3</td>
<td>14.8 ± 3.2</td>
</tr>
<tr>
<td>PbBP + Pb (37°C, +GSH)</td>
<td>13.3 ± 0.8</td>
<td>4.5 ± 1.4</td>
<td>17.8 ± 2.2</td>
<td>23.9 ± 4.8</td>
</tr>
<tr>
<td>PbBP (4°C, +GSH)</td>
<td>15.2 ± 1.3</td>
<td>3.5 ± 0.3</td>
<td>18.7 ± 1.6</td>
<td>18.8 ± 0.7</td>
</tr>
<tr>
<td>PbBP (4°C, -GSH)</td>
<td>13.7 ± 0.9</td>
<td>0.9 ± 0.2</td>
<td>14.6 ± 1.0</td>
<td>6.1 ± 1.1</td>
</tr>
<tr>
<td>PbBP (37°C, +GSH)</td>
<td>13.7 ± 0.4</td>
<td>1.5 ± 0.7</td>
<td>15.3 ± 0.5</td>
<td>9.0 ± 4.1</td>
</tr>
<tr>
<td>PbBP (37°C, -GSH)</td>
<td>13.4 ± 1.2</td>
<td>0.0 ± 0.0</td>
<td>13.4 ± 1.2</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

* Amount of zinc bound was not affected by pH, temperature, GSH or Pb.
* Factors influenced the amount of free zinc in the following order: pH (P = .01); GSH (P = .03); and Pb (P = .09).
* Total amount of zinc was not affected by any of the four factors.
* Factors influenced the percentage of free zinc in the following order: pH (P = .001); GSH (P = .01); and Pb (P = .09).
be such an example and has been shown to bind several divalent cations, including cadmium and beryllium (Price and Joshi, 1983).

An unexpected finding in this study was that the reversal of lead inhibition of ALAD by PbBP may be mediated, in part, by the donation of zinc to ALAD with subsequent activation of the enzyme. This possibility came to our attention when it was discovered that addition of PbBP alone increased hepatic ALAD activity by approximately 20% (table 1). The activation of ALAD by zinc ion (Komai and Neilands, 1968; Abdulla and Haeger-Aronson, 1971; Cheh and Neilands, 1973) and the reversal of zinc of lead-induced inhibition of ALAD activity in vitro (Finelli et al., 1975; Border et al., 1976; Cerlewski and Forbes, 1976; Haeger-Aronson et al., 1978; Davis and Avram, 1978, 1980) have been described. Our data provide evidence for potential zinc donation to ALAD in that the zinc concentrations of the PbBP is 30 μM; the zinc concentration was 6 μM upon addition of a 600-μl aliquot (189 pmol of lead-binding equivalents) to reaction mixtures. In addition, Sephadex G-25 chromatography disclosed that approximately 15% of the zinc bound to the PbBP is released during the ALAD reaction at pH 6.8 (fig. 5). The amount of zinc released is sufficient to replicate ALAD and to reverse inhibition of both hepatic and renal ALAD by approximate IC50 concentrations of lead (fig. 4, A and B). Despite a small increase in free zinc, the release of zinc from PbBP is not significantly affected by the presence of lead, i.e., lead does not appear to displace zinc markedly by competition for binding sites (table 3). Furthermore, it appears that zinc release from PbBP is primarily dependent on the pH of the ALAD reaction mixture. The fact that zinc is released from the PbBP fraction at pH 6.8 but not pH 7.4 supports the concept of "pH microdomains" within cells that facilitate transfer of essential metal ions between protein molecules (Fowler et al., 1984).

The release of zinc from the PbBP with possible donation to and subsequent activation of ALAD is plausible in light of recent evidence postulating a similar role for other metal-binding proteins. In addition to a possibly fortuitous role in metal detoxication, metallothionein is believed to be involved in the homeostasis of essential metals, such as zinc and copper. Because these metals must be supplied continually for proper development and maintenance of physiological processes, e.g., synthesis of metalloenzymes, but are toxic at the cell, organelle and molecular level, metallothionein could fulfill a sequestering and dispensing task to maintain metal homeostasis. Recently, it has been shown that zinc and copper in mammalian and fungal metallothioneins reactivates several enzymes in vitro, including mammalian apoperoxidase dismutase (Geller and Winge, 1982), mammalian apocarboxylic anhydrase (Li et al., 1980; Udom and Brady, 1980) and fungal tyrosinase (Lerch, 1980), which suggests that metallothioneins indeed function as physiological metal ion reservoirs and/or donors to metalloenzymes in vivo.

Because semipurified PbBP was utilized in this study and both renal PbBP and Zn,Cu-metallothionein elute with similar retention coefficients after gel filtration chromatography, the possibility exists that zinc, in the donation mechanism discussed above, may in fact be released from the constitutive renal Zn,Cu-metallothionein (Petering et al., 1984). Although zinc has a very high affinity for thionein from equine kidney and liver with an apparent dissociation constant (Kd) of approximately 10^-14 to 10^-12 M, metal ions are released from metallothionein most readily when subjected to acidic pH conditions or by other metals with higher stability constants (Kagi and Vallee, 1960; Vasak and Kagi, 1983) and many metals, including lead and cadmium, displace zinc from metallothionein in vitro at pH 7.0 (Waalkes et al., 1984). Thus, metallothionein could readily release zinc ions under the strong reducing (+GSH) and pH (6.8) conditions of the ALAD assay. These observations are in agreement with previous reports (Li et al., 1980; Udom and Brady, 1980; Geller and Winge, 1982) where it was demonstrated that zinc and copper from metallothionein are donated to zinc- or copper-dependent apoenzymes at relatively neutral pH.

Because the 11,500 PbBP and metallothionein possess similar molecular weights, i.e., similar retention coefficients, it could be argued that the PbBP is indeed metallothionein. Several lines of evidence now suggest that this is possibly the case. First, although PbBP does not fractionate into the two well-described isometallothioneins after anion-exchange chromatography (Oskarsson et al., 1982), it is possible that lead bound to metallothionein confers different charge properties to the protein or is "striped off" the protein during purification. Second, while the induction of renal metallothionein by cadmium treatment does not affect binding of lead to the PbBP (Oskarsson et al., 1982), induction of metallothionein in kidney by zinc increases the amount of lead that can bind this fraction and protects against lead inhibition of ALAD (P. L. Goering and B. A. Fowler, manuscript in preparation). This apparently stems from the fact that cadmium is more tightly bound to metallothionein than zinc (Kagi and Vallee, 1960). Third, although lead is a poor inducer of metallothionein in most tissues, including liver and kidney (Eaton et al., 1980; Waalkes and Klaassen, 1985), it may displace zinc in vitro from hepatic Zn-metallothionein, which had been induced and subsequently isolated from zinc-pretreated rats (Waalkes et al., 1984).

Data presented in this study and kinetic data from a previous study (Goering and Fowler, 1984) showing that addition of 11,500 renal PbBP to liver ALAD reaction mixtures reverses the inhibition of ALAD by lead, suggest that this PbBP regulates the in vivo intracellular bioavailability of lead to renal ALAD and accounts for the insensitivity of this enzyme to lead in the kidney (Fowler et al., 1980; Woods and Fowler, 1982). The present study demonstrates that the mechanism by which PbBP reverses lead-induced inhibition of ALAD in vitro results from a combination of two effects acting in concert. First, the PbBP sequesters lead in the cytosol with less potentially available to interact with ALAD. The capacity of the PbBP to bind approximately one fourth of the total cytosolic lead under the in vitro conditions used in this study implies that the protein is markedly reducing the pool of free lead ion, thus altering the steady-state interaction of lead with ALAD. Second, because PbBP readily releases zinc under optimal conditions of the ALAD assay, it can potentially serve as a zinc donor to ALAD, with subsequent activation of the enzyme. Therefore, the previously reported in vivo and in vitro differences in various tissues with respect to the sensitivity of ALAD to lead inhibition (Fowler et al., 1980; Silbergeld et al., 1982; Goering and Fowler, 1984) are most likely a function of the relative tissue concentrations of PbBP.

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