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PRELIMINARY PURIFICATION AND CHARACTERIZATION STUDIES OF A LOW MOLECULAR WEIGHT, HIGH AFFINITY CYTOSOLIC LEAD-BINDING PROTEIN IN RAT BRAIN

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Carrier-free ^{203}Pb has been used to label high affinity lead-binding proteins in rat brain cytosol to allow their initial characterization. The low molecular weight ^{203}Pb-protein complex collected from a Sephadex G-75 column eluate has been further purified by Sephadex DEAE chromatography and then partially characterized. The protein has a molecular weight of 23,000 daltons as determined by SDS polyacrylamide gel electrophoresis and significant levels of glutamic acid (9.3%), aspartic acid (10.8%) and cysteine (9.4%). Western blot studies conducted using the polyclonal antibody to the renal lead-binding proteins showed a lack of reactivity, indicating that the brain protein is immunologically distinct from that found in the kidney.

Previous studies from this laboratory (1-6) have demonstrated the presence of high affinity lead-binding proteins (PbBP) in kidney and brain cytosol. These molecules have been shown to markedly alter the sensitivity of the enzyme δ-aminolevulinic acid dehydratase (E.C. 4.2.1.24) to Pb inhibition in both kidney (2,3) and brain (4). In kidney, the renal PbBP has also been shown to facilitate the intranuclear movement and chromatin binding of Pb (5,6). Recent studies (7) have shown the renal PbBP is a cleaved form of α_2-microglobulin with the first 9 N-terminal amino acid residues removed.

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There is a growing concern with the low level effects of Pb on the central nervous system (8) and hence interest in intracellular binding components which mediate Pb bioavailability at these exposure levels in the central nervous system. Basic scientific comparisons of the renal and brain PbBP are also essential towards understanding tissue-specific differences in the bioavailability of this element in these two target organs following low level Pb exposure. This report presents the results of initial purification/characterization studies on the brain cytosolic PbBP and compares the physical properties, amino acid composition and immunological cross-reactivity of this molecule with those of the now characterized PbBP from kidney.

METHODS AND MATERIALS

Animals and Materials

Male Sprague-Dawley rats (CD strain, purchased from Charles River Laboratories, Boston MA) weighing 250-350 gms were used in the experiments. Enzyme grade TRIS (BRL, Gaithersburg, MD), ACS certified sodium acetate (Mallindkrodt), analytical reagent grade glacial acetic acid (Mallindkrodt) and monothioglycerol (Sigma) were used. Phenylisothiocyanate, sequanal grade triethylamine and amino acid standard H were purchased from Pierce Chemical Co. Bromphenol blue, acrylamide, bisacrylamide, coomassie blue, dithiothreitol, TEMED and ammonium persulfate were all electrophoresis purity grade reagents from Bio-Rad Laboratories. $^{203}PbCl_2$ (specific activities 10-500 mCi/μMole) was obtained from New England Nuclear.

Preparation of PbBP

The procedures of Goering and Fowler (2), and Mistry, Lucier and Fowler, (5) were followed for the preparation of rat brain cytosol by homogenization of rat brain in an equal volume of 10 mM Tris/Acetate, 0.5 mM monothioglycerol pH 8.6 buffer. Cytosol was labeled with ^{203}Pb by incubation on ice for one hr. The labeled cytosol was applied to a 2.5 x 55.0 cm Sephadex G-75 (Pharmacia) column equilibrated in 10 mM Tris/Acetate, 0.5 mM monothioglycerol pH 8.6 buffer and fractions collected and counted using a 200-400 keV window setting. The PbBP peak fractions were pooled, passed over a Sephadex G-25 column equilibrated in homogenization buffer to remove free lead that may have dissociated from the protein-lead (PbBP) complex PbBP complex and then applied to a 1.0 x 35.0 cm Sephadex A-25 DEAE column equilibrated in homogenization buffer. The PbBP complex was eluted from the column with a 10 to 700 mM Tris/Acetate gradient and the fractions counted using a 200-400 keV window setting.

SDS Polyacrylamide Gel Electrophoresis

The method of O'Farrell, 1975 (9) was used to prepare 14% acrylamide slab gels with 5% stacking gels. The electrophoresis was run at 40 mA per gel and the proteins stained with coomassie blue. Protein standards (Sigma Chemical Company) were run in lanes adjacent to the lead-binding protein.

Amino Acid Analysis

A modified procedure of Heinrickson and Meredith (10) was used for amino acid analysis. Samples previously desalted into distilled water were measured into 6 x 50 mm test tubes and dried under vacuum. The dry sample vials were placed in sealed vials with 200 µl of 6N HCl containing 1% (v/v) phenol, evacuated and then flushed with nitrogen six times before being left evacuated. The samples were hydrolyzed at 150°C for 1 hr and then dried under vacuum. PTC amino acid derivatives were prepared by adding 20 µl of a solution of 70% (v/v) absolute ethanol, 10% (v/v) distilled water, 10% (v/v) triethylamine and 10% (v/v) phenylisothiocyanate to each dried sample, the reaction allowed to proceed for 15 min at room temperature and then the sample dried under vacuum. Chromatography was performed on a Beckman Model 345 Ternary HPLC with a 4.6 x 150 mm Ultrasphere ODS column equilibrated in 0.15 M sodium acetate, .08% (v/v) triethylamine pH 6.5 buffer in 6% (v/v) acetonitrile. Samples were eluted with a 0→46% convex gradient of 60% (v/v) acetonitrile in HPLC grade water. Amino acid standards and metallothionein protein standards were run with each set of samples to allow evaluation of amino acid recoveries.

Tissue Western Blot Analyses

Western blot analyses using the polyclonal antibody to the purified renal Pb were conducted according to the method of Towbin (11).

RESULTS AND DISCUSSION

The Sephadex G-75 elution profiles of lead-labeled cytosol, shown in Figure 1, both have a void volume lead peak as well as a lead-protein complex with a molecular weight of approximately 23,700 daltons. When cytosol was labeled with high concentrations of lead (20.0 µMolar, Figure 1B) there was a third lower molecular weight lead peak not seen when the samples were labeled with low concentrations of lead (56.0 nMolar, Figure 1A). The low molecular weight bound ^{203}Pb peak (fractions 39-44 of Figure 1) was collected and further purified by ion-exchange chromatography.

After removal of free lead from the Sephadex G-75 purified low molecular weight PbBP (see Methods), this lead-protein complex could be resolved from a Sephadex DEAE anion exchange column (see Figure 2) as a single peak. Best results were obtained when the PbBP was applied to the DEAE immediately upon elution from the Sephadex G-75 column. After standing for a day or more, at room temperature or at 0-5°C a symetrical well resolved peak could not be obtained in the DEAE elution profiles.

The elution of the lead-protein complex from DEAE columns was also dependent upon pH of the elution buffer. In data not shown, lowering the pH

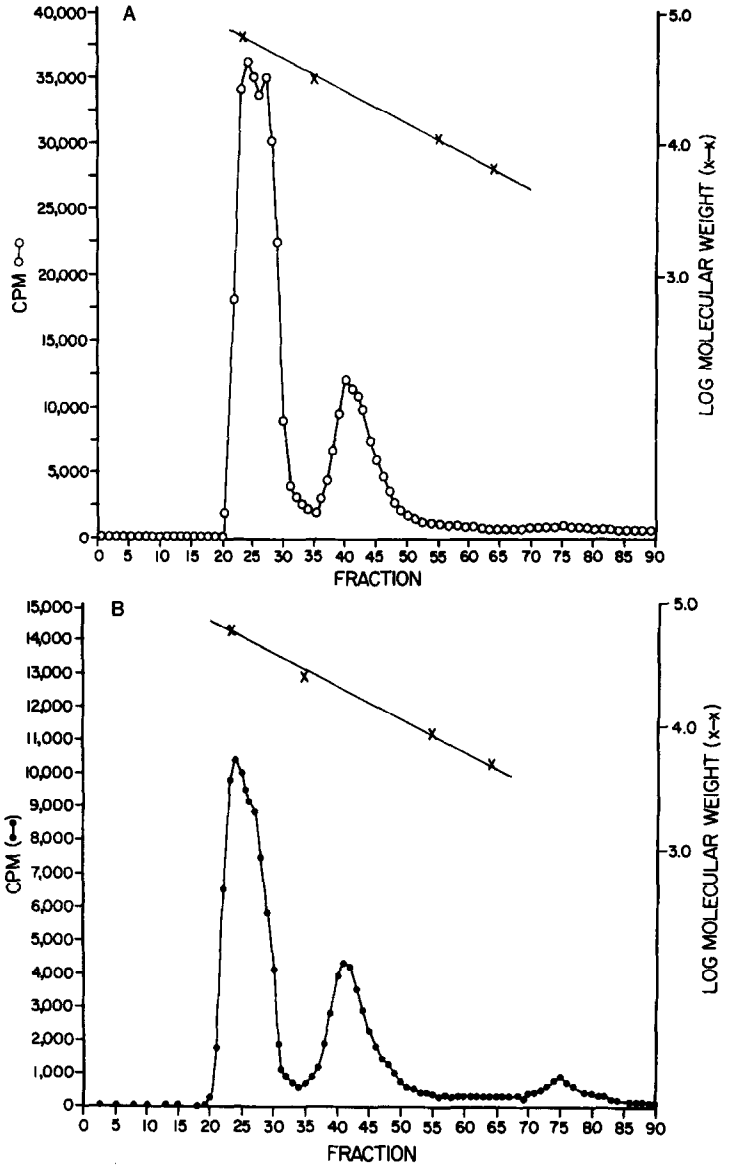

Figure 1. Sephadex G-75 chromatography of male rat brain cytosol. Cytosol was prepared, incubated with ^{203}Pb and fractioned over a Sephadex G-75 column as described in Methods. The final concentrations of lead with which the cytosol samples were incubated were 56.0 nMolar for Figure 1A (0-0, open circles) and 20.0 μMolar for Figure 1B (•-• closed circles). Bovine serum albumin, bovine erythrocyte carbonic anhydrase, horse heart cytochrome C and bovine lung aprotinin protein standards were used to calibrate the column, this calibration curve (X-X) is included on both Figure 1A and 1B. In both Figure 1A and 1B, the low molecular weight lead-binding protein eluted as a 23,700 dalton protein.

of the column buffer from 8.6 to 7.8 greatly decreased the affinity of the protein-metal complex for the resin. The elution of rabbit liver MTII standards was not affected as dramatically as the brain PbBP by alterations in

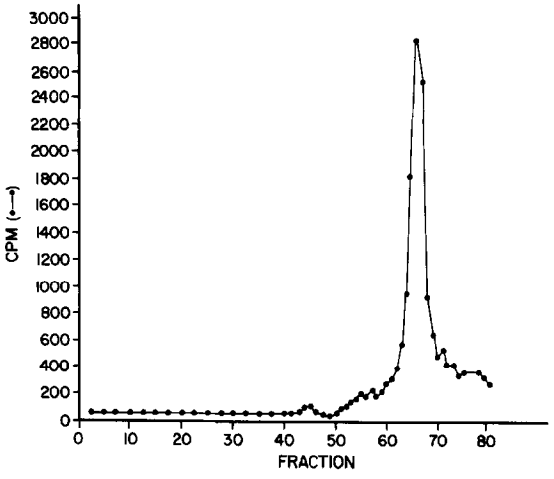

Figure 2. Sephadex DEAE ion-exchange chromatography of male rat brain lead-binding protein. Lead-binding protein prepared by Sephadex G-75 chromatography of ^{203}Pb-labeled male rat brain cytosol (Fractions 39-44 from Figure 1A) was applied to and eluted from a Sephadex DEAE column as described in Methods. Fractions 64-68 were collected and subjected to amino acid analysis and SDS-polyacrylamide gel electrophoresis.

column buffer pH. At pH 8.6, the two proteins could be readily resolved from one another with the MTII eluting first. Samples of the PbBP purified by DEAE chromatography at pH 8.6 were collected for further analysis.

SDS-polyacrylamide gel electrophoresis of the DEAE purified PbBP showed it to be a 23,000 dalton protein, as seen in Figure 3. These data agree well with the apparent molecular weight determined for the elution of the native protein from the G-75 column (Figure 1).

The amino acid composition of the DEAE purified PbBP is shown in Table 1. The preparation had high levels of aspartic acid (10.8%), glutamic acid (9.3%) and cysteine (9.4%) with significant levels of tyrosine (6.6%) and phenylalanine (4.4%). Determinations of tryptophane, glutamine and asparginine were not conducted. The high acidic amino acid contents and low basic amino acid contents (lysine 3.8% and arginine 4.9%) are consistent with the high affinity of the protein for the DEAE resin.

Western blot analyses conducted on tissue homogenates of brain and kidney (Figure 4) with polyclonal antibodies to the purified renal PbBP (α_2-microglobulin - 9 N-terminal residues) showed no reactivity of this antibody in the brain homogenate.

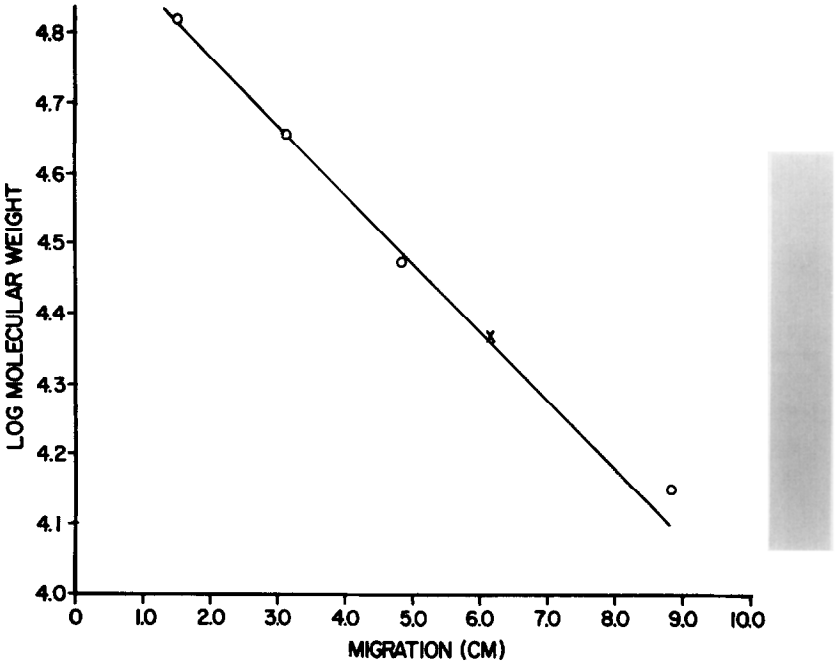

Figure 3. SDS-polyacrylamide gel electrophoresis of male rat brain lead-binding protein purified by ion-exchange chromatography. Lead-binding protein was collected from the Sephadex DEAE column eluate (fractions 64-68 of Figure 2), desalted by gel filtration into distilled water and then concentrated. The samples were run on SDS-PAGE as described in Methods. Bovine serum albumin, bovine erythrocyte carbonic anhydrase, horse heart cytochrome C and bovine lung aprotinin were run on the gel with the lead-binding protein preparation and used to draw the standard curve (O-O). The migration distance of the lead-binding protein (X) corresponds to a molecular weight of 23,000 daltons. The lead-binding protein as visualized on the SDS-PAGE by staining with coomassie blue, is shown to the right of the standard curve.

Table 1. Amino acid analysis of rat brain lead-binding protein isolated by Sephadex G-75 and DEAE ion-exchange chromatography

AMINO ACID[A]	MOLE %
CYST	9.41
ASP	10.80
GLU	9.26
SER	7.61
GLY	5.13
HIS	4.04
ARG	4.87
THR	5.15
ALA	5.88
PRO	6.24
TYR	6.56
VAL	5.92
ILE	4.93
LEU	6.18
PHE	4.39
LYS	3.75

[A]PTC amino acid derivatives were prepared and assayed as described in Methods. Separate analyses were not made for tryptophan, glutamine, and asparginine.

Figure 4. Western blot of kidney, (Lane 1), and brain (Lane 2), homogenates from male rats showing marked reactivity of cleaved renal form of α_2 microglobulin, the higher molecular weight native form which is present in both liver and kidney and the absence of reactivity in the brain homogenate.

The precise identity of the low molecular weight lead-binding protein is presently not known. The possibility that it is a low affinity lead-binding protein present in high concentrations in male rat brain is not likely as the amounts of protein recovered were consistent with a concentration of 1-5 μM in whole brain (data not shown). Several lines of evidence suggest that it is not the renal PbBP or a metallothionein-like protein. The absence of reactivity of brain homogenates with polyclonal antibody to the renal PbBP on Western blots (Figure 4) demonstrated that the brain PbBP is immunologically distinct from the renal protein. The pH-dependence of the elution of the 23,000 dalton lead-protein complex was clearly different from that of rabbit liver MTII standards with the lead-binding protein having a far greater pH-dependence in its affinity for Sephadex DEAE. The molecular weight of 23,000 daltons is also higher than that typically reported for metallo-

thioneins (12). In addition, the amino acid composition is markedly different from that of metallothioneins purified from rabbit kidney or liver (12), or the "metallothionein-like" protein purified from rat brain (13). The precise amino acid composition of the brain PbBP will be refined from further purified preparations of more rigorously demonstrated homogeneity.

It should be noted that a similar acidic lead-binding rat brain protein has also been found to accumulate in rat kidney after chronic exposure of animals to lead in drinking water (14-15). The molecular weight of this protein has been reported to be 32,000 daltons which would appear to make it different from the 23,000 dalton protein studied here after a one hour incubation of rat brain cytosol with ^{203}Pb. Further comparative studies are planned to determine if there is a relationship between these two molecules in rat brain.

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