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The Isolation of a High Affinity Zinc Binding Protein from *Limulus Polyphemus*

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Parvalbumins have been isolated from the skeletal muscles of numerous vertebrate species [1, 2]. These low molecular weight (mol. wt. ≅ 12,000), acidic (pI ≅ 4.0) proteins have the ability to bind two calcium ions with high affinity ($pK_d \cong 5$ to 7). The precise function of the muscular parvalbumins remains unknown; however, they appear to be modulated by calcium ion [2]. It is of particular interest that Potter *et al.* [3] has recently shown that parvalbumins isolated from carp can activate rat brain phosphodiesterase in a calcium-dependent manner. Thus far no muscle proteins possessing the most distinctive features of the vertebrate parvalbumins have been identified from an invertebrate source. Employing procedures very similar to those used for the isolation of vertebrate parvalbumins [4], we have identified four acidic, low molecular weight mol. wt. ≅ 13,000) proteins from an invertebrate source: *Limulus polyphemus* (the horseshoe crab). Two of the four proteins identified have been purified to homogeneity, by the criterion of single band migration on both anionic and sodium dodecyl sulfate–urea polyacrylamide disc gel electrophoresis [5]. The most acidic (pI ≅ 3.0) of the purified proteins was unexpectedly found to contain a tightly bound zinc ion following the isolation procedure. No zinc was found in any of the other proteins. Surprisingly, only trace amounts of calcium (*i.e.*, less than 0.1 mol of calcium per mol of protein by atomic absorption spectroscopy) were bound to each of the Limulus proteins following the isolation procedure. In this communication we report on the procedures for isolating the low molecular weight, acidic proteins from the horseshoe crab, the amino acid composition of the two components purified to homogeneity and the results of zinc(II) and cadmium(II) exchange experiments performed on a specially designed apparatus, employing the Limulus zinc binding protein.

Experimental

Live horseshoe crabs (*Limulus polyphemus*) were obtained from Northeast Marine Specimens Co. (NEMSCO), Woods Hole, Massachusetts (U.S.A.). Sephadex G-75-40 was obtained from Sigma Chemical Co., St. Louis, Missouri (U.S.A.), and DEAE-cellulose, Whatman DE-52, anion exchanger was purchased from Whatman Biochemicals Ltd., Kent, England. For the zinc exchange experiments, 99.9% carrier free ^{65}zinc(II) in HCl was supplied by New England Nuclear, Boston, Massachusetts (U.S.A.). All other chemicals were high grade commercial products. Effluent protein during gel fitration and anion exchange chromatography was monitored by ultraviolet absorbance at 280 nm with a Cary-14 recording spectrophotometer. Zinc(II) ion was determined on a Perkin Elmer Model/360 atomic absorption spectrometer. Amino acid analyses were performed using a Glenco AS-100 amino acid analyser, equipped with DC-1A resin (Durrum Chem. Co.).

Horseshoe crabs (500 to 1500 g in weight) were killed and the five pairs of legs were dissected and the muscle removed. The transverse hinge muscle between the prosoma and the ophisthosoma and the telson hinge muscle were also removed. The muscle was minced and then extracted in an equal volume of cold (4 °C), deionized water. The muscle extract was separated from the tissue by centrifugation at 12,000 g for 20 minutes and dialyzed against cold deionized water overnight. Solid ammonium sulfate (40 g/100 ml) was added to the supernatant to the 60% saturation level (or to the 70% level, see Figure 1). The salt-precipitated proteins were removed by centrifugation at 8,000 g for 15 minutes. The supernatant from the 60% salt saturation step was brought up to 100% ammonium sulfate saturation. The suspension was centrifuged at 15,000 g for 20 minutes, the pellet removed, dissolved in deionized water and dialyzed exhaustively against 0.1 *M* ammonium bicarbonate (pH = 7.8). The lyophilized proteins from the 100% saturated salt precipitation step were separated into high and low molecular weight fractions by gel filtration on Sephadex G-75. Columns (2.5 cm × 145 cm) were run at an elution rate of 20 ml/hr with 0.1 *M* ammonium bicarbonate (pH = 7.8) as eluant. Samples (5 ml) containing about 140 mg protein/ml were applied to the column. Fractions (about 5 ml/fraction) from the second peak, containing the low molecular weight protein fraction from the gel filtration step were next applied (0.73 g of protein, by weight, in 10 ml buffer) to a DEAE-cellulose anion exchange column (2.5 cm × 70 cm), equilibrated at 4 °C with 0.015 *M* HCl brought to pH = 5.7 with

piperazine. Proteins were eluted (17 ml/hr), in order of decreasing pI, by applying a linear chloride ion gradient.

^{65}Zinc(II) exchange experiments were performed in a two-compartment (7 ml/compartment volume) flow dialysis cell constructed in our laboratory. The flow cell was positioned 5 cm in front of a γ-ray scintillation spectrometer, in order to facilitate the continuous monitoring of ^{65}zinc(II) release from previously labeled protein. For all experiments a flow rate of approximately 250 ml per hour was maintained through the flow cell. The buffer employed for all exchange experiments was 10 m*M* PIPES (piperazine-N,N′-bis(2-ethane-sulfonic acid)), 20 m*M* KCl, pH = 7.0. Protein was labeled with ^{65}zinc(II) in the following manner: Zinc bound to the protein following the isolation procedure was removed by exhaustive dialysis against 10 m*M* dipicolinic acid (DPA). The apo-protein was next incubated with 99.9% carrier-free ^{65}zinc(II) chloride, and finally, exhaustively dialyzed against 10 m*M* PIPES buffer (pH = 7.0) to remove free ^{65}zinc(II). A typical experiment involves placing ^{65}zinc(II)-labeled protein, dissolved in the PIPES buffer (pH = 7.0), in the protein side of the flow dialysis cell. The protein solution is constantly agitated with a mechanical stirrer. Buffer in the buffer side of the flow cell, separated from the protein side by a semi-permeable cellulose membrane (pore size = 23 Å), is rapidly and constantly replaced (about 36 changes per hour) by fresh buffer *via* an inlet port at the bottom of the apparatus and an outlet port at the top. A constant flow rate is maintained with a peristaltic pump. Following equilibration of the system by passing buffer through the flow cell for many hours, 10 m*M* metal ion (*e.g.*, zinc(II) or cadmium(II)), dissolved in the PIPES (pH = 7.0) buffer, is passed through the flow cell. ^{65}Zinc(II) exchange was followed by monitoring the γ-ray decay of ^{65}Zn ($t_{1/2}$ = 244 days) remaining in the protein compartment as follows: the γ-ray detector employed was a 3″ × 3″ cylindrical Harshaw NaI (Tl) scintillation crystal, optically coupled to a photomultiplier tube. Bias voltage of 1.5 kV was applied to the photomultiplier using an Ortec #456 power supply. The output of the photomultiplier tube was amplified using an Ortec #113 preamplifier and then further amplified and shaped using an Elscint CAV-3 linear amplifier. In order to minimize the effect of background radiation and to effectively integrate the ^{65}Zinc(II) 1.115 MeV γ-ray peak, a single channel analyzer (SCA) was used to select pulses corresponding to 1.115 ± 0.050 MeV. The unit employed was an Ortec 420-A timing SCA. SCA logic pulses were shaped using an Ortec #416 gate and delay generator and fed to a Nuclear Data ND-2200 multichannel pulse hight analyzer (MCA). The MCA was used in the multiscale mode which allows the analyzer to sweep memory in time wile storing input pulses. One thus obtains a time spectrum of the source activity. In order to obtain a sufficiently long "dwell" time for each memory location an Ortec #719 timer was used as an external clock. "Dwell" was set at 400 seconds in all experiments. Data was read out using an IBM computer typewriter and also punched onto paper tape, to facilitate plotting using a Wang Laboratories 720-B computer. Half-lives were calculated from the data using CLSQ, the Brookhaven least squares decay curve analysis program [6] on a Xerox 530 computer.

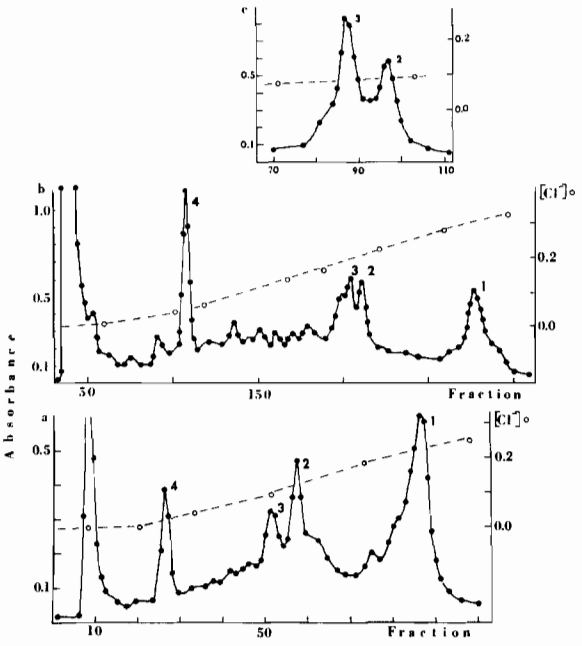

Figure 1. DEAE-cellulose anion exchange chromatography of low-molecular weight proteins from a) 70–100% $(NH_4)_2SO_4$ fractionated myogen, 107 mg were applied to the column (1 cm × 57 cm). Elution was at 18 ml/h, volume of fraction was 5.2 ml. b) 60–100% $(NH_4)_2SO_4$ fractionated myogen. 730 mg of protein were applied to the column (2.5 cm × 70 cm). Elution was at 17 ml/h, volume of fraction was 4 ml. Ultraviolet absorption was recorded at 280 nm (solid line). Cl^- concentrations were determined by conductivity measurements (broken line). c) Re-chromatography (column 1 cm × 56 cm) of LC-2 and LC-3 from the 60–100% $(NH_4)_2SO_4$ fraction. Elution was at 1.7 ml/h, volume of fraction was 4.2 ml.

Results and Discussion

The elution profile from the final DEAE-cellulose anion exchange step of the Limulus preparation is shown in Figure 1. In the order of increasing acidity, the proteins are identified as components 4, 3, 2 and 1. Limulus component 1 (LC-1) was found to contain about 0.75 mol of zinc per mol of protein following the isolation procedure. No zinc was found in any of

TABLE I. Amino Acid Compositions.

Residue	LC-1[a]	LC-4[a]	Carp[b] 4.25	Carp[b] 3.95	Hake[b] 4.36	Ray[b] 4.65	Rabbit[c] 4.9	Cod[b] 4.4	Frog[b] 4.5
Gly	4	8	8	9	12	7	9	9	9
Val	4 - 5	6	5	4	4	4	5	5	7
Ile	3	4	5	6	7	6	6	5	7
Leu	8	7 - 8	9	9	8	10	9	8	9
Ser	5 - 6	6	5	6	5	11	8	7	10
Thr	7	6	5	4	5	7	5	2	2
Cys	5 - 6	3	1	1	1	2	0	2	0
Asp/Asn	13	11	17	16	12	17	12	12	13
Glu/Gln	18	12	8	10	10	8	13	9	12
His	4 - 5	3	1	0	1	4	2	0	0
Lys	10	10	13	11	12	13	16	12	11
Arg	2	5	1	1	1	1	1	1	3
Thp	3	2	0	0	0	0	0	1	0
Tyr	6	5	0	1	0	1	0	1	1
Phe	4	5	10	9	10	8	9	10	9
Pro	2	9	0	1	0	1	1	0	0
Met	3	3 - 4	0	0	1	1	3	0	0
Ala	9 - 10	7 - 8	20	20	19	12	11	23	15

[a]Proteins samples were hydrolysed at 108 °C for 24 hours in 6 N HCl or 3 N *p*-toluenesulfonic acid containing 0.2% tryptamine (T. Y. Liu and Y. H. Chang, *J. Biol. Chem., 246,* 2842 (1971)). For the HCl hydrolysates standard single-column methodolgy was employed. Tryptophan present in the *p*-toluenesulfonic acid hydrolysates was determined using the short-column procedure of Spackman *et al.* (D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem., 30,* 1090 (1958)) on a column (0.9 × 10 cm) of Durrum DC-1A resin. [b]Data obtained from the tabulation presented in reference 1. [c]Data obtained from reference 7.

TABLE II. Half-lifes of Exchange Processes.

	HCAB Half-Life (hours)	FIT[a]	LZBP Half-Life (hours)	FIT[a]
Cadmium	368	1.152	26	1.066
Zinc	29	1.254	11	1.291
Dipicolinic Acid	11	2.473	16	1.081

[a]FIT as calculated in the analysis program is essentially $(\chi^2)^{1/2}$ for the number of degrees of freedom involved.

the other components. None of the Limulus proteins were found to contain more than about 0.1 mol of calcium per mol of protein following the isolation procedure. Preliminary equilibrium dialysis experiments, employing ^{45}calcium(II) indicate a $pK_d(Ca^{2+}) < 4.0$ for both LC-1 and LC-4. The amino acid compositions of the two components purified to homogeneity (*i.e.*, LC-1 and LC-4) are presented in Table I. Molecular weights for the two purified components, based on the amino acid composition data assuming a molecular weight in the range of 12,000 to 14,000 from the results of SDS-urea electrophoresis, are as follows: 13,073 ± 250 for LC-1 and 12,862 ± 160 for LC-4.

The amino acid composition of the two purified Limulus proteins are compared to the compositions of parvalbumins from seven different vertebrate sources (1, 7) in Table I. Inspection of the table reveals many similarities and a few interesting differences. In both LC-1 and LC-4, the levels of valine, isoleucine, leucine, serine and threonine are all very similar to the levels found in the seven vertebrate parvalbumins shown. The total number of acidic residues is somewhat higher in LC-1 (*i.e.*, 31 total acidic residues) than in the vertebrate parvalbumins shown (*i.e.*, 24 total acidic residues), consistent with the greater acidity of LC-1. LC-4 contains 23 total acidic residues, a value very close to the vertebrate parvalbumin level. Although the histidine level in LC-1 is high compared to the parvalbumin levels, the 16 to 17 total basic residues is comparable to the average of 15.6 for the parvalbumins shown. LC-4 contains 18 total basic residues. The principal differences in the amino acid composition between LC-1 and the vertebrate parvalbumin is the relatively low glycine and phenylalanine and the unusually high cysteine and tyrosine level. For LC-4 the principle differences relate to the relatively low alanine and phenylalanine content, the relatively high tyrosine content and the unusually high proline content.

Figure 2 presents data on the exchange of protein bound ^{65}Zn(II) for competing non-radioactive Zn(II) and Cd(II), obtained on the flow dialysis apparatus

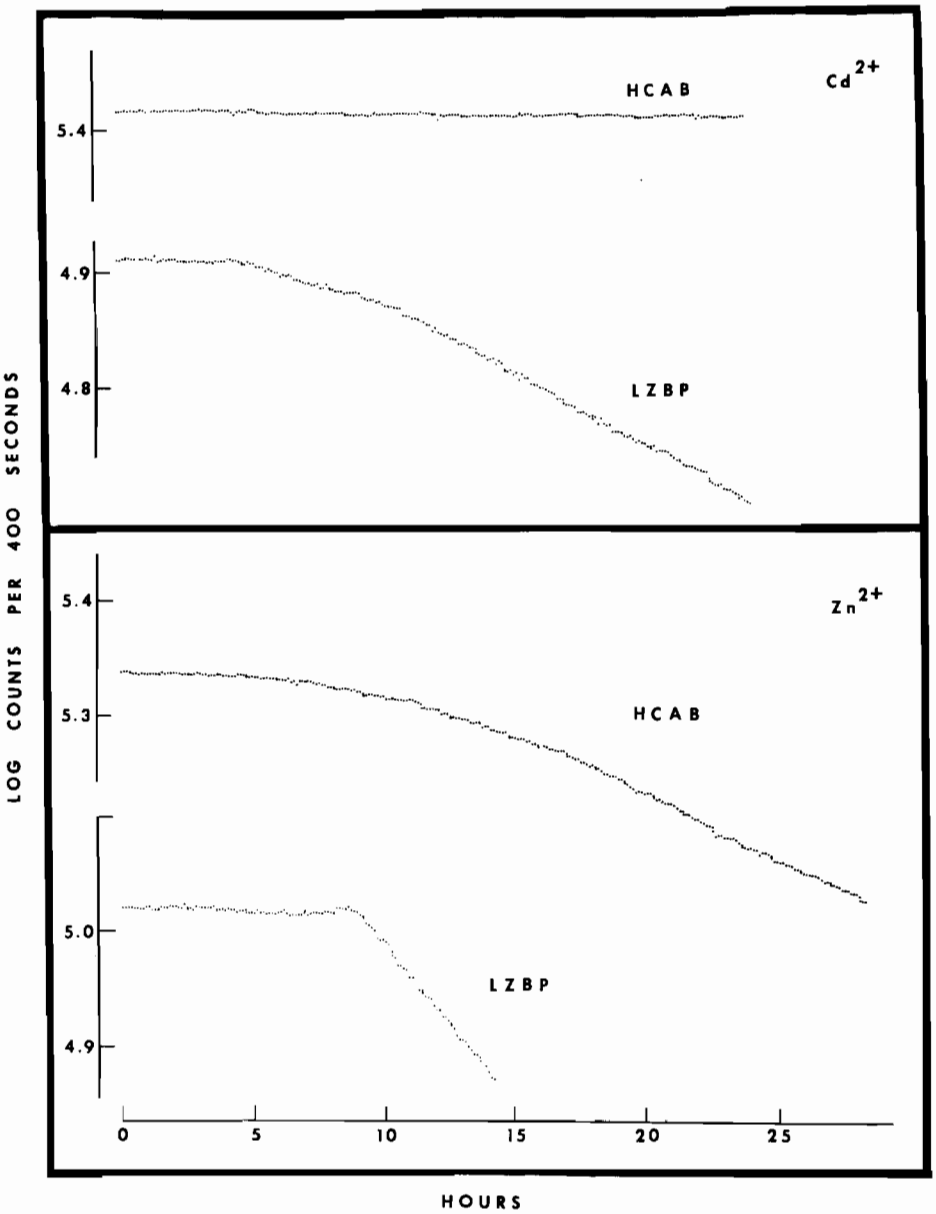

Figure 2. Time–activity spectra for the exchange of protein bound $^{65}Zn(II)$ with competing Zn(II) and Cd(II). Each point corresponds to 400 seconds of data acquisition. See text for further details.

described above. Data for both LC-1 and a well studied, zinc-binding reference protein, human erythrocyte carbonic anhydrase-B (HCAB), are illustrated (HCAB was prepared according to the procedure of Khalifah *et al.* [8]. Half-lifes for the exchange processes are given in Table II. It is important to note that in each of the experiments depicted in Figure 2, only a single exchange process is being followed, since the PIPES buffer causes essentially no $^{65}Zn(II)$ to be released from the proteins (*i.e.*, all curves are flat prior to the addition of competing metal ions to the wash buffer). Cd(II) does compete for bound zinc in HCAB; however, the exchange half-life is quite long, about 368 hours (Table II). Note that Cd(II) displaces bound zinc from CP-1 over an order of magnitude faster than from HCAB. Table II also reveals that Zn(II) exchange for bound $^{65}Zn(II)$ proceeds with a half-life about two and one-half times shorter for LC-1 than for HCAB. Finally, it is interesting to note that the exchange half-lifes, at least for HCAB, seem to be related to the binding affinity of the protein for Zn(II) and Cd(II), since the published stability constant for Cd(II) binding to human carbonic anhydrase is about one order of magnitude lower than that for Zn(II) [9].

Table II also presents the results of half-life measurements for exchange experiments employing the zinc chelating agent, dipicolinic acid (DPA). The half

life for $^{65}Zn(II)$ exchange out of protein is significantly *shorter* for HCAB than for LC-1 (11 hours *versus* 16 hours). These results are particularly interesting since Figure 2 indicates tha Zn(II) exchange for $^{65}Zn(II)$ is significantly *longer* for HCAB than for LC-1 (29 hours *versus* 11 hours). The results of Table II are consistent with a higher binding affinity for HCAB than for LC-1, but a more "solvent accessible" binding site in HCAB *versus* LC-1, which might be expected to facilitate the interaction between the chelating agent and the zinc ion bound to HCAB.

We have shown in this communication that the low molecular weight, acidic proteins isolated from the muscle of the ancient arthropod, *Limulus polyphemus,* exhibit significant deviations in the amino acid composition as well as the metal binding properties when compared with the vertebrate parvalbumins. It is important to recall that the isolation procedure is essentially identical to that employed for the isolation of the vertebrate parvalbumins. Are the Limulus proteins, in fact, homologues of the vertebrate parvalbumins, or are they totally unrelated proteins? If the Limulus proteins are homologues, can we associate their altered properties with the >350 million year evolutionary gap which separates this arthropod species from the vertebrate species with which they are compared. It is hoped that the results of current high resolution carbon-13 nuclear magnetic resonance and three dimensional X-ray difference Fourier experiments, designed to compare conformational aspects of the Limulus proteins with those of parvalbumins from the common mirror carp, will provide the solutions to these intriguing questions.

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