Identification of Metallothionein in *Pleurodeles waltl*

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The characterization of metallothionein in the Urodele amphibian species *Pleurodeles waltl* was achieved. A simple and rapid method for identification of metallothionein, based on its strong affinity for cadmium (^{109}Cd), was used. We were able to show that metallothionein is constitutively synthesized in liver, ovary and brain. The property of metallothionein to strongly bind essential (Zn, Cu) as well as toxic (Cd, Hg) metals is consistent with a dual role in cellular metabolism, *i.e.* homeostatis and detoxification of heavy metal ions.

Introduction

Metallothioneins are small cysteine-rich proteins able to bind heavy metal ions with high affinity (Hamer, 1986). Since their discovery (Margoshes and Vallee, 1957), metallothioneins have been extensively studied in many groups, and particularly in Mammals. The role of metallothioneins is not yet fully understood, but several data strongly suggest they play an important role in the control of zinc and copper homeostasis. They likely serve as a source of zinc for newly synthesized apoenzymes. Zinc is a cofactor for nearly 300 enzymes (Vallee, 1991) and is also bound to protein domains in many DNA-binding transcription factors (Schmiedeskamp and Klevit, 1994). Recent experiments have shown that thiolate ligands in metallothionein confer redox activity on zinc clusters. This strongly suggests that metallothioneins would control the cellular zinc distribution as a function of the cellular energy state (Maret and Vallee, 1998). Metallothioneins would also play an important role in detoxification processes implicating toxic heavy metals such as cadmium and mercury (Kägi and Nordberg, 1979; Foulkes, 1982; Suzuki *et al.*, 1993).

We are the only laboratory to date working on metallothionein from *Pleurodeles waltl*, an Urodele amphibian species. As a first approach, we purified *Pleurodeles* metallothionein from liver, ovary and brain. We combined the standard techniques of chromatography and polyacrylamide gel electrophoresis to show that *Pleurodeles* metallothionein can be easily identified using a radioactive tracer (^{109}cadmium). Our future investigations will focus on the regulation of metallothionein synthesis during *Pleurodeles* development.

Materials and Methods

Animals

The *Pleurodeles waltl* (amphibia, Urodela) is living in Morocco. Males and females were kept in the Maâmora (Rabat) and raised in our laboratory. They are maintained in large aquariums with twice weekly feeding of beef liver and occasional *Tubifex* (Gallien, 1952). A natural mating occur typically between the months of February and Mai. Embryos are collected and allowed to develop in the laboratory. Animals used in this study were from our breeding stocks.

Heat stable protein preparations

Livers, ovaries and brains isolated from *Pleurodeles* were homogenized in 10 volumes of 0.1 M ammonium formate buffer, pH 8.6, containing 0.1% mercaptoethanol. The homogenate was centrifuged at 130,000 × *g* for 4 h. The supernatant was heated to 65 °C for 10 min, then cooled to 0 °C and centrifuged at 10,000 × *g* for 30 min. The supernatant thus obtained was concentrated by

lyophilization and dissolved in ammonium formate buffer. Total protein concentration was determined by the method of Bradford (1976) using gamma globulin as the standard.

Gel chromatography

Heat stable proteins (10 mg) were applied to a Sephadex G-75 column (1.6 × 120 cm) equilibrated with 10 mM ammonium formate, pH 8.6, containing 0.1% mercaptoethanol. The column was eluted with the same buffer and 5 ml fractions collected. Yeast alcohol dehydrogenase (150 kD), bovine serum albumin (67 kD), ovalbumin (43.5 kD) and carbonic anhydrase (29 kD) were used for column calibration. Metallothionein was traced by its ability to bind ^{109}cadmium *in vitro*. This radioactive isotope was added to the samples (3.7 kBq $^{109}CdCl_2$, 3.22 GBq/μM, Amersham) prior to the chromatographic analysis. The radioactivity was monitored on 1 ml aliquots using a Beckman g-4000 gamma spectrometer.

Polyacrylamide gel electrophoresis

Proteins were electrophoresed on 20% polyacrylamide gels containing 0.1% SDS (Laemmli, 1970). Gels were run on a miniature vertical slab gel unit from Hoefer Scientific Instruments (San Francisco, USA). Electrophoreses were performed in duplicate, one gel being used for protein vizualisation (Coomassie Brilliant Blue) and the other for autoradiography. After electrophoresis, proteins were stained with Coomassie Brillant R-250. ^{109}Cd used to trace metallothionein was added (2.22 kBq) to the heat-stable protein samples prior to electrophoresis. Radiolabeled metallothionein was visualized by exposing overnight the dried gel to X-Ray film. Apparent molecular weights were determined by measurement of relative mobilities using the broad range SDS-PAGE molecular weight standards from Biorad (myosin 200 kD, β-galactosidase 116 kD, phosphorylase-b 97 kD, serum albumin 66 kD, ovalbumin 45 kD, carbonic anhydrase 31 kD, trypsin inhibitor 21.5 kD, lysozyme 14.4 kD, aprotinin 6.5 kD).

Protein electroelution

After electrophoresis, the acrylamide band containing metallothionein was excised, and metallothionein electroeluted (3 h, 200 V) in 0.1% SDS, 10 mM ammonium bicarbonate buffer, pH 8.6, using a Biotrap apparatus (Schleicher and Schüll), then dialysed overnight against 1 l of ammonium formate buffer.

Reduction and carboxymethylation assay

The procedure used was adapted from Crestfield *et al.* (1963). Briefly, purified metallothionein electroeluted from SDS-page gel was denatured and reduced in the presence of 5 mM dithiothreitol (DTT), 6 M urea, in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 8.6, then carboxymethylated for 1 h in the dark using a freshly prepared solution of 0.1 mM iodoacetic acid.

Results and Discussion

We have used chromatography and polyacrylamide gel electrophoresis to characterize metallothionein from *Pleurodeles waltl*, an amphibian Urodele species. Heat stable liver proteins from adult animals were fractionated by chromatography on Sephadex G-75 columns and metallothionein traced by its capacity to bind ^{109}Cd. Figure 1 shows a typical elution profile of the *P. waltl* liver proteins binding ^{109}Cd. They were recovered in a

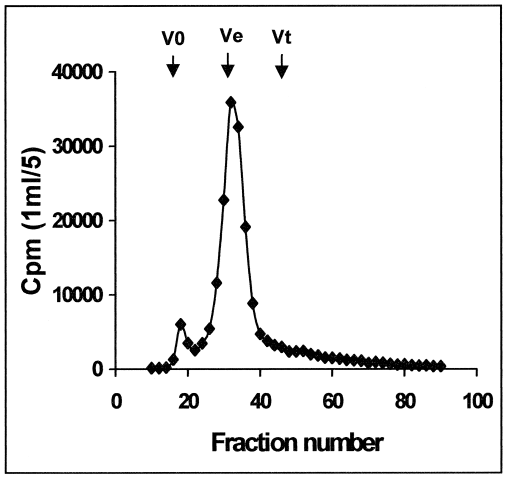

Fig. 1. Sephadex G-75 elution of ^{109}Cd labeled liver metallothionein. Elution was carried out as described in Materials and Methods. Five ml fractions were collected and 1 ml aliquots counted (see Methods). Vo is the void volume at which blue dextran elutes from the column and Ve the metallothionein exclusion volume.

single peak eluting at a Ve/Vo ratio of 1.9, suggesting the presence of a cadmium binding protein of approximately 10 kD. Similar results were obtained when heat stable proteins isolated from ovary and brain adult animals were similarly analyzed (data not shown). These elution properties are those expected for metallothionein (Debec *et al.*, 1985).

Further purification of metallothionein was achieved using gel electrophoresis. Radioactive Sephadex G-75 fractions (fractions 28 to 36, Fig. 1) were pooled, lyophilized and analyzed on SDS polyacrylamide gels. Figure 2 shows the electrophoretic protein patterns at each purification step. The location of metallothionein in the stained gel was determined by comparison with the radioactive profile of a duplicate gel run in parallel. Figure 3 illustrates the typical pattern we repeatedly obtained with ^{109}Cd labeled metallothionein. Although numerous bands were resolved on the stained gel, only a single band was observed in the autoradiogram. The corresponding protein displays a relative mobility of 0.45 and an apparent molecular weight of 28 kD. This result does not fit well with the expected 6 kD metallothionein molecular weight and also contrasts with the 10 kD estimation following Sephadex G-75 gel filtration (Fig. 1). These discrepancies can be explained taking into account the unusual properties of metallothionein. The multiple sulfhydryl groups of metallothionein in fact lead to the formation of multimeric complexes, and then to an overestimation of its molecular weight. This interpretation was tested by using dithiothreitol and iodoacetamide which abolish protein interaction by reduction and alkylation of sulfhydryl groups. Purified liver metallothionein was thus fully reduced, denatured and carboxymethylated, then reelectrophoresed under denaturing conditions on 20% acrylamide, 6 M urea, 0.1% SDS. It is to be noted that metallothionein, following this chemical modification that alter the cysteine residues, is no more able to bind ^{109}Cd. As shown in Fig. 4, carboxymethylation modified the migration properties of *P. waltl* metallothionein since the apparent molecular weight of the protein was shifted by this treatment to around 8 kD, *i.e.* in the range of the expected molecular weight for metallothionein. These data fit our previous results on *Xenopus* and

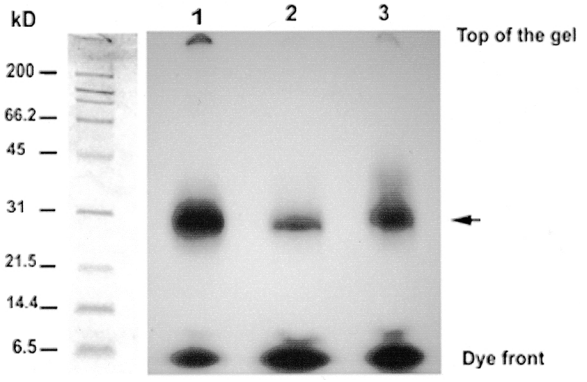

Fig. 3. ^{109}Cd metallothionein isolation by polyacrylamide gel electrophoresis. Thermo-stable proteins (10 μg) from liver (lane 1), ovary (lane 2) and brain (lane 3) were fractionated by SDS-polyacrylamide gel electrophoresis after *in vitro* addition of ^{109}Cd. The gel was dried and the position of metallothionein determined by autoradiography (arrow on the right). Molecular weight markers stained with Coomassie Brilliant Blue are indicated on the left side.

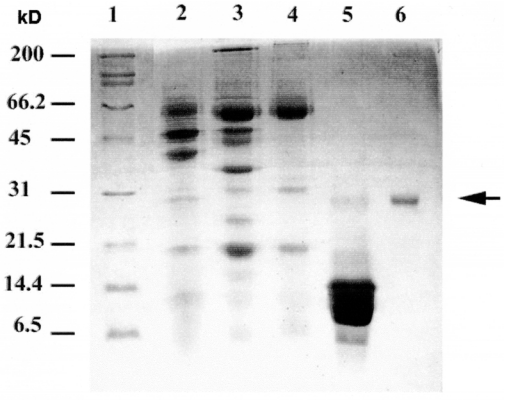

Fig. 2. Purification of metallothionein from *Pleurodeles walt*. Proteins corresponding to the different purification steps were fractionated by SDS-PAGE electrophoresis on a 20% polyacrylamide gel and stained with Coomassie Brilliant Blue. Thermo-stable proteins were loaded in lanes 2–4 [lane 2: liver (25 μg), lane 3: ovary (25 μg), lane 4: brain (15 μg). Lane 5 corresponds to the pooled fractions (40 μg) eluting at the metallothionein Ve (Fig. 1). Lane 6 shows the electrophoretically pure metallothionein obtained after electroelution from a polyacrylamide gel. The molecular weight markers were loaded on lane 1 [myosin (200 kD), β-galactosidase (116 kD), phosphorylase-b (97 kD), serum albumin (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21.5 kD), lysozyme (14.4 kD), aprotinin (6.5 kD)]. Molecular weights are indicated in kilodaltons. The band corresponding to metallothionein is indicated by an arrow on the right side.

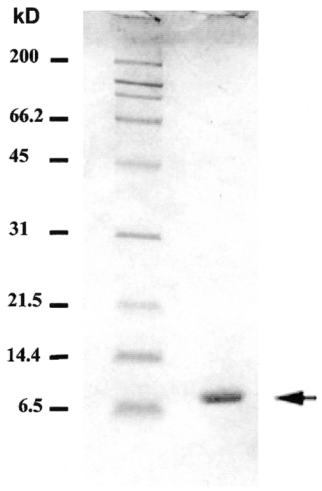

Fig. 4. Electrophoretic properties of alkylated metallothionein. The pooled fractions eluting at the metallothionein Ve (Fig. 1) were electrophoresed on SDS-PAGE gels and the band corresponding to metallothionein cut out and electroeluted from the gel. Metallothionein was then reduced with DTT, carboxymethylated and electrophoresed on a 20% polyacrylamide gel under reducing conditions. The position of metallothionein, stained with Coomassie Brilliant Blue, is indicated by an arrow on the right side. Molecular markers are those used in Fig. 2.

Drosophila metallothionein, and illustrate the difficulty to determine the molecular weight of certain proteins whose biochemical properties induce the formation of multimeric aggregates. Although additional studies are needed to characterize further the *P. waltl* metallothionein, the present analysis has enabled us to perform the first characterization of that protein by using a simple and rapid method. This method is also useful to analyze metallothionein synthesis during ontogeny as well as after metal intoxication.

Given the fact that metallothionein is able to bind heavy metal ions such as Zn^{2+}, Cd^{2+} and Hg^{2+}, it is generally considered that these proteins play a role both in metal homeostasis and detoxification processes. A number of data suggest that metallothionein would play in vertebrates an important role in metal transfer reactions in the liver, kidney and central nervous system. Metallothionein was also shown to be involved in developmental processes in sea urchins (Nemer *et al.*, 1984, 1991), *Drosophila* (Silar *et al.*, 1990) and Mammals (Andrews *et al.*, 1987, 1991). One metallothionein isoform (62 amino acids, 20 cysteines) has been characterized in the liver of *Xenopus laevis* (Yamamura and Suzuki, 1983), and the corresponding metallothionein cDNA cloned (Muller *et al.*, 1993; Saint-Jacques and Séguin, 1993). All of these results are consistent with the idea that metallothionein plays an important role in animals. Zinc is most commonly bound by metallothionein isolated from many vertebrates in normal conditions (Suzuki *et al.*, 1993). The ability of cadmium to displace this essential metal on purified metallothionein and to bind it stronger than zinc (Waalkes *et al.*, 1984) may explain a role in cellular detoxication. The high basal level of metallothionein that we have detected in various organs of *P. waltl* strongly suggests that this protein may be used in this species primarily for zinc storage.

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