Rainbow Trout Metallothionein

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Received December 4, 1984

Abstract

Two forms of hepatic metallothionein were isolated and purified from rainbow trout injected intraperitoneally with cadmium chloride. Both forms showed similarities with mammalian metallothioneins, had a high cystein content (30 mol%), and were void of aromatic amino acids and histidine. The molecular weight was estimated to be about 6000 dalton for the apothioneins, and the thiol groups of the cysteine residues complexed with the heavy metals (Cd, Cu, Zn) in a SH/Me^{++} ratio of about 2.4. The amount of copper in metallothionein from rainbow trout was very high, greater than the amount of cadmium and zinc after injections of 3 mg cadmium/kg body weight. The total metal content of cadmium, copper and zinc in metallothionein 1 and 2 were about 7 and 8 atoms per molecule respectively.

Introduction

Metallothionein (MT) is characterized as a low molecular weight protein, weighing around 6000 daltons [1, 2], that binds zinc, copper, cadmium and mercury. MT contains large amounts of cystein, usually about 30% [3], and lacks aromatic amino acids and histidine [3, 4]. The isolation of MT has been performed on a wide variety of mammalian species [5, 6], as well as on lower vertebrates [7] and invertebrates [8]. High levels of MT can be induced in animals by administration of the appropriate heavy metals [6, 9]. Following induction, the bulk of MT is found in kidney and liver.

The complete isolation and characterization of MT from several species of teleosts have recently been accomplished [7, 10–12]. In these teleosts MT appears to be very similar to mammalian MT. Generally, MT has been shown to exist in at least two isoforms in most studied teleosts [7, 12–15]. However, in rainbow trout (*Salmo gairdneri*) injected with zinc, Ley *et al.* [9] found evidence of only one single form of MT.

The existence of at least two isoforms of hepatic metallothionein in rainbow trout has been indicated by previous work done at our laboratory [14] and by Thomas *et al.* [16]. The aim of the present study was to make a more complete isolation and characterization of hepatic MT from rainbow trout.

Experimental

Chemicals

Sephadex G-50, G-75 and DEAE A-25 were from Pharmacia (Sweden). YM-2 membranes were from Amicon (U.S.A.). Ovalbumin, chymotrypsinogen, ribonuclease and blue dextran were from Pharmacia (Sweden). Albumin, bacitracin, insulin A-chain, Lima bean trypsin inhibitor Type II L, dithiothreitol and ammonium bicarbonate were from Sigma (U.S.A.), guanidine from Fluka (Switzerland) and Tris and Coomassie brilliant blue R-250 from Merck (F.R.G.). All other chemicals were of analytical grade.

Experimental Performance

Juvenile rainbow trout, weighing about 100 g, were purchased from a local fish hatchery (Antens Laxodling AB) and were held in 50 litre aquaria in the laboratory (10 °C, 6 fish in each). Each fish was injected intraperitoneally twice a week during three weeks to yield a total dose of 3 mg Cd/kg body weight. One week after the last injection, at day 28, fish were stunned by a blow on the head and the livers were removed and washed in a few ml of 50 mM Tris-HCl buffer (pH 8.1) prior to homogenization in the same buffer (20% w/v), using a glass–teflon homogenizer.

Isolation Procedure

The homogenates were centrifuged at 10,000 × g for 20 min at 4 °C. The supernatants were pooled and recentrifuged at 105,000 × g for 60 min at 4 °C. Portions of the ultrasupernatant were run

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0020-1693/85/$3.30

through a calibrated Sephadex G-75 column (2.6 X 60 cm) equilibrated with 50 mM Tris-HCl (pH 8.1). Absorbance was measured at 254 nm and 280 nm in each fraction using an UV spectrophotometer (Perkin–Elmer Lambda 3). The Sephadex G-75 column was calibrated with albumin, ovalbumin, chymotrypsinogen, ribonuclease and bacitracin.

The major cadmium-binding fractions (Fig. 1) were pooled and concentrated by ultrafiltration using

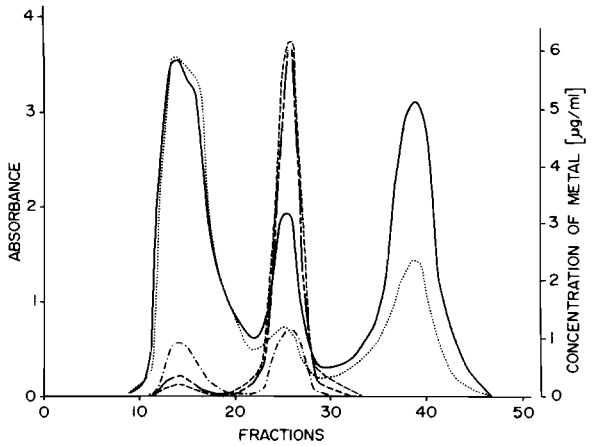

Fig. 1. Elution profile from Sephadex G-75 run of rainbow trout liver ultrasupernatant. Abs 254 nm (————); Abs 280 nm (·····); cadmium (-------); copper (----) and zinc (-·-·-).

YM-2 membranes. A portion of the concentrate was subjected to anion exchange chromatography on a DEAE Sephadex A-25 column (2.6 X 20 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.1). The column was eluted with a 800 ml linear Tris-HCl gradient (50–200 mM, pH 8.1). The absorbances at 254 nm and 280 nm were monitored in each 6 ml fraction. The major cadmium-binding peaks were pooled and concentrated by ultrafiltration on YM-2 membranes. The concentrated samples were applied to a Sephadex G-50 column (2.6 X 90 cm) equilibrated with 10 mM ammonium bicarbonate (pH 8.1). After final purification, the two isoforms of cadmium-binding protein were lyophilized and stored at −20 °C.

Molecular Weight Determination

Molecular weight estimates of unfolded, oxidized polypeptide chains were performed on a Sephadex G-50 column (1.3 X 90 cm) equilibrated with 6 M guanidine chloride (pH 8.0) [17]. Blue dextran 2000 (V_o) and bacitracin (V_t) were used as reference substances. Samples containing 1 to 5 mg protein were first oxidized with performic acid overnight [18]. After removal of the performic acid on a rotary evaporator, the samples were dissolved in 0.5 ml 6 M guanidine chloride buffer and applied to the column. Fractions of 1.0 ml were collected and monitored at 280 nm. The distribution coefficients, K_D, were evaluated from the relationship $K_D = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume of the unknown sample, V_o the void volume and V_t the total volume. The proteins used as standards were; chymotrypsinogen (M_w 25,700), ribonuclease (M_w 13,700), Lima bean trypsin inhibitor Type II L (M_w 8,400) and insulin A-chain (M_w, 2,500).

Chemical Characterization

The purified isoproteins were hydrolyzed at 110 °C in 6 M HCl in vacuum-sealed tubes for 20 h and 90 h. Samples for cystein determination were treated by oxidation. The quantitative amino acid analysis was performed by ion-exchange chromatography.

Cadmium, copper and zinc were determined by atomic absorption spectrophotometry on an IL Video 12 instrument. Homogenate samples and supernatant fractions were first digested with 70% nitric acid and then hydrogen peroxide was added until the digestion mixture was colorless. Chromatography fractions were analysed without prior digestion.

The purity of metallothionein was assessed by SDS disc-gel electrophoresis, according to the method described by Laemmli [19]. A 10% acrylamide concentration was used. The gels were stained with Coomassie brilliant blue R-250 and were scanned on a Gelman DCD-16 digital computing densitometer at 560 nm.

Results and Discussion

Metallothionein was isolated from livers of rainbow trout treated repeatedly with cadmium during three weeks (total dose 3 mg Cd/kg body weight). The elution pattern after chromatography on a Sephadex G-75 column is shown in Fig. 1. The majority of the cadmium and the copper was found associated to proteins with an apparent molecular weight of 10,000 dalton. A minor portion of both metals eluted together with high-molecular weight components. Zinc was distributed in equal amounts in the high-molecular weight fractions and in the 10,000 dalton fractions. There were no heavy metals (Cd, Cu and Zn) present in the peak corresponding to free hydrated metal ions. After further isolation of the pooled 10,000 dalton fraction on ion-exchange chromatography (Fig. 2), two cadmium-copper containing peaks were observed, and they were numbered metallothionein 1 (MT-1) and metallothionein 2 (MT-2).

The presence of at least two isoforms of MT is consistent with observations on other vertebrates [2, 12, 13, 20, 21]. There are, however, a few species

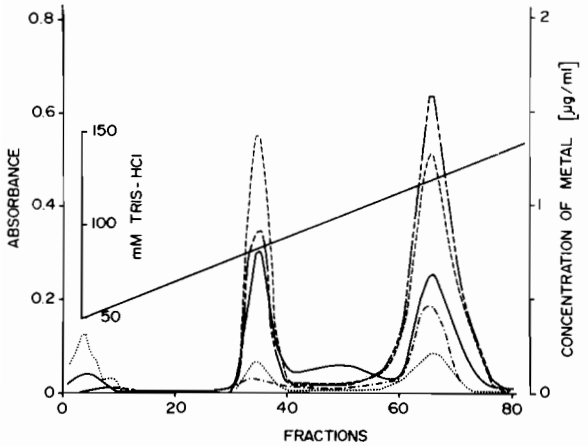

Fig. 2. Elution profile of ion-exchange chromatography on DEAE Sephadex A-25 of the 10,000 dalton peak from Sephadex G-75 runs. Abs 254 nm (———); Abs 280 nm (·····); cadmium (-------); copper (—·—) and zinc (—·—·—). The Tris gradient is shown by filled line.

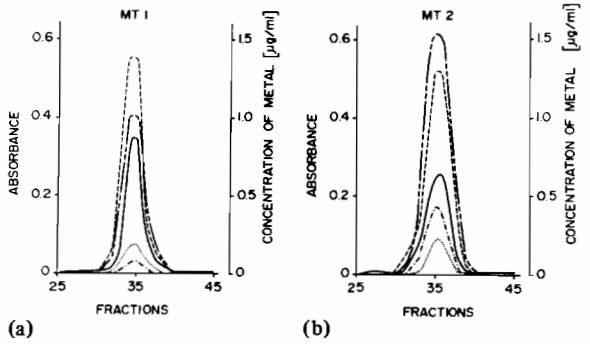

Fig. 3. Elution profiles of the separated MT-1 (3a) and MT-2 (3b). Abs 254 nm (———); Abs 280 nm (·····); cadmium (-------); copper (—·—) and zinc (—·—·—).

of teleosts in which other results have been obtained. In the liver of staghorn sculpin, *Leptocottus armatus* [11], and in the liver of plaice, *Pleuronectes platessa* [10], only one form of MT appears to be present. On induction of hepatic MT with zinc, Ley *et al.* [9] also obtained results that indicated the presence of only one form of MT. Sokolowski and Weser [22] showed that, under certain circumstances, zinc–thionein would elute in one peak on ion-exchange chromatography.

The present work shows that there are two isoforms of MT present in rainbow trout liver after induction with cadmium. To ascertain that the second peak was not due to disulphide linkages of metallothionein, 0.03% dithiothreitol was introduced in the Tris buffer. Under these conditions two peaks of metallothionein were still obtained on ion-exchange chromatography.

Although there were minor variations in metal distribution between MT-1 and MT-2 from individual experiments, a general pattern could be distinguished. MT-2 contained proportionally more copper and zinc, and less cadmium, than MT-1. On chromatography of metallothionein on an ion-exchanger, MT-1 eluted at a Tris-HCl concentration of 80 mM, while MT-2 eluted at 120 mM Tris-HCl. Final purification and buffer change were performed on a Sephadex G-50 column equilibrated with 10 mM ammonium bicarbonate. Both forms appeared to be already highly purified after the ion-exchange chromatography step. Elution profiles of the MT peaks from Sephadex G-50 runs are shown in Figs. 3a and 3b. After concentration on YM-2 membranes, the two MTs were lyophilized and used in characterization of the proteins.

The typical recoveries in the chromatographic steps were 90–95%. About 70% of the cadmium originally present in the liver could be accounted for in the metallothionein fractions on Sephadex G-75 chromatography. These data are in good agreement with results obtained when ^{109}Cd was used as a tracer [14].

Polyacrylamide disc-gel electrophoresis was used to ascertain the homogeneity of purified MT-1 and MT-2. Both forms appeared as single bands and had slightly different R_f values. MT-1 had a R_f value of 0.80 while MT-2 had a R_f value of 0.82, indicating that MT-1 is slightly larger than is MT-2 (Fig. 4b).

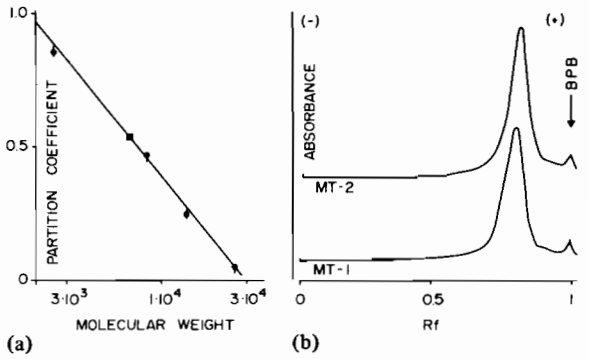

Fig. 4. Molecular weight estimations shown in 4a. Standards (•) and metallothionein (♦). SDS disc-gel electrophoresis of MT-1 and MT-2 is shown in 4b. BPB, bromphenol blue.

The criteria used to characterize hepatic MT from rainbow trout were molecular weight, amino acid composition, metal content and ratio of cystein to heavy metals. These characteristics of MT from rainbow trout were generally in good agreement with previously characterized MT from mammalian species [1–4].

The molecular weight of performic acid oxidized MT was estimated to be about 6500 daltons for both isoforms (Fig. 4a). This would correspond to a molecular weight of 6000 daltons for the unmodified apothionein.

From the amino acid analysis (Table I) the chain weight was calculated to be 5989 daltons for MT-1 and 5955 daltons for MT-2, under the assumption that only one residue of methionine was present in the protein (Table II). Different methods have been employed to determine the molecular weight of metallothionein. The most satisfying results are obtained using chromatography of the proteins in 6 M guanidine chloride or 8 M urea. Electrophoresis apparently gives values for MT that are far in excess of the actual molecular weight. Using chromatography in 6 M guanidine chloride, the molecular weight for the unmodified apothionein was calculated to be about 6000 for both MT-1 and MT-2. These results are in good agreement with results obtained by others who have used the same technique [1, 21], as well as with values from sequence determinations of MT in other vertebrates [23, 24].

TABLE I. Amino Acid Composition of Rainbow Trout Hepatic Metallothionein.

Amino acid	Metallothionein 1		Metallothionein 2	
	Mol %	No. res.[a]	Mol %	No. res.[a]
ASP	10.1	6	12.5	8
THR	6.8	4	7.3	4
SER	14.0	8	15.8	10
GLU	4.2	3	3.5	2
PRO	3.1	2	3.0	2
GLY	9.6	6	9.8	6
ALA	4.8	3	4.9	3
VAL	1.9	1	1.7	1
CYS[b]	32.8	20	28.4	17
MET[c]	2.3	1	2.2	1
LYS	10.4	6	10.8	6
ILE	0	0	0.1	0
LEU	0	0	0.1	0

[a]Calculated on the basis of 1 MET residue per mol. [b]Determined from cysteic acid content in performic acid oxidized protein. [c]Determined as methionine sulfone.

TABLE II. Characteristics of Metallothionein 1 and Metallothionein 2.

	Metallothionein 1	Metallothionein 2
Chain weight[a]	5989	5955
Molecular weight[b]	6638	6506
Cadmium[c]	3.08	1.95
Copper[c]	4.49	4.09
Zinc[c]	0.28	1.10
Total metal[c]	7.85	7.14
Cystein to metal[a]	2.55	2.38

[a]Calculated from quantitative amino acid analysis. [b]Based on actual metal content in MT-1 and MT-2. [c]Gram atom of metal per mole of metallothionein.

The amino acid composition of rainbow trout metallothionein was similar to metallothionein from mammalian species [3] and to fish MT [10, 12]. Rainbow trout MT contained high amounts of cystein (32.8% in MT-1 and 28.4% in MT-2) and were devoid of aromatic amino acids, histidine and arginine. Traces of isoleucine and leucine were observed in MT-2 but not in MT-1. Further, the two isoforms differed in the content of four amino acids. MT-1 contained more glutamic acid and cystein and less aspartic acid and serine than MT-2. The total number of residues was calculated to be 60 in both forms. The absence of aromatic amino acids, histidine, arginine, isoleucine and leucine suggests a high degree of purity in the isolated MT. A cystein content of about 30% is considered normal for MT present in other vertebrates [1, 2, 21], but is higher than that reported for some teleosts. Thus, values from 20% to 25% were found in eel, *Anguilla anguilla* [7], in staghorn sculpin [11] and in rainbow trout [9].

The metal content and the cystein-metal ratios are shown in Table II. Rainbow trout injected intraperitoneally with 3 mg Cd/kg body weight reached a cadmium content of 3.08 and 1.95 gram atoms per mole of MT-1 and MT-2, respectively. Copper was found in higher amounts, 4.49 and 4.09 gram atoms per mole of MT-1 and MT-2, respectively. Zinc was found in small quantities in both isoforms, 0.28 gram atoms in MT-1 and 1.10 gram atoms in MT-2. The molar ratios of cystein to metal were 2.55 in MT-1 and 2.38 in MT-2.

After the induction of MT by a specific heavy metal, there are almost always small amounts of other heavy metals bound to MT. Thus, after the induction with cadmium in rainbow trout, zinc and copper were found in the isolated MT. When all the involved heavy metals have been measured, the amount of zinc was usually greater than the amount of copper in metallothionein after induction with cadmium [1, 21]. However, in a few teleosts [12, 13] treated with cadmium, the amount of copper was greater than the amount of zinc. This is also the case in rainbow trout, and the reason seems to be the high levels of copper in the livers of normal rainbow trout [9]. Cadmium and zinc are known to bind in a 1:3 arrangement with cystein, while copper appears to prefer binding to two cystein molecules [24, 25]. Assuming these binding characteristics, the theoretical cystein-heavy metal ratio was calculated

to be 2.43 in both MT-1 and MT-2. The actual ratios that were derived from the quantitative amino acid analysis were 2.55 and 2.38 respectively for MT-1 and MT-2. Thus, the proposed binding patterns are in agreement with the cystein-heavy metal ratios found in the present study.

The role of metallothionein in heavy metal detoxification is yet to be elucidated. The biological half life of cadmium is unusually long in higher vertebrates [27] and it has been postulated that metallothionein is involved in this phenomenon, as well as in the accumulation of cadmium in the target organs. It has also been proposed that metallothionein plays a protective role in heavy metal poisoning [3]. In the case of rainbow trout, the role of metallothionein in environmental sequestering of cadmium has been questioned [16]. It seems evident that different heavy metals may be detoxified in different manners. Whether rainbow trout metallothionein can reduce the toxic influence of cadmium remains to be answered. Further work is needed in order to ascertain the role of metallothionein in the detoxification of cadmium.

Acknowledgements

We gratefully acknowledge Dr. Lars Strid at the Department of Biochemistry, University of Göteborg, Sweden, for performing the amino acid analyses, Dr. Åke Larsson at the Brackish Water Toxicology Laboratory, Studsvik, Sweden, for critical reading of the manuscript, and Mrs. Birgitta Vallander, for drawing the figures. This study was financed by the Research Committee of the National Swedish Environmental Protection Board, Kungliga Hvitfeldtska Stipendieinrättningen and Hierta–Retzius fond för vetenskaplig forskning.

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