White-sided dolphin metallothioneins: purification, characterisation and potential role☆

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Abstract

Metallothioneins (MTs) were characterised in the kidneys of a white-sided dolphin *Lagenorhynchus acutus* stranded along the Belgian coast, displaying high levels of cadmium (Cd) and mercury (Hg) in liver and kidney. The protein has two isoforms: MT-1 and MT-2. MT-1 binds Cu, Zn, Hg and Cd, while MT-2 only binds Zn, Hg and Cd. This suggests different metabolic functions for the two isoforms: MT-1 is mainly involved in Cu homeostasis; MT-2, which was four-fold more abundant than MT-1, detoxifies most of the accumulated cadmium. © 2002 Elsevier Science Inc. All rights reserved.

1. Introduction

Since their first discovery by Margoshes and Vallee (1957), metallothioneins (MTs) have been detected studied in both prokaryotes and eukaryotes (Roesijadi, 1992, 1996) through more than 5000 research papers. MTs received their designation from their high metal and sulfur content. The mammalian forms are characterised by a low molecular mass of 6000–7000 Da, containing 60–68 amino acid residues, among which 20 cysteines. MTs bind a total of 7 equivalents of bivalent metal ions. However, the natural function of metallothioneins remains elusive. Previous studies on structure, function and molecular regulation have established a central role for these small proteins

in the homeostatic regulation of essential metals, including zinc (Zn) and copper (Cu), and detoxification of non-essential metals, such as mercury (Hg) or cadmium (Cd) (Klaassen et al., 1999). In order to understand the functions of MTs, the functionality and structure of each MT isoform need to be considered. Indeed, in various mammal organs, such as liver or kidney, two major isoforms, distinguishable by their charge, are present at low basal levels, MT-1 and MT-2 (Kägi, 1993). To date, however, no functional difference between the two isoforms has been firmly established (Moffat and Denizeau, 1997).

In the framework of heavy metal ecotoxicology, marine mammals appear as a choice material, since high levels of Hg and Cd can be naturally encountered in these animals (Das et al., in press). The potential role of metallothioneins in marine mammals has recently been reviewed by Das et al. (2000). It appears that very few characterisations of marine mammal metallothioneins have been attempted. The first attempt was realised on sea lion, *Zalophus californianus*, and kidney and liver

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of sperm whale, *Physeter macrocephalus* (Ridlington et al., 1981). Thereafter, MT I and II isolation and characterisation have been performed in the kidney of striped dolphin, *Stenella coeruleoalba* (Kwohn et al., 1986, 1988). More recently, Pillet et al. (2000) have described the presence of both isoforms in the blood leucocytes of the grey seal, *Haliochoerus grypus*.

In order to obtain a better understanding on the role of metallothioneins in the detoxification and dynamics of trace metals in marine mammals, detection, purification and trace metal content of white-sided-dolphin renal metallothioneins have been performed and compared with published data.

2. Materials and methods

The kidney and liver of an adult male white-sided dolphin, *Lagenorhynchus acutus*, found dead along the Belgian coast on 22 September 1999 were sampled and stored at -20 °C. These dolphin tissues were remarkably fresh (Jauniaux, personal communication), allowing protein analysis. The renal tissue contained 78% water.

2.1. AcA 54 chromatography

A sample of 3–4 g of kidney was homogenised using an Ultra-Turrax homogeniser in 0.01 M ammonium formate (pH 7.4) containing 10 mM sodium azide and 0.01% dithiothreitol, and centrifuged at $26\,000 \times g$ (60 min, 4 °C). The supernatant was filtered on an AcA 54 gel column (1.6×64 cm) at 4 °C. Fractions were collected and the absorbance was read at 215, 254 and 280 nm.

2.2. Heavy metals analysis and MT concentration assessment

After adding nitric acid (65%) to each fraction and to the pellet, homogenate and supernatant, all were slowly heated to 100 °C until digestion was complete. Samples were diluted using deionised water and filtered prior to heavy metal analysis (Zn, Cu and Cd) by atomic absorption spectrophotometry (ICPS: ARL 3510). Hg was analysed by flameless atomic absorption (Perkin Elmer MAS-50A) as described by Joiris et al. (1991). Quality control measurements for total mercury included replicate analysis, resulting in coefficients of vari-

ation <10% and analysis of certified material (DORM-1, NRC, Canada) as described by Debacker et al. (1997).

The Hg absolute detection limit is 10 ng, corresponding to 0.13 μg g^{-1} fresh weight (FW) for an average of 1.5 g FW. Detection limits for Cu, Cd and Zn were 0.18, 0.18 and 0.17 μg g^{-1}, respectively. Quality of the analyses was controlled through participation in an intercalibration program Quevauviller, 1997. Recovery rates ranged from 94, 97, 100 and 102% for Hg, Cu, Zn and Cd, respectively. Metal concentrations are expressed as μg g^{-1} FW. From the heavy metal analyses, the MT concentration was estimated considering that Cu occurs in the divalent form and that 1 mole MT with a molecular mass of 6800 Da typically binds seven divalent metal ions (7 g atom per MT mole) (Kägi, 1993; Kojima et al., 1999).

2.3. DEAE Sephadex chromatography

Anion exchange chromatography was performed according to Kwohn et al. (1986). Briefly, after ACA 54 gel chromatography, the fractions with low absorbance at 280 nm and high absorbance at 254 nm were pooled and lyophilised (VIRTIS, 12XL). To eliminate the NaCl resulting from the lyophilisation, the proteins were chromatographed on a Sephadex G25 medium column (1.5×28 cm). Metallothionein separation was performed on an anion-exchange Sephadex A25 (1.6×70 cm) column equilibrated with 1 mM Tris–HCl buffer (pH 8.6). Elution was carried out in gradient mode with 1–250 mM Tris–HCl buffer, pH 8.6.

The ultraviolet spectra of renal MT-1 and MT-2 were recorded using the highest resolution of 0.2 nm. DEAE Sephadex chromatography, Cd, Zn and Cu analyses, and UV spectral measurements were carried out in triplicate.

3. Results

AcA-54 gel chromatography (Fig. 1a) of the supernatant reveals a large band of metal (Zn, Cd, Cu) in the 10-kDa region of the profile, consistent with the presence of metallothioneins. This metal peak is associated with high absorbance at 254 nm and low absorbance at 280 nm, related to the lack of aromatic amino acids of these proteins. This metallic peak shows remarkably high temperature
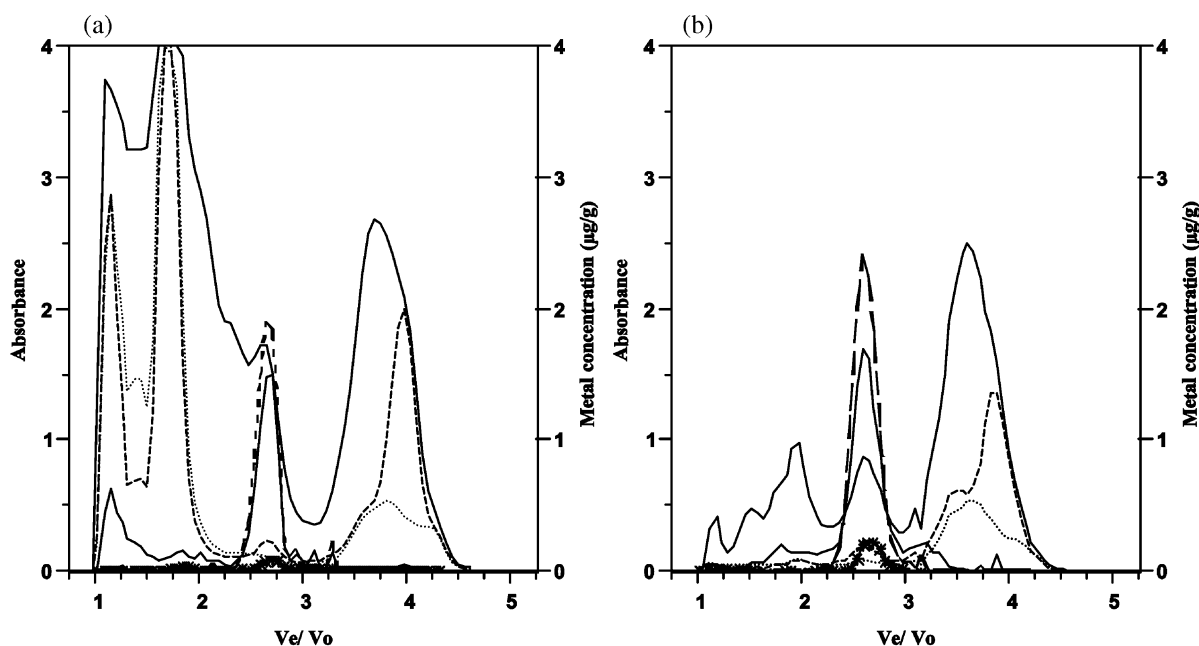
Fig. 1. Elution profile on AcA 54 column of the water-soluble fraction extracted from kidney of the white-sided dolphin *Lagenorhynchus acutus* (a) before and (b) after 89 °C heat treatment of the cytosolic fraction. The three types of bold lines represent the metal concentrations (-, Zn; --, Cd; **, Cu) of the fractions. The normal lines represent the protein absorption (-, 215; --, 250; …, 280 nm); Ve, elution volume; Vo, void volume of the column; mean volume per fraction, 2 ml.

tolerance after a 3-min heat treatment at 89 °C followed by centrifugation (Fig. 1b).

Table 1 shows the metal (Cd, Zn, Hg, Cu) distribution in the insoluble and soluble fractions, including the metals bound on high molecular-weight-proteins, metallothionein-like proteins and smaller molecules. High cadmium concentrations were found in the kidney (more than 12 μg g^{-1} FW) and liver (5 μg g^{-1} FW; data not shown). Almost 60% of the total Cd, 30% of the total Zn and 12% of the total copper are bound to cytosolic metallothioneins. The amount of metallothionein in kidney was calculated from the metal content, assuming that the protein was fully saturated (seven equivalent of divalent ions or 7 g atom per MT mole) with a molecular mass of 6800 kDa. A metallothionein concentration of 172 μg g^{-1} FW was calculated. After DEAE Sephadex A25 chromatography, the level of both isoforms estimated from trace metal data differed (Fig. 2): the MT-2 concentration was four-fold higher than that of MT-1.

Fig. 2 shows the elution profile of the DEAE Sephadex A-25 ion exchange chromatography. The

Table 1
Zn, Cd, Cu, and Hg renal content in the insoluble fraction (pellet), cytosol (soluble proteins) containing the high molecular-weight-proteins (HMWP), metallothioneins (MTs) and lower molecular-weight-proteins (LMWP)

Metal	Metal content (μg g^{-1} FW)					
	Total	Insoluble fraction (pellet)	Cytosol (supernatant)	Protein type		
				HMWP	MTs	LWMP
Zn	19 (100%)	8 (42%)	11 (58%)	4	6.6 (35%)	0.7
Cd	12 (100%)	3 (25%)	9 (75%)	<0.2	8.30 (71%)	0.3
Cu	2.2 (100%)	1.6 (72%)	0.6 (18%)	0.14	0.4 (18%)	<0.2
Hg	2 (100%)	1.5 (75%)	0.6 (25%)	ND	ND	ND

ND, not determined.

profile reveals three metal-containing fractions, indicating that the MT was resolved into two isoproteins along the NaCl gradient. The first metal-containing fraction (labelled I in the figure) did not bind to the column matrix and was eluted with 1 mM Tris–HCl (pH 8.6) before the gradient started. This peak contains Zn, Cd and Hg. The last two fractions were, in accordance with earlier experiments on mammalian liver, kidney and intestine, designated as MT-1 and MT-2 (Kwohn et al., 1986; Suzuki, 1991; Moffat and Denizeau, 1997). Hg concentrations were determined in pooled fractions (0.016 and 0.03 μg atom for MT-1 and MT-2, respectively).

Furthermore, we observed that the metal composition of white-sided dolphin isometallothioneins varied in the different isoforms: MT-1 contained Zn, Cd, Hg and Cu, while MT-2 contained Zn, Cd and Hg. Copper could not be detected in the second isoform (Table 2).

The ultraviolet spectrum reveals very low

Table 2
The molar ratio of metal/thionein in striped (after Kwohn et al., 1986) and white-sided dolphins (mean ± standard deviation of the measure, this work; n = number of replicates)

	Metal/thionein ratio (g-atoms 6000 g^{-1})		n
	Striped dolphin	White-sided dolphin	
MT-1			
Cd	2.41	2.57 ± 0.3	3
Hg	0.06	0.39	1
Zn	2.09	2.47 ± 0.2	3
Cu	0.98	1.82 ± 0.7	3
MT-2			
Cd	2.93	2.99 ± 0.4	3
Hg	0.04	0.22	1
Zn	2.86	3.88 ± 0.5	3
Cu	0.16	<0.04	3

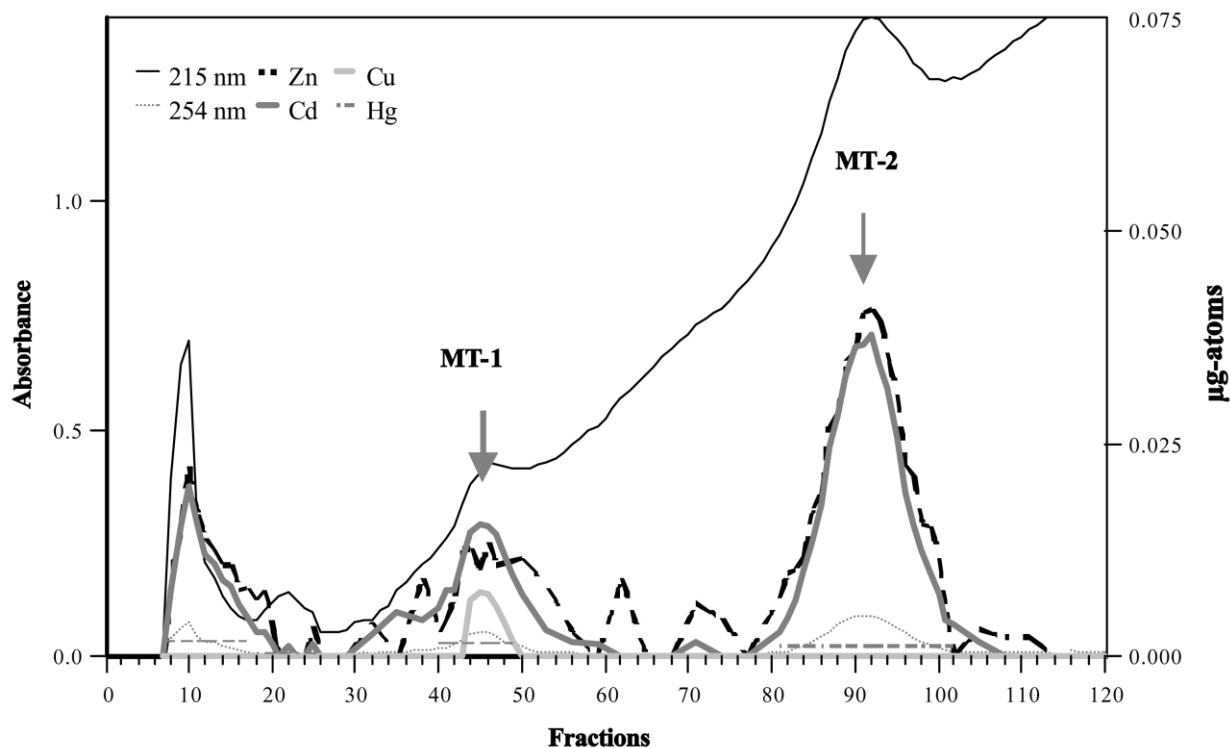absorbance at 280 nm and a shoulder at 254 nm, mainly for MT-2 (Fig. 3). The absorbance values

Fig. 2. DEAE Sephadex A-25 chromatography profile of white-sided-dolphin metallothioneins. Pooled and concentrated metallothionein fractions from AcA 54 column were applied to a DEAE Sephadex A-25 column. The three types of bold lines represent the Zn, Cd, and Cu concentrations of the fractions. Metal concentrations are expressed in μg-atoms (concentration expressed in μg g^{-1} divided by the respective metal atomic mass). The horizontal dotted lines represent the Hg concentration in the three pooled fractions. The normal lines represent the 215- and 250-nm protein absorption.
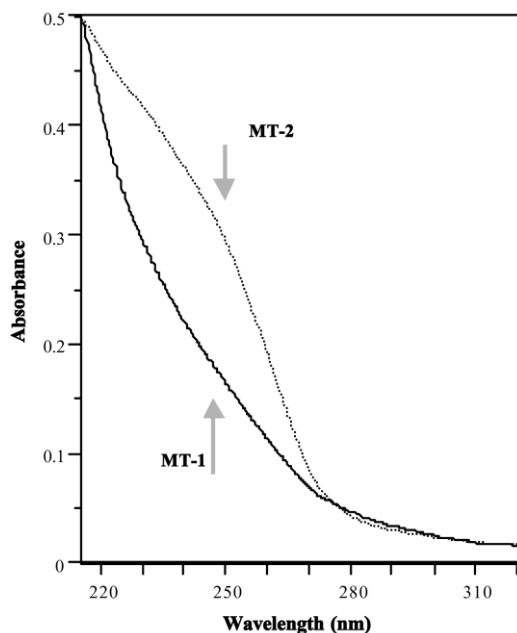
Fig. 3. Ultraviolet spectrum of white-sided dolphin MT-1 and MT-2.

at 230, 250, 270, and 310 nm reflect Zn–S, Cd–S, Cu–S and Hg–S binding.

4. Discussion

Cadmium is known to preferentially accumulate in the kidneys, bound in large amounts to MTs, which also contain Zn and Cu. Renal and hepatic Cd concentrations measured in the male white-sided dolphin kidney are high (Table 1). Indeed, the hepatic Cd concentration is 10-fold higher than the 0.5 μg g^{-1} FW previously measured in the liver of an adult white-sided dolphin from the US Atlantic coast (Kuehl et al., 1994).

Of the renal cadmium, 81% was found in the soluble fraction of the tissue, mostly bound to low-molecular-weight, soluble and heat-stable proteins (Fig. 1, Table 1) with high absorbance at 254 nm and a lack of absorbance at 280 nm, in agreement with MT-specific features. The heat tolerance of these proteins is remarkable and implies that a single dose of Cd, Zn, Cu and Hg in the heat-treated supernatant, followed by centrifugation, could allow the determination of the MT-bound metals, and hence the renal MT con-

centrations. Complementary studies on other species have been carried out in our laboratory to test the accuracy of such a method to quantify MTs in marine mammals.

Mercury is preferentially distributed in the insoluble fraction of the kidney (pellet, Table 1), probably resulting from the well-known formation of tiemannite (HgSe) in marine mammal liver and kidney (Das et al., 2000; Das et al., in press).

Mammal kidney metallothioneins consist of two isoforms, MT-1 and MT-2, named in the order of their elution from an anion-exchange column (Suzuki, 1991). The resolution of white-sided dolphin renal MT into two isoproteins (MT-1 and MT-2, Fig. 2) by ion exchange chromatography corroborates the observations made on kidney and liver MT of monkey (Paliwal et al., 1982, 1986), human (Hellemans et al., 1999) and striped dolphin (Kwohn et al., 1986).

Cd, Zn and Hg bound to both isoforms, while copper was not detected on MT-2. These results are consistent with a previous study on striped dolphin, *Stenella coeruleoalba,* which showed that MT-2 bound Cu in lower amounts than MT-1 (Kwohn et al., 1986; Table 2). These results seem to indicate a specific role of renal MT-1 in copper homeostasis, copper being an essential metal involved in many enzymatic processes (Eisler, 1997).

MT-2 is the major isoform present in white-sided dolphin kidney, with a MT-2/MT-1 ratio concentration equal to 4.2. A ratio of 16 has been measured in striped dolphin kidney and 6.2 in equine kidney (Kwohn et al., 1986). However, it is worth noting that Wagemann and Hobden (1986) reported MT-1 as the major form of MTs in the liver and kidney from a narwhal. In fact, the relative abundance of each isoform can differ according to species, age, tissue and the nature of the inducers (Moffat and Denizeau, 1997). The higher MT-2 concentration probably results from a difference in the rate of synthesis of hepatic MT-1 and MT-2, which leads to a greater and more prolonged induction of MT-2 following administration of trace metals (Lehman-McKeeman et al., 1991) and a faster degradation rate of MT-1 compared to MT-2 (Mehra and Brenmer, 1985). As a whole, this leads to a more important role of MT-2 in the sequestration of Cd.

5. Conclusions

White-sided dolphin metallothioneins display two renal isoforms, MT-1 and MT-2, characterised by different metabolic functions in relation to copper cellular homeostasis (MT-1) and cadmium detoxification (MT-2). MTs appear to play a minor role in the binding and detoxification of Hg by marine mammals. Moreover, after appropriate heat treatment, trace metals measured in the supernatant were those bound to MTs, leading to a potential rapid and simple method to quantify these proteins in marine mammal tissues. Further experiments are needed to test the suitability of this method to others tissues and species.

Acknowledgments

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