The presence of metallothionein-like proteins in the liver of two species of flatfish: *Limanda limanda* and *Microstomus kitt*

Flatfish
Metallothionein-like protein
hepatic
Cadmium
Chromatography

Poisson plat
Protéine métallothionéine
Hépatique
Cadmium
Chromatographie

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ABSTRACT

Two species of flatfish, *Limanda limanda* (dab) and *Microstomus kitt* (lemon-dab) were injected intraperitoneally with Cd to determine the nature of the metal-binding proteins induced in their livers. The supernatants were analysed by gel filtration chromatography and the levels of metal and sulfhydryl groups, as well as the spectral behaviour of the low molecular weight proteins, provided evidence that the hepatic metal-binding proteins induced in the two flatfish belong to the family of metallothionein-like proteins.

RÉSUMÉ

Protéines de type métallothionéine dans le foie de deux espèces de poissons : *Limanda limanda* et *Microstomus kitt.*

Deux espèces de poissons, *Limanda limanda* (limande) et *Microstomus kitt* (limande-sole) ont reçu une injection intrapéritonéale de Cd afin de déterminer la nature des protéines liant les métaux, induites au niveau du foie. Les surnageants ont été analysés par chromatographie de filtration sur gel et les teneurs en métal et en groupements sulfhydryles, ainsi que le comportement spectral des protéines de faible poids moléculaire, mettent en évidence que les protéines hépatiques liant les métaux induites chez les deux espèces de poissons plats appartiennent à la famille des protéines de type métallothionéine.

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INTRODUCTION

Pollution of the littoral zone by heavy metals leads to an increase of their concentration in marine organisms. The fact that these pollutants are persistent in the environment adds a further hazard which stimulates research activities devoted to the evaluation of the effects they exert at the population, organism and cellular levels.

Metallothionein (MT) levels in marine organisms have been proposed as biochemical indicators of heavy metal pollution (Viarengo *et al.*, 1980; Roch *et al.*, 1982; Bayne *et al.*, 1988). They play a role in the homeostasis of essential metals and in the detoxification at the cellular level (Webb and Cain, 1982; Klaverkamp *et al.*, 1984). MT have a high metal content, with 7 g atoms of bivalent metal ions or up to 12 g atoms of monovalent ions per

mole bound to MT as metal-thiolate complexes (Kägi and Schäffer, 1988). This content is equivalent to one bivalent metal atom per three cysteine residues, or one monovalent metal ion per two cysteine residues. These low molecular weight proteins have an unusual amino acid composition, as they lack aromatic amino acids, and cysteine accounts for 30 % of the residues. Consequently, they are characterized by a low absorbance at 280 nm (absence of aromatics) and an absorbance at 254 nm due to the presence of Cd-thionein, the metal-thionein linkage being reversible (Kägi and Nordberg, 1979). The levels of MT increase after exposure of fish to class IIB metals (Noël-Lambot *et al.*, 1978; McCarter and Roch, 1984; Kito *et al.*, 1986) but in some instances, the characteristics of the metal-binding proteins so induced appear to be different to MT (Thomas *et al.*, 1983; Brown *et al.*, 1990).

We carried out experiments to demonstrate the presence of hepatic metal-binding proteins in the two pleuronectides *Limanda limanda* (dab) and *Microstomus kitt* (lemon-dab) after intraperitoneal injection of cadmium.

MATERIAL AND METHODS

Chemical

Sephadex G75-Superfine was from Pharmacia (Sweden). DTNB (dithionitrobenzoic acid) and gluthatione were from Sigma (USA).

Experimental animals

Limanda limanda and *Microstomus kitt* were collected in the south of the North Sea between Calais and Dunkerque. The fish were kept alive, transported to the Marine Station and maintained in flowing sea water aquaria. Each fish was injected intraperitoneally 4 times a day over 2 or 3 days with $CdCl_2$ at a dose of 1 mg Cd per kilogram body weight at each injection. They were killed by cutting behind the gills and dissected to obtain the livers which were frozen at – 20°C.

Isolation procedure

The livers (about 1.5 g) were homogenized using an Ultra-Turrax homogenizer in 2 vol of 0.5 M sucrose at 0°C for 2 minutes. The homogenates were centrifuged at 37 000 × g at 4°C for 3 hours. Approximately 3 ml of the supernatant was applied to a column of Sephadex G75 (2.6 × 85 cm) equilibrated with 0.01M ammonium formate buffer (pH 7.4). This buffer was selected because of its ease of removal, when necessary, at the completion of chromatography. The absorbance at 254 nm was recorded and fractions (6 ml) were collected of which 2 ml was used to assay for metal content and 300 µl to determine sulfhydryl groups. The fractions containing low molecular weight proteins were monitored for absorbance from 200 to 300 nm using a Beckman model 24 spectrophotometer and measured again after addition of hydrochloric acid (pH 2).

Colorimetric determination of the sulfhydryl groups

Sulfhydryl groups were quantified by the method used by Tukendorf and Baszynski (1985) and based on the observations of Ellman (1959). Microtitre plates (for immunoassays) were used allowing a quick measurement of the absorbance of 96 very small samples as follows:

The 300 ml solution were placed in each well of the microtitre plate and 50 ml of DTNB reagent (40 mg of DTNB in 10 ml of 0.1M phosphate buffer, pH 7.8) were added. The reaction occurred over 30 min and the absorbance at 405 nm was determined on a microtitre plate reader. Triplicate samples were measured and controls were provided by 3 wells containing buffer solution. Glutathione, diluted in ammonium formate buffer (0.01M) was used to develop a standard curve (1 mole SH/mole). The quantity of MT was determined using the ratio 20 moles SH/mole MT (Kägi and Norberg, 1979).

Heavy metal analysis

Zinc, copper and cadmium concentrations in fractions from gel filtration chromatography were measured using a Perkin-Elmer HGA 500 flame atomic absorption spectrophotometer. Detection limits were 0.04, 0.04 and 0.03 mg/l for the measurement of Zn, Cu and Cd respectively. Deuterium background correction was used for Cd and Zn analyses. The quantities of MT were estimated by the ratio 7 g atom/mole MT for Cd and Zn, and 12 g atom/mole MT for Cu (Kägi and Schäffer, 1988).

RESULTS AND DISCUSSION

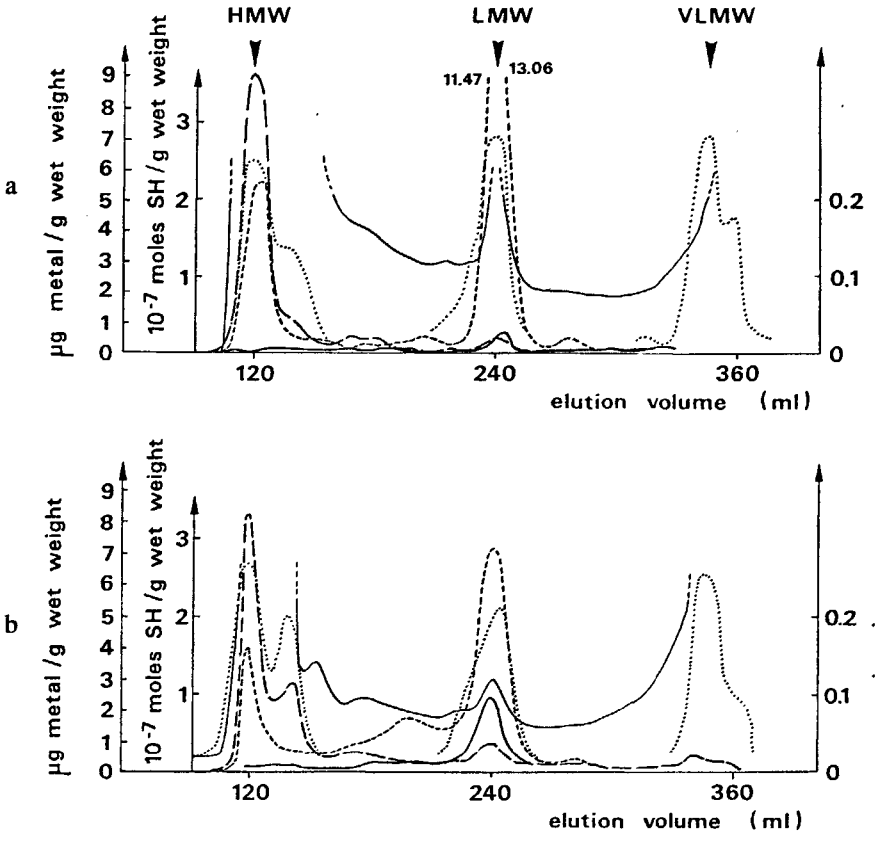
Three absorbance peaks (254 nm) were obtained after chromatography of hepatic supernatants from Cd-injected fish (Fig. 1*a* and *b*). They correspond to high molecular weight proteins (HMW; > 60 kDa), low molecular weight proteins (LMW; 8-12 kDa) and very low molecular weight components (VLMW; < 5 kDa).

Heavy metal distribution in the different molecular weight ranges: The distribution of Cd, Cu and Zn in the hepatic supernatants showed high levels of Zn and Cd and lower levels of Cu eluted with the HMW proteins. The presence of metals in the HMW proteins was expected and consistent with their role in the normal metabolism of teleost fish (*i.e.* in enzymes, functional proteins, etc.). Significant amounts of metal were measured in fractions containing the LMW proteins. This is consistent with the presence of MT or other metal-binding proteins (MBP) of similar apparent molecular weight. No metals were detected in the VLMW fractions, which correspond to small molecules and peptides (*e.g.* glutathione). This absence of metals in the VLMW fractions has also been noted in the livers of the fish *Mugil cephalus* and *Salmo gairdneri* (Wofford and Thomas, 1984; Olsson and Haux, 1985). Similar partition of heavy metals in the different molecular weight ranges have been reported for different species of fish (Kito *et al.*, 1982; Olsson and Haux, 1985).

Figure 1

Sephadex G75 elution profiles of hepatic supernatant from Limanda limanda *(a) and* Microstomus kitt *(b), injected with Cd.*

(——), Cu; (— — —), Zn; (-----) Cd; (·····) SH; (—), Abs $_{254nm}$



Presence of sulfhydryl groups associated with different molecular weight ranges: The sulfhydryl groups were associated with the three absorbance peaks. The SH groups detected in the HMW peak and the VLMW peak were associated with normal structural or/and functional proteins, and with presence of glutathione and/or cysteine respectively, whereas SH measured in the LMW peak were assumed to be related to MT as they were found in metal-containing fractions .

Concentrations of metal-binding proteins as indicated by metal and sulfhydryl ratios: The concentrations of "equivalent MT" within the LMW fractions from chromatography of liver from *L. limanda* and *M. kitt* injected with Cd are presented Table 1. They were derived from the expected ratio of metal to MT, and sulfhydryl groups to MT. For the two species of fish, the quantities of MT obtained from either methods were similar. These results indicate that the metal-binding proteins have similar metal-binding abilities to MT and a similar content of sulfhydryl groups (*i.e.* 20 moles SH/mole protein).

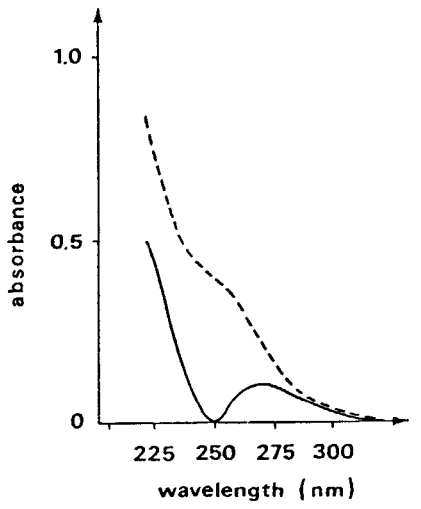


Figure 2

Ultraviolet absorption spectra of the low molecular weight proteins from Limanda limanda *obtained after separation by Sephadex G75 chromatography (— — —) at pH 7.4; (——) at pH 2.0.*

Table 1

Amounts of "equivalent metallothioneins (MT)" in livers from Cd-injected individuals (Limanda limanda *and* Microstomus kitt), *estimated by the quantities of metals and SH groups, eluted in the low molecular weight fractions by Sephadex G75 chromatography.*
(MT and metals, expressed in µg/g wet weight; SH, expressed in 10^{-8} moles/ g wet weight).

Species indiv.		Cu	MT	Cd	MT	Zn	MT	MT (total)	SH	MT
L. limanda	1	1.2	10.2	35.6	294.5	1.4	19.9	325	109.3	355
	2	7.6	64.5	20.7	171.2	2.8	39.9	276	82.7	269
M. kitt	1	6.9	58.8	24.0	198.3	2.9	41.2	298	88.9	289
	2	5.3	45.2	26.7	220.6	2.5	35.5	301	96.9	315

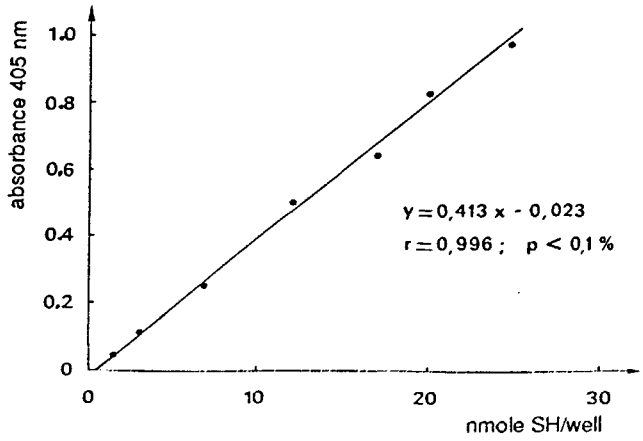


Figure 3

Standard curve of glutathione.

Spectral behavior: The ultraviolet absorption spectra of the LMW fractions from *L. limanda* showed an elevated absorbance at 254 nm (which decreased significantly after acidification of the solution) and consequently yielded a high ratio A_{254}/A_{280} (Fig. 2). Those spectral properties of LMW proteins under neutral and acidic conditions are characteristic of MT (Kagi and Nordberg, 1979).

The spectral behaviour of the hepatic LMW metal-binding proteins and their concentrations in metals and sulfhydryl groups strongly suggest that those proteins belong to the family of MT-like proteins.

Characteristics of the colorimetric method for the quantification of the sulfhydryl groups: The standard curve obtained with glutathione was described by the formula: y = 0.413 x – 0.023 (Fig. 3) and the lowest detectible quantity of thiol groups was 1.76 nmole SH per well (which corresponds to 88 pmole MT). Based on its molecular weight, the detection limit of MT is thus 0.62 µg/well *i.e.* 1.77 µg/ml. Considering the characteristics of the gel filtration column, the minimal quantity of MT detected by this method is in the range of 100 µg/g wet weight. Lower detection limits of MT are obtainable by methods designed to measure the proteins directly in the supernatants. The limit for measurement of MT by differential pulse polarography analysis is about 10 µg MT/g wet weight (Hogstrand and Haux, 1992). Perch MT can be detected at 9 ng/g by radio-immunology (Hogstrand and Haux, 1990) and the metal saturation assay technique is sensitive enough to measure MT concentrations accurately above 40 µg/g wet weight (Onosaka and Cherian, 1982). The colorimetric method, however, has the advantage of requiring neither specific equipment nor the utilization of antibodies or the manipulation of radioactive substances. Consequently, although not suitable for routine analysis of MT in biological samples, it is useful for the detection of sulfhydryl-rich containing proteins such as MT, previously separated by chromatography, as the amounts of MT in feral fish are usually higher than 100 µg/g wet weight.

CONCLUSION

This study indicates that the major low molecular weight metal-binding proteins in liver of the two species of teleosts *Limanda limanda* and *Microstomus kitt*, injected intaperitoneally with Cd, belong to the family of metallothionein-like proteins. These proteins might be a suitable candidate for monitoring the effect of environmental pollution on commercially important food stocks. Before such monitoring can occur, however, it will be necessary to demonstrate the link between environmental metal concentrations and levels of MT-like proteins in the fish, and to select a suitable method for routine measurements. The results of such studies will be reported shortly.

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REFERENCES

Bayne B.L., R.F. Addison, J.M. Capuzzo, K.R. Clarke, J.S. Gray, M.N. Moore and R.M. Warwick (1988). An overview of the GEEP Workshop. *Mar. Ecol. Progr. Ser.* **46**, 235-243.

Brown D.A., S.M. Bay and G.P. Hershelman (1990). Exposure of scorpionfish (*Scorpaena guttata*) to cadmium: effects of acute and chronic exposure on the cytosolic distribution of cadmium, copper and zinc. *Aquat. Toxicol.* **16**, 295-310.

Ellman G.L. (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70-77.

Hogstrand C. and C. Haux (1990). A radioimmunoassay for perch (*Perca fluviatilis*) metallothionein. *Toxicol. Appl. Pharmacol.* **103**, 56-65.

Hogstrand C. and C. Haux (1992). Evaluation of differential pulse polarography for the quantification of metallothionein- a comparison with radioimmunoassay. *Anal. Biochem.* **200**, 388-392.

Kägi J.H.R. and M. Nordberg (1979). Metallothionein. Proceedings of the first international meeting on metallothionein and other low molecular weight metal binding proteins. *Experientia supplementum*, **34**, Zurich, July, 1978, 17-22.

Kägi J.H.R. and A. Schäffer (1988). Biochemistry of metallothionein. *Biochem.* **27**, 8509-8515.

Kito H., T. Tazawa, Y. Ose, T. Sato and T. Ishikawa (1982). Protection by metallothionein against cadmium toxicity. *Comp. Biochem. Physiol.* **73C**, 1, 135-139.

Kito H., Y. Ose and T. Sato, (1986). Cadmium-binding protein (metallothionein) in carp. *Envir. Health Perspect.* **65**, 117-124.

Klaverkamp J.F., W.A. McDonald, D.A. Duncan and R. Wagemann (1984). Metallothionein and acclimation to heavy metals in fish: a review. In *Contaminant Effects on Fisheries* (Cairns V.W., Hodson P.V. and Nriagu J.O. Eds), Wiley, New York, 99-113.

McCarter J.A. and M. Roch (1984). Chronic exposure of coho salmon to sublethal concentrations of copper. III Kinetics of metabolism of metallothionein. *Comp. Biochem. Physiol.* **77C**, 1, 83-87.

Noël-Lambot F., C. Gerday and A. Distèche (1978). Distribution of Cd, Zn and Cu in liver and gills of the eel *Anguilla anguilla* with special reference to metallothioneins. *Comp. Biochem. Physiol.* **61C**, 177-187.

Olsson P.E. and C. Haux (1985). Rainbow trout metallothionein. *Inorg. Chim. Acta* **107**, 65-71.

Onosaka S. and M.G. Cherian (1982). Comparison of metallothionein determination by polarographic and cadmium-saturation methods. *Toxicol. Appl. Pharmacol.* **63**, 270-274.

Roch M., J.A. McCarter, A.T. Matheson, M.J.R. Clark and R.W. Olafson (1982). Hepatic metallothionein in rainbow trout (*Salmo gairdneri*) as an indicator of metal pollution in the Campbell river system. *Can. J. Fish. Aquat. Sci.* **39**, 1596-1601.

Thomas D.G., J.F. de Solbe, J. Kay and A. Cryer (1983). Environmental cadmium is not sequestered by metallothionein in rainbow trout. *Biochem. Biophys. Res Comm.* **110**, 2, 584-592.

Tukendorf A. and T. Baszynski (1985). Partial purification and characterization of copper-binding protein from roots of Avena sativa grown on excess of copper. *J. Plant Physiol.* **120**, 57-63.

Viarengo A., M. Pertica, G. Mancinelli, G. Zanicchi and M. Orunesu (1980). Rapid induction of copper-binding proteins in the gills of metal exposed mussels. *Comp. Biochem. Physiol.* **67C**, 215-218.

Webb M. and K. Cain (1982). Functions of metallothionein. *Biochem.* **31**, 137-142.

Wofford H.W. and P.Thomas (1984). Interactions of cadmium with sulfhydryl-containing compounds in striped mullet (*Mugil cephalus* L.). *Mar. Environ. Res.* **14**, 119-137.