Metal-binding proteins in bream (*Abramis brama* L.) caught in the River Elbe

Ulrike Kammann, Jürgen Grymlas, Wolfgang Hein and Hans Steinhart

Metal-binding proteins were investigated in livers of bream caught in the River Elbe from Steti (Czech Republic) to Hamburg (Federal Republic of Germany). A major zinc and copper binding protein fraction with a low molecular weight of 10 000 to 12 000 Da and with properties similar to mammalian hepatic metallothionein was isolated from bream livers using gel filtration chromatography. Two protein isoforms could be separated by reversed phase-high performance liquid chromatography (RP-HPLC), however, mercury was associated with only one isoform. The possibility of different detoxification potentials of the isoforms is discussed. Maximal concentrations of metal-binding protein were detected in samples from Dresden. If metal-binding proteins are to be included in a biological monitoring study, further investigations are required.

Keywords: *metal-binding protein, bream, Elbe, mercury, pollution-effects.*

Introduction

Metallothioneins (MT) are low-weight, heat stable proteins characterized by high cysteine and heavy metal contents. MT appears to be ubiquitous in vertebrate tissues and is readily induced by a variety of agents including the metals mercury and cadmium to which it binds (Kägi and Kojima 1987, Stillman *et al.*, 1992). MT and metal binding proteins (MBP) have been isolated from tissues of several fish species (George and Langston 1994, Roesijadi 1992, Kammann *et al.* 1996). The major role of MT under normal metabolism is presumed to be the homeostatic regulation of intracellular zinc- and copper-availability (Olsson *et al.* 1987). By binding excess metal, MT appears to act as a detoxifying agent. Thus analysis of fish liver MT might provide a measure of sub-acute metal exposure (Roesijadi 1992, Cosson, 1994).

MTs have been proposed as biomarkers for marine ecosystems in international frameworks (ICES 1995) and some field investigations indicate the usefulness of their application (Galgani *et al.* 1992, Hylland *et al.* 1992, Schlenk *et al.* 1995). For most aquatic species studied, at least two chromatographically distinct isoforms have been reported (Weber *et al.* 1992, Kammann *et al.* 1996). However, there are some fish species with only one MT isoform. It has been suggested that different MT isoforms may have different biological functions or detoxification potentials (Vallee 1991, Chu *et al.* 1994). However, quantification of the isoforms is not included in some commonly used methods for determination of MT. An MT determination which is specific for isoforms may provide valuable information for interpretation of monitoring data.

The River Elbe is one of the most polluted rivers in Europe. According to the political changes in the eastern part of Europe a reduction of the pollution load and a commencement of the process of clean-up of the river ecosystem is expected (Müller 1996) and may be monitored using biomarkers (Jedamski-Grymlas *et al.* 1995). The present study was undertaken to contribute to the discussion in what way MT-like MBP could be used as biomarkers of exposure in the River Elbe.

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METHODS

Chemicals

All chemicals were of analytical grade and obtained from Sigma Chemicals Co. (Deisenhofen, Germany) unless otherwise noted. Sephadex G-75 was purchased from Pharmacia (Uppsala, Sweden) and acetonitril, far UV-quality, from Fisons (Leicestershire, Great Britain). HNO_3, suprapur was purchased from Merck (Darmstadt, Germany).

Collecting the fish

Male bream were caught in October 1992 with nets (40 mm mesh width) positioned for approximately 1 h at 7 stations in the River Elbe. The locations of the sampling stations extended from the city of Steti (Czech Republic) at the upper stream to the downstream city of Hamburg (Federal Republic of Germany) covering a distance of 800 km (Figure 1). Immediately after the catch bream were killed by a blow to the head. The fresh weight and standard length were registered. Livers were removed, rinsed with ice-cold 0.15 M KCl, divided into subsamples and shock frozen in cryo tubes with liquid nitrogen. All bream were adults with mean body lengths between 24 and 45 cm.

Isolation of MBPs

A subsample of 1 g liver tissue was homogenized in 2 ml 10 mM Tris-HCl buffer (pH 7.5 containing 5 mM 2-mercaptoetanol and phenylmethansulfonylflourid) using a glass-teflon homogenizer. The homogenates were centrifuged (1 h, 105 000 × g, 4°C) followed by a heat treatment in a water bath (10 min, 59°C) and subsequent centrifugation (30 min, 3500 g, 4°C). In the resulting cytosol, zinc, copper and cadmium contents were determined by atomic absorption spectrophotometry (AAS) after acid hydrolysis. The protein content of cytosol was determined by the method of Bradford (1976). A subsample of the cytosol was subjected to gel filtration chromatography (GFC) employing a column (25 × 280 mm) filled with Sephadex G-75 and connected to a peristaltic pump, and using the homogenization buffer as mobile phase. The column was eluted at a flow rate of 30 ml/h. Fractions (3.5 ml) were collected by a fraction collector attached to a cryostat (4°C) and monitored for the UV-absorption by 220, 254 and 280 nm at an UV-photometer. The thiol contents were determined in the GFC fractions according to Ellman (1959) with 2,2′-dithio-5,5′-dinitrodibenzoic acid using rabbit liver MT I as standard substance. The MBP containing fractions were concentrated

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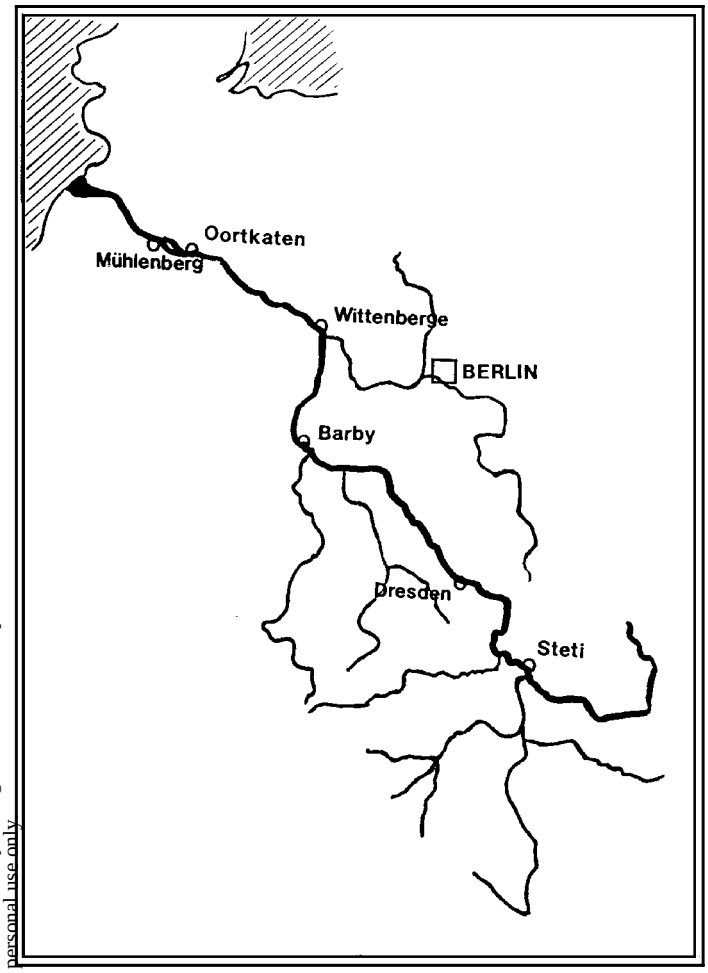

Figure 1. Sampling locations at the River Elbe in the Federal Republic of Germany and in the Czech Republic.

via ultrafiltration (Macrosep D-3000, Filtron, FRG), filtered through a 0.2 μm membrane and stored in liquid nitrogen until HPLC-analysis if not used immediately. All steps of sample preparation were performed at 4°C.

Spiked samples and standard solutions of rabbit liver MT I were used to test the recovery of single steps of MBP isolation procedure. Loss of MT during the ultrafiltration step was 26%, probably due to absorbtion of the protein to the membrane, however, in samples cytosolic protein seems to have a protective effect. During GFC or HPLC treatment only 5% or less were lost. A total recovery of 70% was determined. HPLC recovery of rabbit liver MT I standard substance was calculated over metal content in the eluate. The recovery ranged from 90 to 95%.

Molecular weight (MW) estimation

The MW of the metal-binding protein was estimated using GFC. Samples were applied to the GFC-system described above. The following protein standards were used for column calibration: blue dextran (MW 2 000 000 Da), egg albumin (MW 45 000 Da), carbonic anhydrase (MW 29 000 Da), α-lactalbumin (MW 14 200 Da), cytochrome C (MW 12 400 Da), and rabbit liver MT (apparent MW 10 000 Da).

HPLC-analysis

The HPLC-analysis was carried out according to the method described by Richards (1989) with modified buffer conditions resulting in an improved resolution of the MT or MBP isoforms in the chromatogram. The Merck-Hitachi HPLC-system consisted of a gradient pump, a UV/VIS-detector adjusted at 220 nm, a Rheodyne injection system equipped with a 100-μl-sample loop and a Nucleosil RP-8 column (4 × 250 mm, 300 μm mesh) maintained at 25°C by a column thermostate. For gradient elution a 1 mM Tris-HCl buffer (pH 7.7 [buffer A]) and a mixture of buffer A with acetonitrile (40:60, v:v [buffer B]) were used. The linear gradient of 0 to 30% buffer B was run within 20 min at a flow rate of 1 ml/min. The MBP content was determined with external standard solutions of rabbit liver MT. The linearity of the HPLC-method ranged from 1.6 to 25 μg MT injected. The correlation coefficient was greater than 0.999.

Metal determinations

Copper and zinc were analysed by flame-AAS after acid hydrolysis of the cytosol with HNO_3. Mercury concentrations were determined according to Hatch and Ott (1968) by cold vapour-AAS in fresh tissues and in HPLC eluates (detection limit 0.04 μg/l). Metal contents in GFC and HPLC fractions were determined directly.

Results

A protein containing mainly zinc and copper (MW range of 10 000–12 000 Da) was detected in the heat treated cytosol eluting from the Sephadex G-75 column (Figure 2). The metal-binding protein contained high amounts of thiol, indicating a high cysteine content of 25–30 residues per molecule (calculated from the protein and thiol contents of the 10 000–12 000 Da fraction) which is typical for MT. The properties of the protein (molecular mass, heat stability, metal binding and high thiol-content) lead to the conclusion that the MBP isolated from bream liver tissues was of the MT type. Two isoforms of MBP could be separated by RP-HPLC (Figure 3a). In both MBP isoforms the metals copper and zinc could be detected, however, mercury was only associated with one isoform (Fig. 3b).

Zinc, copper and mercury were quantified in male fish from every station. Mean MBP concentrations in heat treated bream liver cytosols ranged from <2 mg/g protein (limit of determination for the sum of both MBP isoforms) to 10.3 mg/g protein in samples from Dresden (Table 1). Although the data base is too small to draw statistically significant conclusions, we can provide an indication of the presence of differences in MBP isoform relations in field samples (compare Table 1).

Discussion

Mercury is a potent inducer for MT in fish liver. Application of mercury led to higher increases of MT levels in carp than observed by treatment with cadmium or zinc (Cosson 1994). The presence of mercury in only one isoform of the MBP of bream (Figure 3b) could have been caused by a mercury induced synthesis of the isoform or by a higher affinity for the metal, respectively. A combination of both causes is possible as well.

Chu *et al.* (1994) detected differences in the binding capacity of mercury between the two major MT isoforms of rabbit liver: The ability of apo-MT1 to bind to Hg^{2+} ion is stronger than the ability of apo-MT2. Apo-MT1 was able to bind more Hg^{2+} ions and exhibited a more stable conformation than the apo-MT2. Jiang *et al.* (1994) reported different structural parameters for

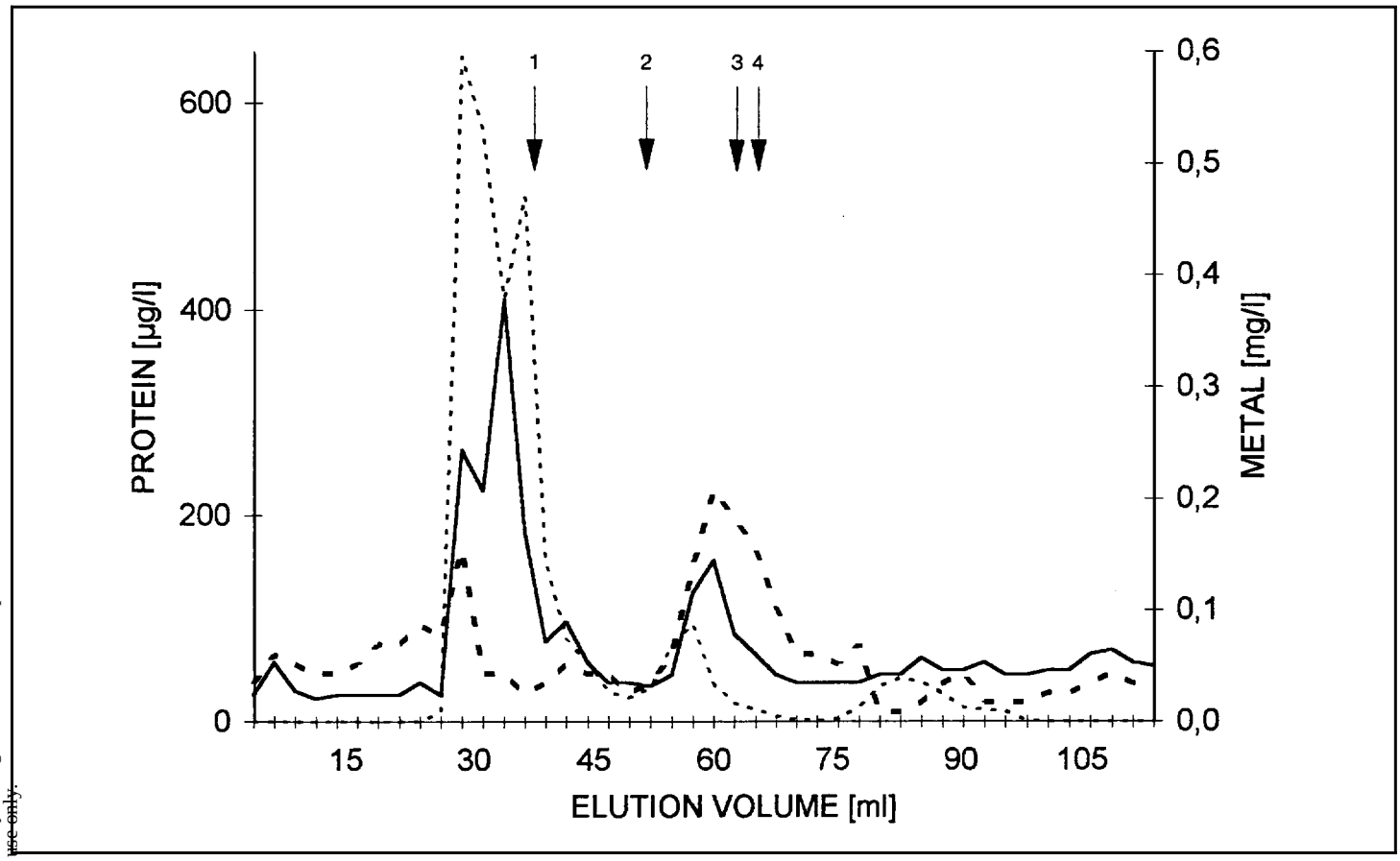

Figure 2. GFC elution profile of heat treated bream liver cytosol. Protein: light dashed line; zinc: solid line; copper: dark dashed line. Arrows indicate elution volumes of standard proteins.1: egg albumin. MW 45 000 Da; 2: carbonic anhydrase, MW 29 000 Da; 3: α-lactalbumin, MW 14 200; 4: cytochrome C, MW 12 400 Da coeluting with hepatic rabbit MT, apparent MW 10 000 Da.

mercury-containing MT than for MT containing other metals. The authors proposed a new structure of Hg_7-MT (formed from Zn_7-MT at pH 7) in which each Hg^{2+} is coordinated by four thiolates but with two unusually short bonds and two unusually long bonds.

In accordance with Chu *et al.* (1994) our results underline the possibility for differences in interaction of MBP isoforms and mercury in bream caught in the River Elbe. If single MBP isoforms are inducible by potentially toxic metals or have a stronger affinity for these metals the isoforms may be involved in different detoxification processes. Chu *et al.* (1994) predicted different detoxification potentials for the two MT isoforms of rabbit liver. Therefore the knowledge of metal affinity of the MBP isoforms may be important for the interpretation of data from monitoring studies if isoform specific data are available.

Elevated levels of cytosolic zinc and copper concentration, as presented in Table 1 (sampling site Dresden), are typical for

		Cu [mg/g prot.]		Zn [mg/g prot.]		Hg [µg/g FW]		MBP [mg/g prot.]			
Station	n	mean	s.d.	mean	s.d.	mean	s.d.	M_a	M_b	sum	Hg, Igeo class sediment
Steti	4	0.69	0.32	0.94	0.48	0.79	0.30	3.0	1.9	4.9	4
Dresden	5	1.32	0.75	1.94	1.16	5.41	3.68	6.5	3.8	10.3	4
Barby	5	0.87	0.48	0.73	0.28	0.60	0.24	3.5	2.2	5.7	5
Wittenberge	5	0.93	0.97	0.50	0.50	–	–	1.7	2.3	4.0	5
Oortkaten	9	0.56	0.30	0.62	0.28	1.40		<1.0	<1.0	<2.0	4
Mühlenberg	5	0.73	0.40	0.58	0.17	–	–	1.1	2.1	3.2	4

Table 1. Mean values and standard deviations (s.d.) of metal and metal-binding protein (MBP) concentrations in hepatic cytosols of male bream (*Abramis brama*) from the River Elbe.

Data are expressed on the base of cytosolic protein (prot.) or fresh water weight (FW). n: number of fish analysed individually; –: not determined; M_a, M_b: mpb isoforms compare Figure 3a. Hg Igeo classification for sediments (<20 µm sediment fraction) according to Müller and Furrer (1994): Igeo class 0 (non polluted): <0.6 mg Hg/kg; Igeo class 4 (high polluted): 4.8–9.6 mg Hg/kg; Igeo class 5 (high to excessive polluted): 9.6–19.2 mg Hg/kg.

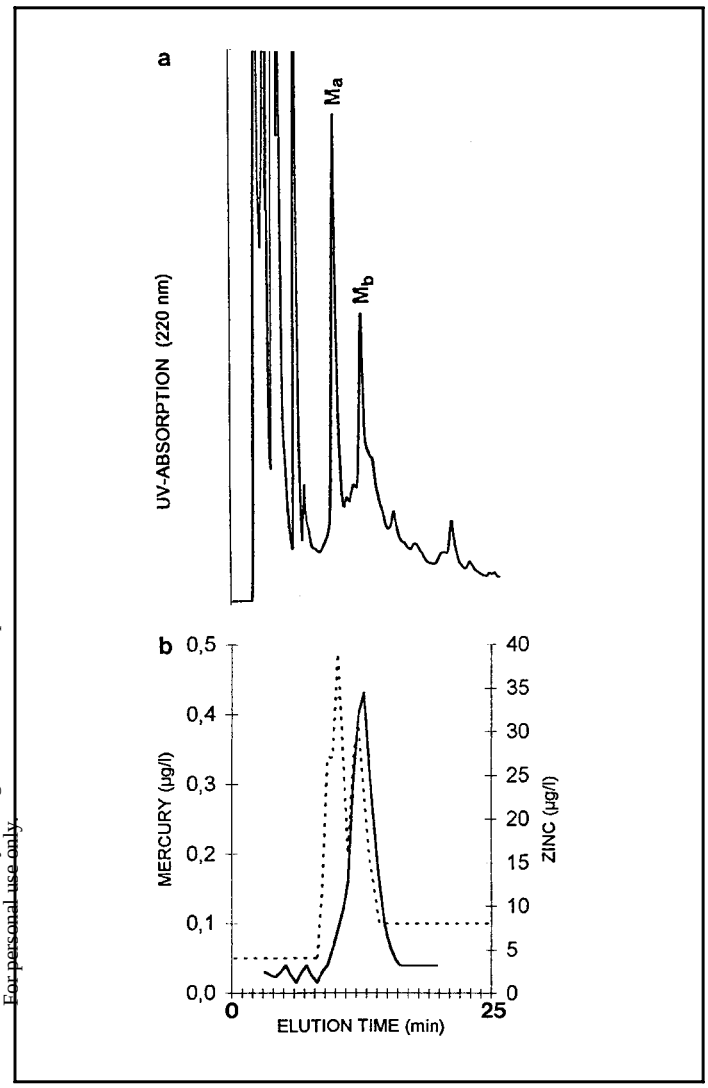

Figure 3. RP-HPLC elution profiles. a: UV-absorption (220 nm), labelled peaks are MBP isoforms; b: zinc (dashed line) and mercury concentrations (solid line) determined in the RP-HPLC eluate.

MT induction (George and Langston 1994). Mercury concentration in liver tissue may have promoted the MBP synthesis in bream caught at Dresden; however, other causes for elevated MBP levels cannot be excluded. The mercury content of bream from the River Elbe is probably due to discharges of chemical and pharmaceutical industries which have been partly closed during the last years. Consequently mercury discharges have decreased over the last years (Müller 1996). In October 1992, when the fish for the present study were caught, mercury concentration in sediment of the River Elbe was analysed by Müller and Furrer (1994). The authors classified the sediment contamination at the sampling site as 'high polluted' or 'high to excessive polluted' compared to background values (Table 1).

For integrating MBPs in a bioeffect monitoring concept, information is incomplete. The physiological function and factors influencing the basal MBP status of the fish are not sufficiently known. Further studies are necessary to clarify the biological significance of the isoforms. If there are different metal binding abilities of the MBP isoforms, as discussed above, they should be assayed separately. Therefore it will be necessary to separate the isoforms by suitable chromatographic techniques.

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