Purification and Properties of Plaice Metallothionein, a Cadmium-Binding Protein from the Liver of the Plaice (*Pleuronectes platessa*)

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A low-molecular-weight protein induced in the liver of the plaice (*Pleuronectes platessa*) by exposure to cadmium was purified and characterized. It is closely similar to mammalian metallothioneins in all of its properties in that it is a single-chain cadmium-binding protein of approx. 7000 mol.wt. with a high cysteine content (31 mol %) and no aromatic amino acid residues. The thiol groups of the cysteine residues complex with the cadmium in a SH/Cd molar ratio of 3:1 and produce a characteristic absorption maximum at 250 nm. Unlike the mammalian metallothioneins, however, metal analyses reveal only traces of zinc and copper in addition to cadmium. The presence of carbohydrate previously assumed from a positive reaction with periodic acid/Schiff reagent has now been disproved, and the positive reaction attributed to interaction with the thiol groups in the protein.

Studies on the metabolism of cadmium in mammals have shown that this heavy metal is detoxified in liver and kidney by being complexed to thionein, a protein of low mol.wt. (6000) containing up to 30 % cysteine and no aromatic residues. The cadmium is bound to the thiol groups of the cysteines in an SH/Cd molar ratio of 3:1 (Kägi *et al.*, 1974; Kojima *et al.*, 1976). Such metallothioneins have now been characterized from human, rabbit, chicken, rat, horse, mouse and a sea-bird (Pulido *et al.*, 1966; Nordberg *et al.*, 1972; Weser *et al.*, 1973; Kägi *et al.*, 1974; Nordberg *et al.*, 1975; Kojima *et al.*, 1976; Osborn, 1978). A restricted study on the uptake of cadmium by a teleost fish, the plaice, suggested that fish may also detoxify cadmium by a similar mechanism (Coombs, 1975), and in a preliminary communication the partial purification of a metallothionein from plaice liver was described (Overnell *et al.*, 1977). Since that time, the presence of a metallothionein-like protein has been demonstrated in the liver and kidney or their equivalent organs in a number of other aquatic organisms, including the rock-fish (Olafson & Thompson, 1974), goldfish (Marafante, 1976), carp (Yamamoto *et al.*, 1978), the swimming crab (Olafson *et al.*, 1979), the edible crab (J. Overnell & E. Trehwella, unpublished work), the common mussel (Noël-Lambot *et al.*, 1978; S. G. George, unpublished work) and the limpet (Howard & Nickless, 1977). The further purification and characterization of plaice metallothionein are now reported and its properties are compared with those of mammalian metallothioneins.

Experimental

Induction of metallothionein

Mature plaice (200–400 g), caught locally off the Aberdeenshire coast, were maintained unfed in a seawater aquarium at 5–15°C. The fish were injected intraperitoneally with increasing concentrations of CdCl₂ in 1.1 % (w/v) NaCl (0.2 mg of Cd/kg of fish on day 1, 0.6 mg/kg on day 8 and 2.0 mg/kg on day 15). On day 22 the fish were killed and the livers were dissected out and stored deep-frozen.

Isolation of metallothionein

The initial isolation methods are based on those of Kägi *et al.* (1974). A 20 g wet wt. portion of combined livers was homogenized in 40 ml of 1.1 % (w/v) NaCl for 30 s in a Polytron rotating-blade homogenizer (Kinematica G.m.b.H., Luzern, Switzerland). The clear supernatant obtained after centrifugation of the homogenate at 100 000 g for 1 h at 4°C in an MSE 2406 rotor (r_{av} = 7.62 cm) contained 70 % of the original cadmium, and it was applied to a column (5 cm × 80 cm) of Sephadex G-75 (Pharmacia, Uppsala, Sweden) and the column was eluted with 20 mM-Tris/HCl buffer, pH 8.6, at a flow rate of 75 ml/h. Fractions (20 ml) were collected and monitored for Cd and A_{250} . The low-molecular-weight cadmium-containing fractions with high A_{250} and where the cadmium concentrations were greater than one-half of the maximum (fractions 58–63) were combined and further purified by ion-exchange chromatography on DEAE-32 cellulose (Whatman, Maidstone, Kent) pre-equilibrated with 20 mM-Tris/HCl buffer, pH 8.6,

and the column (2.5 cm × 26 cm) was eluted with a linear gradient from 20 mM-Tris/5 mM-HCl buffer, pH 8.6, to 200 mM-Tris/50 mM-HCl buffer, pH 8.6. Fractions (20 ml) were again collected and monitored for Cd and A_{250} . Fractions 102–106, containing the cadmium, were pooled and concentrated by Diaflo ultrafiltration with a UM-2 membrane (Aminco, Lexington, MA, U.S.A.). The concentrated Cd-protein was finally added to a column (1.5 cm × 80 cm) of Bio-Gel P-30 (Bio-Rad Laboratories, Watford, Herts, U.K.) and eluted with 50 mM-Tris/12.5 mM-HCl buffer, pH 8.6; 5 ml fractions were collected and A_{250} and Cd in each fraction were measured as before. Blue Dextran, myoglobin, cytochrome *c* and KCl were used as markers for calibrating the column.

Determination of metals

This was performed on a Varian Techtron AA5 atomic absorption spectrophotometer (Varian Associates, Walton-on-Thames, Surrey, U.K.) calibrated with standard solutions (BDH Chemicals, Poole, Dorset, U.K.) after appropriate dilution with 1 M-HCl (BDH AristaR ultra-pure acid). Eluent fractions were aspirated directly into the air/acetylene flame. Tissue samples were either wet-ashed by addition of 70 % (w/v) HNO₃ (AristaR ultra-pure acid) in sealed Teflon screw-topped bombs (Techni Verre, Paris, France) at 100°C for 1 h or dry-ashed in platinum dishes at 500°C in a silica-lined muffle furnace and the ash was dissolved in 1 M-HCl before aspiration.

Determination of protein

Protein was measured by the method of Miller (1959), with horse heart cytochrome *c* (BDH Chemicals) as a standard. The concentration of the cytochrome *c* was calculated from its A_{410} (an isobestic point) (Margoliash *et al.*, 1959). The protein concentration of metallothionein was also determined for one sample by quantitative amino acid analysis of the performic acid-oxidized material for calibration.

Determination of thiol

Thiol groups were measured spectrophotometrically with *p*-hydroxymercuribenzoate (Sigma Chemical Co., Poole, Dorset, U.K.) by following the procedure of Coombs *et al.* (1964) or amperometrically with Ag⁺ (Benesch *et al.*, 1955).

Amino acid and hexosamine analyses

These were performed on an automated amino acid analyser (model JLC-6AH; JEOL, London, U.K.). The unmodified protein was hydrolysed with 3 M-toluene-*p*-sulphonic acid + 0.2 % (w/v) tryptamine at 100°C for 24 h and for 72 h according to the method of Liu (1972). No losses were detected between the 24 h and 72 h hydrolyses. This procedure also allows the determination of tryptophan and

hexosamines to be made on the same samples. Cysteine was determined as cysteic acid after prior oxidation of the protein with performic acid followed by hydrolysis in 6 M-HCl in sealed tubes at 100°C for 24 h. The final amino acid analyses were calculated from the combined results of the two methods.

Polyacrylamide-gel electrophoresis

This was performed by using the system of Davis (1964) with 5 %, 10 % and 15 % (w/v of monomer) gels prepared from acrylamide (95 %) and *NN'*-methylenebisacrylamide (5 %) specially purified for electrophoresis (BDH Chemicals). Bromophenol Blue was used as a marker for migration distance. After electrophoresis the gels were fixed in 12.5 % (w/v) trichloroacetic acid for 30 min and then stained for protein by steeping in 0.04 % (w/v) Coomassie Blue G250 (Serva, Heidelberg, Germany) in 3.5 % (w/v) perchloric acid. The final protein colour was developed by destaining the gel with 5 % (v/v) acetic acid (Holbrook & Leaver, 1976). Duplicate gels were also stained for carbohydrate by the periodic acid/Schiff reagent method of Zacharius *et al.* (1969).

Analytical isoelectric focusing

This was carried out in 10 % (w/v) acrylamide gels containing 3 % (w/v) Ampholine, pH range 3.5–10 (LKB Produkter, Stockholm, Sweden). The Ampholine gels were cast as for the electrophoresis gels except that the Ampholine was substituted for the electrophoresis buffer. The electrophoresis was carried out with 10 mM-NaOH in the cathode compartment at the top and 10 mM-H₂SO₄ in the anode compartment. Samples dissolved in sucrose were applied to the top of the gel.

After focusing the gels were stained for protein with Coomassie Blue and for carbohydrate with periodic acid/Schiff reagent. Before periodic acid/Schiff reagent staining the gels were first washed four times with stirring in 12.5 % (w/v) trichloroacetic acid. For protein the gels were washed four times with stirring with a mixture of 150 ml of methanol and 350 ml of water in which was dissolved 17.25 g of sulphosalicylic acid and 57.5 g of trichloroacetic acid. The gels were then rinsed for 5 min in destaining solution (250 ml of ethanol + 80 ml of acetic acid diluted to 1 litre) before being stained in fresh destaining solution containing 0.1 % (w/v) Coomassie Blue R250 for 15 min at 60°C and then washed in destaining solution until the background was clear.

For determination of the pH gradient a gel was cut into 5 mm sections and the sections were incubated overnight at room temperature with 0.5 ml of water. The pH of each incubation was then measured with a pH-meter fitted with a micro calomel/glass combined electrode. All pH measurements were made at 20 ± 1°C.

For cadmium determinations in the gel slices 50 µl of 6 M-HCl was added to each of the pH-

gradient incubations and the mixture incubated for a further overnight period at room temperature. After incubation the decanted clear gel-free solutions were aspirated directly into the atomic-absorption-spectrophotometer flame.

Materials

Toluene-*p*-sulphonic acid and tryptamine were purified as described by Liu (1972). All other chemicals unless specified were of reagent-grade quality.

Results and Discussion

Isolation and purification

Metallothionein was isolated from the livers of plaice exposed to cadmium as described in the Experimental section. The elution patterns after Sephadex G-75 and DEAE-cellulose ion-exchange chromatography are shown in Figs. 1 and 2. In Fig. 1 the major portion of the cadmium is eluted in the position of low-molecular-weight components and coincides with a small peak of A_{250} , and a minor portion of the cadmium is found in the void volume and is associated with high-molecular-weight components excluded by the Sephadex. There is no cadmium found in the position corresponding to the free hydrated metal ions, where a third large peak of A_{250} occurs.

After ion-exchange chromatography of the pooled low-molecular-weight cadmium-containing fractions,

there is a single peak of cadmium and A_{250} at an ionic strength equivalent to 200 mM-Tris/50 mM-HCl (Fig. 2). This is in contrast with mammalian metallothioneins, where two or more peaks are normally found (Nordberg *et al.*, 1972; Kägi *et al.*, 1974). The exact elution position of the plaice metallothionein was found to vary slightly on repeat experiments and in some instances was found just within the buffer gradient. Further elution of the DEAE-cellulose column with 1 M-NaCl gave no further release of cadmium in any significant amount.

Thiol titrations and amino acid analyses carried out at this stage indicated that further purification was required. This was achieved by rechromatography of the pooled cadmium-containing fractions over Bio-Gel P-30, which produced the elution pattern shown in Fig. 3. The main band of cadmium and A_{250} appeared in fractions 14–16 and was preceded by a small minor peak, which was discarded. This main band was harvested and deep-frozen for subsequent characterization. A 100 g portion of liver (Cd concentration 150 μ g/g wet wt.) yielded 14 mg of metallothionein.

Freezing and thawing

In view of the need of maintaining stocks of purified material in a stable condition for subsequent experiments, the effect of freezing and thawing was investigated. In contrast with horse metallothionein (Kägi *et al.*, 1960), plaice metallothionein appears to be

Fig. 1. Sephadex G-75 chromatography of the 100000g supernatant from the liver homogenate of cadmium-injected plaice

A 20 g portion of liver was homogenized in 40 ml of 1.1 % (w/v) NaCl and the clear supernatant applied to a 5 cm diam. $\times$ 80 cm column of Sephadex G-75. Fractions of volume 20 ml were collected, the column being eluted with 20 mM-Tris/5 mM-HCl buffer, pH 8.6. ----, A_{250} ; —, cadmium. Approx. 40 % of the original amount of cadmium is found in the low-molecular-weight fractions (fractions 58–63).

Fig. 2. Ion-exchange chromatography of the major cadmium-containing peak from the Sephadex chromatography

Fractions 58–63 from the Sephadex chromatography were combined and applied to a 2.5 cm diam. $\times$ 26 cm column of DEAE-cellulose pre-equilibrated with 20 mM-Tris/5 mM-HCl buffer, pH 8.6. The column was eluted with a linear buffer gradient from 20 mM-Tris/5 mM-HCl, pH 8.6, to 200 mM-Tris/50 mM-HCl, pH 8.6. Fractions of volume 20 ml were collected. The fractions between the arrows were combined. Approx. 45 % of the total cadmium added to the column is recovered in these fractions. ----, A_{250} ; —, cadmium; ·····, concn. of Tris.

Fig. 3. Bio-Gel exclusion chromatography of metallothionein after ion-exchange chromatography

Fractions 102–106 from the ion-exchange chromatography were concentrated by ultrafiltration (UM-2 membrane) and the concentrate was applied to a 1.5 cm diam. $\times$ 80 cm column of Bio-Gel P-30. The column was eluted with 50 mM-Tris/12.5 mM-HCl buffer, pH 8.6. Fractions of volume 5 ml were collected. Blue Dextran (BD) and K^+ in arbitrary units mark the positions on the chromatogram of the void volume and the appearance of free metal ions. The recovery of cadmium in the major peak was 75 % of the total amount applied to the column. ---, A_{250} ; —, cadmium.

completely stable to several cycles of freezing and thawing.

Homogeneity and isoelectric-point determination

The homogeneity of the final product was ascertained from polyacrylamide-gel electrophoresis. The densitometer scans of the resultant gels stained for protein, 'carbohydrate' and the distribution of cadmium along the gel length are reproduced in Fig. 4. There are single bands for protein, 'carbohydrate' and cadmium, which occur in identical migration positions. Furthermore a single protein band was obtained after electrophoresis on 5 %, 10 % and 15 % gels, suggesting the probable homogeneity of the metallothionein.

Isoelectric focusing on polyacrylamide gels of the purified metallothionein gave two bands, somewhat diffuse, after protein and periodic acid/Schiff reagent staining, at isoelectric points of 4.5 and 4.6 pH units. After focusing, one gel-length was cut into 5 mm sections and each section analysed for cadmium as described above. These analyses showed there were two bands, one focusing at pH 4.5–4.6 and the other at pH 5.3. The latter band corresponds to free Cd^{2+} ions, which focus at the same pH when electrophoresed under identical conditions. This indicates that there is some release of cadmium from metallothionein during the electrofocusing.

Fig. 4. Gel electrophoresis of metallothionein after Bio-Gel chromatography

Samples of the major cadmium-containing peak from the Bio-Gel chromatography were subjected to electrophoresis in 10 % polyacrylamide gels (5 % cross-linked) with the buffer system of Davis (1964). After electrophoresis the protein was fixed in the gel with 12.5 % (w/v) trichloroacetic acid. (a) Gel stained with Coomassie Blue G250 in 3 % (w/v) $HClO_4$ (reproduced from a densitometer scan). (b) Gel stained with periodic acid/Schiff reagent (reproduced from a densitometer scan). (c) Normalized cadmium concentrations in HCl extracts from 2 mm sections of the gel. R_{BPB} , migration distances relative to Bromophenol Blue.

Determination of free thiol groups and metal composition

Titration of plaice metallothionein with *p*-hydroxy-mercuribenzoate and with silver gave SH/Cd molar ratios of 2.84 ± 0.04 (S.E.M.) (8) and 3.32 ± 0.02 (S.E.M.) (10) respectively. These are similar to the average values of 3.1 and 3.2 found for horse liver and kidney metallothioneins reported by Kägi *et al.* (1974).

Metal analysis of the purified plaice metallothionein gave the following results: Cd, 10.6 %; Zn, 0.04 %; Cu, 0.08 %. Plaice metallothionein contains therefore only traces of zinc and copper, unlike the horse metallothioneins, where zinc and copper are present in significant amounts, and in the

case of the liver metallothionein zinc is the major metal species bound to the thionein (Kägi *et al.*, 1974). The absence of zinc and copper from the plaice metallothionein may be a consequence of the experimental induction of this protein with relatively large doses of cadmium. In support of this hypothesis, livers from untreated control fish with very low cadmium concentrations ($<0.2 \mu\text{g/g}$ wet wt.) were homogenized and the homogenate was treated with excess CdCl_2 to displace any zinc thionein that may have been present. These yielded only a very small amount of metallothionein after Sephadex G-75 chromatography. A similar procedure with the use of radioactive ^{109}Cd is the basis of an assay for metallothionein (Chen & Ganther, 1975). Furthermore, in parallel experiments with cadmium-treated and control fish, which also had been injected intraperitoneally with $[^{35}\text{S}]$ cysteine, a peak of radioactivity coinciding with the position of metallothionein was found only from the cadmium-treated liver cytosols after Sephadex G-75 chromatography. It is concluded that there is only a small pre-existing pool of thionein and that the bulk of metallothionein isolated from cadmium-treated fish results from an increased biosynthesis in response to the increased cadmium load, as is the case for mammals (e.g. see Shaikh & Smith, 1977).

Acidification to pH 2.3 of a solution of the purified protein abolished the characteristic absorption shoulder at 250nm, which arises specifically from a thiol-Cd interaction (Kägi & Vallee, 1961). Re-adjustment of the pH back to 8.6 caused the re-appearance of the 250nm absorbance shoulder. This behaviour is consistent with the hypothesis that the metals specifically complex reversibly with the free thiol groups of the cysteine residues in thionein.

Apparent molecular weight

Estimations of the apparent molecular weight, based on the distribution coefficients obtained after chromatography on Bio-Gel P-30 and P-60 of metallothionein, myoglobin and cytochrome *c* at approximately equal concentrations (by wt.) were made. Three separate preparations of plaice metallothionein gave values of 14000, 13000 and 15000, somewhat higher than those found for other metallothioneins, where a range of 10000–13000 has been reported (Weser *et al.*, 1973; Kägi *et al.*, 1974; Winge *et al.*, 1975; Bremner & Davies, 1976; Noël-Lambot *et al.*, 1978). The molecular weight of plaice metallothionein was also estimated from the migration distances that were obtained when plaice metallothionein, horse kidney metallothionein MT-1B (a gift from Professor J. H. R. Kägi, University of Zürich, Zürich, Switzerland) and myoglobin were each subjected to gel electrophoresis on gels of increasing concentration, by the procedure of Hendrick & Smith (1968). When migration distance

Fig. 5. Estimation of molecular weight of plaice metallothionein from gel-electrophoresis migration distance

Samples of the purified plaice metallothionein, horse metallothionein and myoglobin were subjected to electrophoresis in 5%, 10% and 15% polyacrylamide gels (5% cross-linked) with the buffer system of Davis (1964). After electrophoresis the proteins were fixed in the gel with 12.5% (w/v) trichloroacetic acid and stained with Coomassie Blue G250. R_{BPB} , the migration distance of each protein relative to Bromophenol Blue, is plotted as a function of gel concentration. Δ , Plaice metallothionein; ∇ , horse metallothionein; $\circ$, myoglobin. The slope of the line is proportional to the molecular weight.

is plotted as a function of gel concentration a linear relationship with negative slope is obtained (Fig. 5). The slopes of the lines for plaice and horse metallothioneins are identical and differ from that for myoglobin (mol. wt. 16900). Hence it is assumed that plaice metallothionein has the same molecular weight as that of horse metallothionein. Horse metallothionein is a single-chain protein with a mol. wt. ranging from 6500 to 7000 depending on the metal composition. The much larger and less accurate values obtained from the gel-filtration experiments (10000–13000) are attributed to the deviation from globular shape of this protein, which in its native state occurs in solution as a prolate ellipsoid with an axis ratio of 6 (Kägi *et al.*, 1974).

Amino acid composition

The amino acid composition of the purified protein is given in Table 1. The mole percent values are also expressed as nearest whole unit residues based on 19 half-cystine residues. The analyses show the half-cystine content to be 31mol%. The next most abundant residues are glycine and lysine, 10.9 and 10.2mol% respectively. There is no histidine, tyrosine and tryptophan, and just a trace of phenyl-

Table 1. *Summary of the physical and chemical properties of plaice metallothionein*

Amino acid analysis:

Residue	(mol %)	(residues/mol of mol.wt. 6621)	Residue	(mol %)	(residues/mol of mol.wt. 6621)
Lys	10.24	6	Ala	2.73	2
His	0	0	Cys	31.12	19
Try	0	0	Val	1.84	1
Arg	Trace	0	Met	2.80	2
Asx	9.74	6	Ileu	Trace	0
Thr	9.37	6	Leu	0.76	1
Ser	9.10	6	Tyr	0	0
Glx	5.02	3	Phe	Trace	0
Pro	6.44	4	Glucosamine	Trace	0
Gly	10.85	7			

Metal analysis:

Cd, 10.60 Zn, 0.04 Cu, 0.08 (% of total by wt.)

SH/Cd ratio:

p-Hydroxymercuribenzoate titration 2.86 ± 0.04 (S.E.M.) (8)

Ag⁺ titration 3.32 ± 0.02 (S.E.M.) (10)

Amino acid analysis 2.90

A_{250}/A_{280} ratio: 20

Freeze-thaw: Stable

Carbohydrate assay:

Periodic acid/Schiff reagent +
Total sugars (phenol/H₂SO₄) –
Sialic acid –
Uronic acid –

alanine, in accord with the absence of absorbance at 280nm. A minimum molecular weight calculated from this amino acid composition gives a value of 6621, which is increased to 7406 when the weight of the cadmium is included. This amino acid composition with a high cysteine content is characteristic of a metallothionein and is quite similar to that for horse metallothionein (Kägi *et al.*, 1974).

Carbohydrate content

From our earlier studies (Overnell *et al.*, 1977) plaice metallothionein was considered to be a glycoprotein by virtue of a positive periodic acid/Schiff reagent test. Further evidence to support this hypothesis, however, could not be obtained. Thus examination of the amino acid chromatograms for the presence of amino sugars indicated that only a trace of glucosamine was present. Furthermore the phenol/H₂SO₄ assay for total carbohydrate (Dubois *et al.*, 1956), g.l.c. after mild acid hydrolysis and preparation of alditol acetate derivatives (Metz *et al.*, 1971) and testing for uronic and sialic acids (Bitter & Ewins, 1961; Hammond & Papermaster, 1976) all gave negative results. The positive reaction with periodic acid/Schiff reagent may be a property of the many thiol groups present in this protein and not due to sugar aldehydic groups as is more generally assumed.

Summary and conclusions

The physical and chemical properties of plaice metallothionein are summarized in Table 1. All of these properties closely resemble those seen with mammalian metallothioneins. Fish therefore appear to use this protein also in detoxifying cadmium, and the findings demonstrate a remarkable conservation of primary and tertiary structure in metallothionein during many evolutionary stages.

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