HUMAN HEPATIC METALLOTHIONEINS†

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1. Introduction

Metallothioneins are sulfur-rich proteins first recognized in equine and human renal cortex by their unusual content of cadmium [1,2]. All preparations contained in addition, however, equally appreciable amounts of zinc and the variations in the relative amounts of the two metals suggested even in the earliest studies that they may replace each other. A naturally occurring only zinc-containing form of metallothionein was recently identified in equine liver and recognized as one of the major zinc proteins of this organ [3,4]. In the present study we report the isolation from human liver and characterization of two similar, only zinc-containing forms of metallothionein.

2. Material and methods

2.1. *Extraction procedure*

Human livers were obtained from adult patients of both sexes who had died of causes other than hepatic disease. From each liver an extract was made by buffer extraction and solvent fractionation using a modification of the method described for the isolation of human renal metallothionein [2]. In the new procedure the dialysis step employed previously was omitted and a second solvent fractionation step involving the addition of 2 parts of 95% ethanol, prechilled to −20°C, was introduced to precipitate the metalloprotein fraction. The white precipitate formed was collected by centrifugation (3000 *g*, 20 min) and reextracted for 10 min with 200 ml H_2O. After removal of insoluble material by centrifugation (20 000 *g*, 2 hr) the supernatant was lyophilized.

2.2. *Chromatographic procedures*

Sephadex G-75 and DEAE-Sephadex A-25 were products of Pharmacia, Uppsala, Sweden, and were prepared for use by equilibration with the buffer solutions employed. Gel filtration and ion exchange chromatography were carried out at 4°C in 150 × 4.5 cm and 13 × 1.5 cm columns respectively. Further details of the chromatographic procedures are given in fig. 1. Following chromatography pooled fractions were desalted and concentrated by ultrafiltration at 4°C in an Amicon pressure dialyzer equipped with an UM-2 membrane (Amicon N.V., Oosterhout (N.B.), Holland).

2.3. *Metal analyses*

Metal analyses were performed with an Instrumentation Laboratory IL-353 atomic absorption spectrophotometer after appropriate dilution of the samples in water.

2.4. *Amino acid analyses*

Amino acid analyses were carried out on samples oxidized with performic acid for 16 hr [5] and hydrolyzed in evacuated and sealed tubes in 6 N HCl for 22 hr [6]. Measurements were made on a Beckman Spinco amino acid analyzer, Model 120 B. Cysteic acid and methionine sulfone were determined using the calibration constants for aspartic acid and methionine, respectively. Values for serine and threonine were corrected for standard losses. Tyrosine and tryptophan

† Dedicated to Professor F. Leuthardt, Zürich, on the occasion of his 70th birthday.
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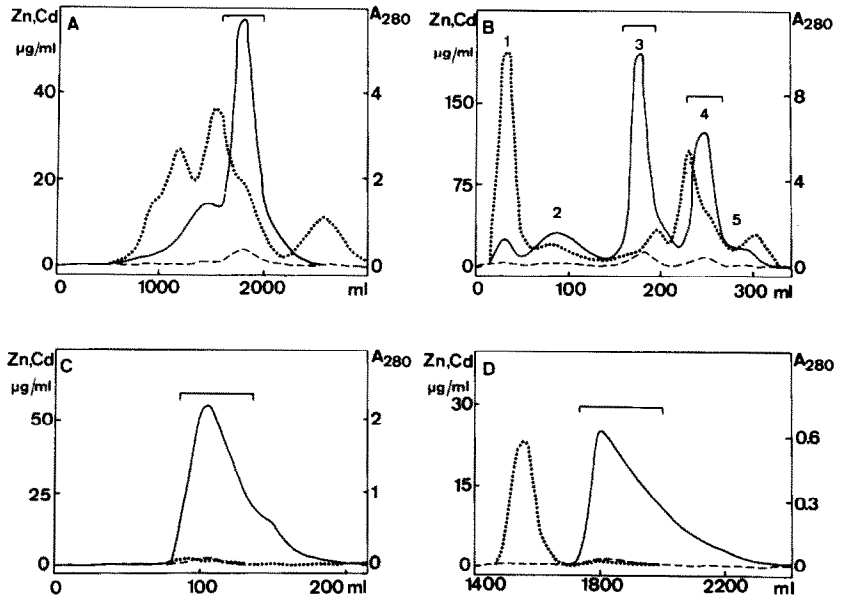

Fig. 1. Chromatographic separation of multiple forms of human hepatic metallothionein. Zinc (——), cadmium (– – –) and absorbance at 280 nm (·····) are measured. The brackets denote effluent fractions pooled for further processing. A. Gel filtration of lyophilized extract of a single human liver on Sephadex G-75 in 0.02 M Tris–0.005 M HCl, pH 8.6. Flow rate: 65 ml/hr; B. Gradient ion exchange chromatography of main zinc-containing peak of fig. A on DEAE-Sephadex A-25 equilibrated with 0.02 M Tris–0.005 M HCl, pH 8.6, as starting buffer. Elution is carried out by passing through the columns 50 ml of starting buffer followed by a 140 ml linear salt gradient and 70 ml limit buffer (0.2 M Tris–0.05 M HCl, pH 8.6). Flow rate: 14 ml/hr; C. Rechromatography of concentrated and desalted fraction 3 of fig. B on DEAE-Sephadex A-25 equilibrated with 0.03 M Tris–0.0075 M HCl, pH 8.6. Elution is carried out with the same buffer without gradient. Flow rate: 15.5 ml/hr. The material pooled is designated metallothionein 1 (= MT-1); D. Rechromatography of concentrated and desalted fraction 4 of fig. B on DEAE-Sephadex A-25 equilibrated with 0.03 M Tris–0.0075 M HCl, pH 8.6. Elution is carried out by passing through the column 1200 ml of the same buffer followed by 1000 ml 0.06 M Tris–0.015 M HCl, pH 8.6. Flow rate: 15.5 ml/hr. The material pooled is designated metallothionein 2 (= MT-2).

were measured spectrophotometrically in the non-oxidized protein. The minimum molecular weight was determined by the statistical method of Delaage [7].

2.5. *Protein concentrations*

Protein concentrations were evaluated from quantitative amino acid analysis data.

2.6. *Reactive mercapto groups*

Reactive mercapto groups were determined by titration with DTNB in 6.0 M guanidine-HCl, pH 8.0, containing 50 mM EDTA [8].

2.7. *Polyacrylamide disc gel electrophoresis*

Polyacrylamide disc gel electrophoresis was carried out at a constant current of 4 mA per tube and approximately 200 V for two hr at 23°C [9].

2.8. *Molecular weights*

Molecular weights were estimated by the gel filtration method of Fish et al. [10] on samples oxidized with performic acid using a 90 X 1.5 cm column packed with Biogel A-5 m, 50-100 mesh, equilibrated with 6.0 M guanidine-HCl (Schwarz/Mann, Orangeburg, N.Y., USA). The partition coefficients K_D were evaluated from the relationship

$$K_D = \frac{V_e - V_o}{V_t - V_o}$$

where V_e is the elution volume of the unknown sample, V_o the void volume and V_t the total volume. As marker proteins were employed: Pancreatic ribonuclease, mol. wt. 13 700 (Calbiochem); trypsin inhibitor from Lima bean, Type II-L, mol. wt. 8400 (Sigma); insulin, oxidized B-chain, mol. wt. 3500; insulin, oxi-

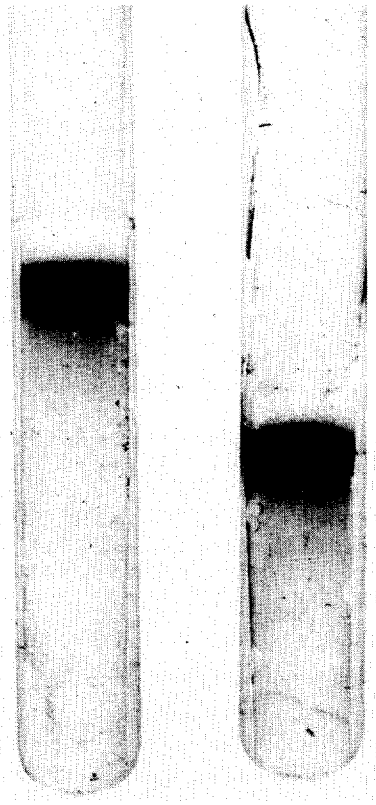

Fig. 2. Polyacrylamide disc gel electrophoresis of main metallothionein fractions. Left: MT-1; right: MT-2.

dized A-chain, mol. wt. 2500 (Mann Research Laboratories).

3. Results

The isolation of zinc-containing proteins from the lyophilized extract of human liver is documented in fig. 1A–D. By gel filtration of the extract on Sephadex G-75 two zinc-containing fractions are separated (fig. 1A). The larger one is characterized also by the presence of small amounts of cadmium. By gradient ion exchange chromatography on DEAE-Sephadex A-25 this material is resolved into five metal-containing fractions (fig. 1B). Peaks 3 and 4 are further purified by rechromatography on DEAE-Sephadex A-25 without gradient (fig. 1C, 1D). The resulting metal-containing fractions exhibit low absorbance at 280 nm and were recognized by their chemical properties (vide infra) as

Table 1
Metal content of human liver metallothioneins (referred to weight of metal-free protein).

	MT-1	MT-2
	%	%
Zinc	6.26	7.58
Cadmium	0.14	0.19
Copper	0.13	0.07
Total	6.53	7.84

variants of metallothionein isolated previously from human kidney [2]. They are designated as human hepatic metallothionein 1 (= MT-1) and metallothionein 2 (= MT-2), respectively. The total yield from one liver ranges for each form between 50 and 100 mg.

Both protein fractions are homogenous as judged by the appearance of a single stainable band on polyacrylamide disc electrophoresis (fig. 2). They differ, however, in electrophoretic mobility, MT-2 moving faster towards the anode than MT-1. On the other hand, both proteins are closely similar in size as judged by their identical behaviour on gel filtration on a 200 cm Sephadex G-50 column.

The metal contents are summarized in table 1. MT-2 contains substantially more metal than MT-1. In both proteins zinc is the predominant metallic species (> 95%). It is accompanied by only small amounts of cadmium and copper.

The amino acid composition of the two forms of metallothionein is also similar (table 2). The most distinctive common features are the abundance of Cys and the total absence of aromatic amino acids as well as of His and Arg. Differences occur, however, in a number of amino acids. Thus, MT-1 contains more Glu, Val and Leu but less Cys, Asp and Ala than MT-2. Titration of mercapto groups in both proteins is in close agreement with the number of Cys suggesting the absence of disulfide bonds.

The number of amino acid residues listed in table 2 is based on the minimum molecular weight of the peptide chains calculated from amino acid analysis data, i.e. 6113 for MT-1 and 6136 for MT-2. A similar value for the chain weight is obtained experimentally by gel filtration of performic acid oxidized MT-1 in 6 M guanidine-HCl using proteins of known molecular weight as standards (fig. 3). If the metal is included

Table 2
Amino acid composition of human liver metallothioneins *.

Amino acid	MT-1 Number of residues**	MT-1 Nearest integral number	MT-2 Number of residues***	MT-2 Nearest integral number
Lys	7.80	8	7.78	8
Asp	2.89	3	4.06	4
Thr	2.45	2	2.08	2
Ser	8.40	8	8.30	8
Glu	3.42	3	2.39	2
Pro	2.40	2	2.07	2
Gly	4.87	5	5.14	5
Ala	5.76	6	6.81	7
Cys†	18.50	18	20.55	21
Val	2.14	2	1.12	1
Met††	0.83	1	0.87	1
Ile	1.18	1	0.95	1
Leu	0.68	1	–	–
Total‡	61.32		62.12	
Mercapto groups‡‡	17.45		21.61	

* Average of 4 analyses.
** Based on the minimum molecular weight 6113.
*** Based on the minimum molecular weight 6136.
† Measured as cysteic acid.
†† Measured as methionine sulfone.
‡ Arg, His, Phe, Tyr and Trp were not found.
‡‡ Measured in the intact protein.

the minimum molecular weight of the native proteins is about 6600.

Based on the chain molecular weight MT-1 contains a total of 6 and MT-2 a total of 7 metal atoms (table 3). This difference in stoichiometry is proportionate to the difference in the numbers of Cys measured by amino acid analysis and of mercapto groups titrated in the intact proteins (table 2). Hence, their ratio to the number of metal atoms bound is the same in both proteins. For each metal atom bound there are three cysteinyl groups available.

4. Discussion

The present data demonstrate that metallothionein is a normal and abundant component of adult human liver. The two proteins which were isolated by a combination of methods previously employed in the preparation of renal metallothioneins are by most criteria identical with the human renal protein [2]. The occurrence of more than one form of hepatic metallothionein is also typical for these proteins as shown previously in human and equine kidney cortex [2,11] and in rabbit and rat liver [12,13].

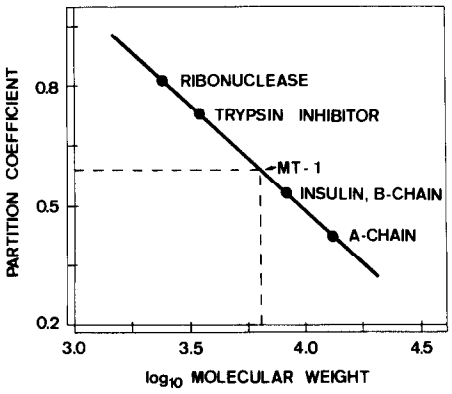

Fig. 3. Determination of chain molecular weight of performic acid oxidized MT-1 by gel filtration in 6 M guanidinium-HCl on Biogel A-5 m. The molecular weight corresponsing to the measured partition coefficient (= 0.59) is 6500. Taking into account an estimated increase in molal volume of 5% on oxidation the molecular weight of the non-oxidized chain is close to 6200.

Table 3
Stoichiometry of metal* and cysteine in human liver metallothioneins.

	MT-1 moles/mole	MT-2 moles/mole
Metal Protein**	6.05	7.24
Cys*** Metal	3.06	2.84
Mercapto groups Metal	2.89	2.99

* Sum of Zn, Cd and Cu.
** Based on minimum molecular weights (see table 2).
*** Measured as cysteic acid by amino acid analysis.

Unlike the human renal metallothioneins which contain both zinc and cadmium in about equal proportions both liver proteins contain zinc as their sole significant metallic constituent (table 1). Their zinc content of 6.3% and 7.6% is the highest of any known zinc metalloprotein comparable only with the total metal content of certain ferredoxins [14] and of ferritin. The total amount of zinc bound to metallothionein in liver represents 5–10% of the total zinc of the organ.

Both hepatic metallothioneins have minimum molecular weights close to the value of 6600 measured for native equine renal metallothionein by sedimentation equilibrium centrifugation [4]. The values are also in agreement with amino acid analysis data reported for rabbit liver metallothionein [12]. The amino acid composition of the two hepatic proteins also resembles that of metallothionein from other sources [3,4,12,15,16]. Their most striking common feature is their extreme content of Cys ranging from 30 to 35% and the total absence of His and of aromatic amino acid residues. A feature shared with the rat and rabbit proteins but not with the equine metallothioneins is the absence of Arg. The differences of MT-1 and MT-2 in six amino acid residues reveal appreciable variation in the primary structure of the two proteins. Sequence studies currently in progress in this laboratory have identified some substitutions and have localized the position of the three additional Cys in MT-2. These studies have also given evidence for a marked microheterogeneity in MT-1, a feature accounting for the substantial deviations of the amino acid composition of this protein from integral values (table 2).

The abundance of cysteine residues offers an obvious explanation for the zinc binding capacity of metallothionein. The stoichiometric relationship of three mercapto groups for each metal atom bound is identical in both MT-1 and MT-2 (table 3). The same ratio of mercapto groups to metal, although the metals vary, is found in all preparations of metallothionein examined thus far [2,3,11] and thus appears to be a fundamental feature of their structure. It suggests that each metal binding site is composed of three mercapto groups, a conjecture also supported by spectroscopic studies [3,11,17]. As a consequence of this stoichiometry each of the polymercaptide complexes formed between Zn^{2+} and three mercaptide ligands should be negatively charged and thus contribute to the total charge of the protein. It seems likely, in fact, that the larger negative charge of MT-2 as compared to MT-1 (fig. 2) is due to its additional metal binding site.

The biological function of metallothionein is not known although some evidence for its participation in cadmium detoxification is available [12,18]. The discovery of solely zinc-containing forms of metallothionein in the present study and in previous work on equine liver [3, 4], suggests, however, that at least in liver the primary biological role of metallothionein must be sought in relation to this metal. An involvement in zinc transport, storage or detoxification are equally plausible possibilities since no physiological system performing such functions for zinc has been identified as yet. Recent reports that in rat liver metallothionein is synthesized in response to parenteral administration of zinc also give credence to a specific role in the metabolism of zinc [19,20].

Acknowledgement

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