CADMIUM-INDUCED SYNTHESIS OF HEPATIC METALLOTHIONEIN IN CHICKEN AND RATS

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1. Introduction

First evidence of a Cd containing protein of rather low molecular weight was obtained by Margoshes and Vallee [1]. In subsequent studies [2—4] an exceptionally high concentration of half cystine was measured in this protein. Due to the possible substitution of Cd either by Zn or Hg and the presence of such large quantities of sulphur the above protein was called metallothionein. It was of interest to note a rapid rise of metallothionein synthesis following the administration of Cd to rabbits [5, 6] and rats [7—10]. For this reason the protein is presumed to act as a metal decontaminating carrier in biological systems.

At the moment different criteria for the homogeneity of metallothionein are reported which may be attributed in part to the elaborate isolation procedures. This is especially the case when the protein is exposed to organic solvents for elongated periods of time during the course of the isolation. We wish to describe the convenient preparation of homogeneous hepatic metallothionein using rats and chicken which were previously injected with Cd. Precipitation steps employing inorganic salts ($(NH_4)_2SO_4$ or KBr) were omitted. Only a very short treatment with organic solvents was performed which was followed by ion exchange and gel chromatography. The metallothionein was monodisperse as shown by gel filtration, discontinuous polyacrylamide electrophoresis and ultracentrifugation. The proteins obtained from either biological source were of striking similarity. The molecular weight was 12 000 ± 500. The total content of metals expressed as the added portions of both Cd and Zn was approx. 7 g atoms per mole of protein or Zn:Cd = 1:2.4 ± 0.1. Furthermore, some physicochemical data like UV absorption, CD properties and X-ray photoelectron spectra were virtually identical whatever metallothionein was examined. From X-ray photoelectron spectroscopy and from circular dichroism measurements substantial evidence was obtained that the major portion of the sulphur containing amino acids can be attributed to cysteine.

2. Experimental

All chemicals employed were of reagent grade quality or better. To minimize metal contamination only plastic or quartz ware was used. Sephadex G-25, G-50 and G-75 (Pharmacia) and DEAE-23 cellulose (Maidstone) were employed in the respective chromatographic steps. Zn and Cd were determined by atomic absorption spectroscopy [11]. In crude fractions protein was assayed using the biuret micro method. The concentration of pure metallothionein was calculated using desalted solutions of known A_{250} which were lyophilized and weighed to constancy. The homogeneity of metallothionein was examined in a way similar to the methods given in [12, 13]. Amino acids were analysed chromatographically after 24 hr hydrolysis of the native protein.

Both white Wistar rats (300 g) and chicken (1500 g) were injected 3—5 times with 10 μmoles of $CdCl_2$ per kg of body weight in 7 day intervals. After the last injection the animals were maintained under normal laboratory conditions for 14 days prior to their sacrifice. Homogenisation of liver tissues and dialysis of the crude preparations was performed using the proce-

dures described elsewhere [1–4] which were modified for the present study. The subsequent application of DEAE-23 chromatography and gel filtration on Sephadex G-50 proved most successful.

3. Results

The treatment of the crude protein fractions with organic solvents employing prechilled chloroform and ethanol was carried out at 0°. The total time interval starting from homogenisation of liver tissues until the dialysis step was finished did not exceed more than 6 hr. In contrast to the procedures already published ion exchange chromatography using DEAE-23 cellulose was introduced. The main bulk of proteins was separated from the Cd-containing protein (fig. 1).

In the subsequent Sephadex G-50 gel filtration the clear separation of metallothionein from other contaminant proteins was accomplished. Additional gel chromatography using G-25 and G-75 yielded exclusively one symmetrical peak. From several trial runs on Sephadex G-50 a molecular weight of 12 000 ± 500 was found for both metallothioneins. One single sedimentation band was also detected during ultracentrifugation (fig. 2). The $s_{20,\omega}$-value was 2.7×10^{-13}.

In addition to the apparent homogeneity of the isolated metallothionein substantial evidence for the presence of a monodisperse protein was obtained from analytical polyacrylamide gel electrophoresis (fig. 3).

As in the case of metallothionein isolated from other biological sources [1–10] the most abundant amino acid was cysteine (table 1). The values expressed in brackets were obtained after oxidation using performic acid.

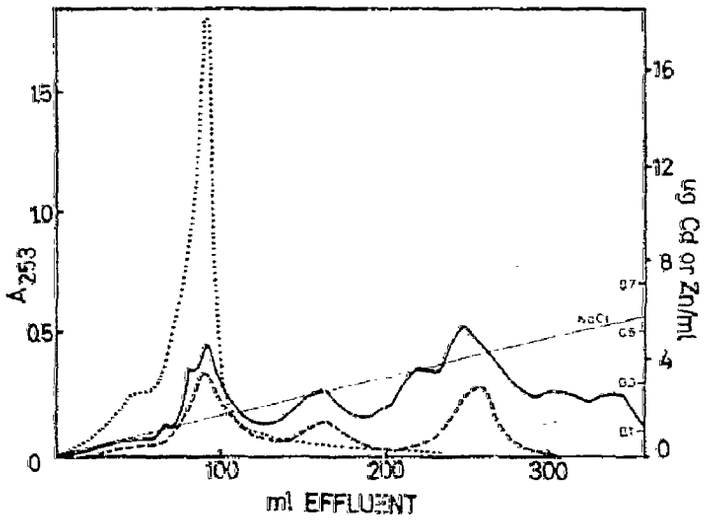

Fig. 1. DEAE-23 chromatography of crude metallothionein from rat liver. (—) Protein, (- - -) Zn (· · ·) Cd. The fraction volume was 6 ml. Elution with a linear NaCl gradient, pH 8.6 (5–600 mM). A rather similar elution profile was obtained using the crude protein fractions from chicken liver.

Fig. 3. Analytical polyacrylamide gel electrophoresis of metallothionein prepared from chicken or rat liver. The applied amount of either metalloprotein was 7 µg. 4% of acrylamide were used in the concentrating gel and 7.5% in the separating gel, respectively. Electrophoresis was carried out at 225 V, 42 mA for 90 min. Staining with Coomassie blue; destaining by diffusion.

Fig. 2. Sedimentation pattern of chicken metallothionein. The employed protein concentration was 3.1 mg/ml, 100 mM potassium phosphate buffer, pH 7.1, 23.5°. Sedimentation direction left to right. Schlieren bar angle 30°. A1 valve synthetic boundary cell. Photographs were taken after reaching full speed (60 000 rpm).

Table 1
Hepatic metallothionein.

	Rat (number of residues per mole)	Chicken
Lysine	15.5	11.7
Histidine	3.6	1.9
Arginine	3.4	5.3
Aspartic acid	7.3	11.1
Threonine	4.7	1.5
Serine	8.6	10.5
Glutamic acid	7.1	4.4
Proline	8.3	10.0
Glycine	9.6	7.8
Alanine	7.5	10.9
Cysteine	24.3 (31.6)	37.1 (36.4)
Valine	4.7	2.0
Methionine	0.5	1.8
Isoleucine	2.9	0.4
Leucine	3.4	0.5
Tyrosine	0.8	0
Phenylalanine	< 1.0	0

24 hr hydrolysates. Values in parentheses determined as cysteic acid.

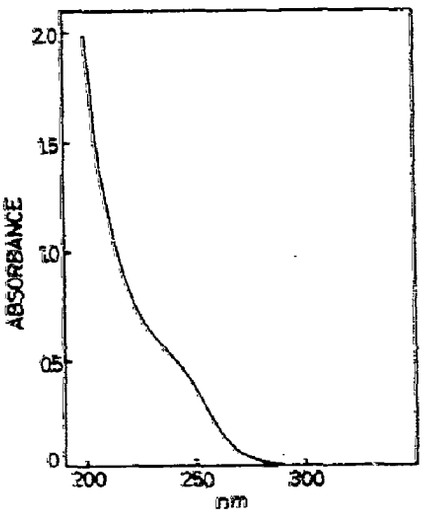

Fig. 4. Ultraviolet absorption of the metallothioneins. Spectra were recorded at pH 6.6 in a Unicam SP 1800. ϵ_{250} was 8.06×10^{-4}; ϵ_{250}: ϵ_{280} was 17.5.

The metal content of the chicken hepatic metallothionein was 2 Zn and 5 Cd per mole of protein or Zn:Cd = 1:2.5. The Zn:Cd ratio for rat liver metallothionein was 1:2.3. For both proteins the maximal number of metal ions was calculated to be 7 g atoms per 12 000 g of protein which is in close agreement with the values reported for human metallothionein [1–4]. The absorption spectrum in the ultraviolet region (fig. 4) is very similar to that published for human and equine metallothionein [1–4]. The virtual absence of aromatic amino acid residues is confirmed by the unusually low absorbance at 280 nm. The slightly expressed peak at 250 nm can be attributed to the metal–S–R bonding. This optical activity is much more expressed in the CD spectrum [15].

4. Discussion

The 6 hr treatment of crude metallothionein fractions with organic solvents and the exclusion of precipitation steps employing KBr, $(NH_4)_2SO_4$ or 2-ethoxy-6,9-diaminoacridine lactate can be considered beneficial for the native state of this protein. As to which degree the 12 000 mol. wt. metallothionein is a dimer consisting of two subunits having a molecular weight of 6000 [4] needs further investigation. Nevertheless it appears that the present metallothionein which was isolated under rather mild conditions could be regarded as the actual native form. This is also confirmed by Winge and Rajagopalan [8] who employed exclusively membrane and gel filtration combined with sucrose gradient centrifugation and found a metallothionein of 12 020 molecular weight. In earlier studies the molecular weight of metallothionein was reported to be in the range of 6000–10 000 [1–7].

An open question of central importance was the elucidation of the nature of the sulphur containing amino acids. From X-ray photoelectron spectroscopic measurements it can be demonstrated that the major portion of the sulphur is attributable to cysteine residues. The measured sulphur 2p binding energy in metallothionein was virtually the same as found for Zn- or Cd-cysteine (table 2).

Table 2

	Measured S 2p binding energy (eV)
CdS	160.5
Metallothionein	161.7
Cd-Cysteine	161.6*
Zn-Cysteine	161.9*
Cd-Cysteine	163.0*
Zn-Cysteine	162.8*

* Taken from [14].

This is further supported by CD data. A positive extremum is clearly visible at 258 nm ($[\theta]_{258}$ = 75 000) which can be assigned to the binding of Cd^{2+} or Zn^{2+} to $^{-}S-R$ moieties [15].

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