^{113}Cd NUCLEAR MAGNETIC RESONANCE OF METALLOTHIONEIN

Non-equivalent CdS_4 sites

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Received 18 July 1978

1. Introduction

The metallothioneins are low molecular weight ($\sim$ 6800) cytoplasmic proteins which may be involved in the metabolism and detoxification of heavy metals, in particular Zn, Cd and Cu [1]. Thioneins of similar amino acid composition occur in large quantities in the liver and kidney, and are probably present in nearly every other tissue, and synthesis can be induced by metal administration [2].

The extremely long half-life of Cd retention in the body ($\sim$ 200 days in rats and 16–33 years in man [3]) can lead to cumulative toxicity, and may be due to the high affinity of thionein for Cd^{2+}. It is therefore important to understand the mechanisms for Cd uptake and release, and in particular to define the coordination stereochemistry of the Cd sites.

Although devoid of aromatic amino acids and histidine, the single thionein polypeptide chain of about 61 amino acids contains about 20 cysteine residues [1,4], and it has been suggested [4] that there are up to 7 equivalent, independent metal binding sites of the type $[metal^{2+}(Cys^-)_3]^-$. We report here ^{113}Cd NMR evidence for 7 magnetically non-equivalent CdS_4 sites in Cd^{2+}-induced rat liver metallothionein.

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2. Materials and methods

2.1. *^{113}Cd-labelled metallothionein*

95% ^{113}CdO (Harwell) was dissolved in the minimum quantity of 2 M HCl and diluted with sodium acetate buffer (pH 5.6) to give an iso-osmotic solution. 4 pairs of female rats (250–300 g Wistar Porton strain) maintained on an Oxoid Pasteurised diet and water ad libitum, were given subcutaneous injections of the enriched ^{113}Cd solution at intervals of 2–3 days: three of 1.5 mg Cd/kg followed by three of 3.0 mg Cd/kg, giving a total dose of 13.5 mg/kg over the 2 weeks of exposure.

Four separate metallothionein preparations were performed. The animals were decapitated and the liver weights recorded. A sample of liver was digested in a 1:4 mixture of $HClO_4$: HNO_3 [5], dissolved in 5 ml 2% HCl and analysed for Cd, Zn and Cu by atomic absorption (Perkin Elmer model 460). The rest of the liver tissue was immediately homogenised in 2 vol (v/w) of prechilled 10 mM ammonium formate buffer, pH 8.0. The homogenate was centrifuged at 15 000 × *g* (10 min) and 110 000 × *g* (60 min) at 2°C. The supernatant was concentrated by dialysis against solid polyethylene glycol (mol. wt 6000, Koch Light Ltd.). The concentrate was centrifuged at 110 000 × *g* (30 min) and fractionated at 4°C on a Sephadex G-75 (fine grade, Pharmacia) column (5 × 85 cm) and eluted with ammonium formate buffer. Fractions, 7.5 ml, were collected at 50 ml/h, monitored at 254 nm and analysed for Cd,

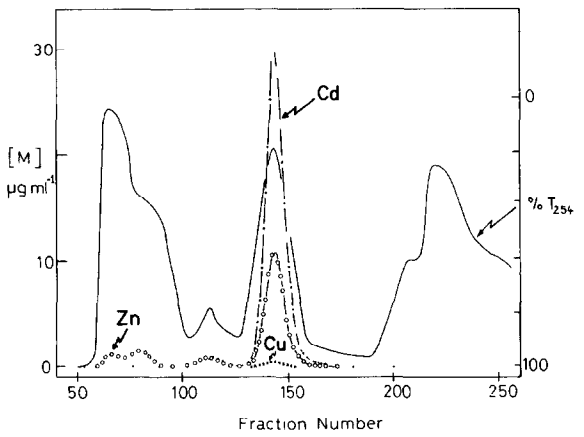

Fig.1. Sephadex G-75 chromatography of the soluble fraction from rat liver homogenate. Key to symbols: metal concentrations [M] in μg/ml. (–●–●–) Cd, (–○–○–) Zn, (–X–X–) Cu, and T is the % transmission at 254 nm (metal–thiolate absorption). Typically, fractions 134–155 were used for the NMR studies.

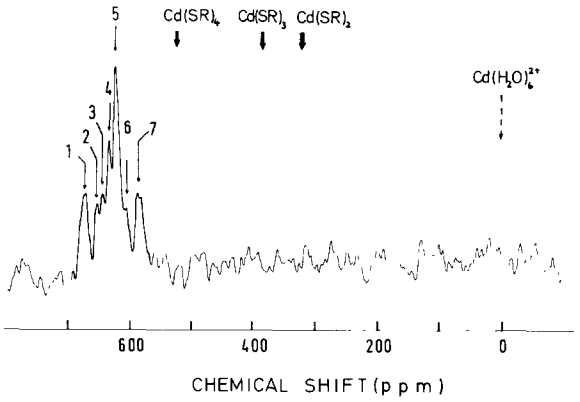

Fig.2. ^{1}H-decoupled ^{113}Cd NMR spectrum of rat liver metallothionein in aqueous solution pH 8 (details given in section 2). The chemical shifts of $Cd(SR)_n$ centres are taken from [6]. Peak 6 was resolved in both runs I and II, but the splitting of peak 7 was not, and is therefore probably due to noise.

Zn and Cu. A typical profile is shown in fig.1. Fractions containing > 2 μg Cd/ml were pooled and lyophilised. NMR sample I (∼ 5 mM) was prepared by dissolving ∼ 67 mg (prep. 1, 2) of thionein in 1.9 ml H_2O/20% D_2O and sample II (∼ 7 mM) 58 mg (prep. 3, 4) in 1.2 ml. Both solutions were slightly cloudy, probably due to the presence of a small amount of aggregated material formed during the lyophilisation step.

2.2. *NMR*

^{113}Cd NMR spectra were obtained on a JEOL FX-100 Fourier Transform spectrometer operating at 22.06 MHz and a probe temperature of 31°C. A spectral width of 20 kHz was used with 16 384 time domain data points, a pulse width of 15 μs (70°) and repetition interval of 0.5 s. ^{1}H-noise decoupling was employed and because of the negative magnetogyric ratio of ^{113}Cd maximum NOE enhancement –2.5 is possible, but no ^{1}H coupling has been observed in small Cd thiolate complexes [6]. An exponential weighting function equivalent to a line-broadening of 120 Hz was applied to the free induction decays. The chemical shift reference contained 40 mM ^{113}Cd and was prepared by dissolving ^{113}CdO in a slight excess of H_2SO_4 and diluting with D_2O.

Transients were accummulated; 120 000 for sample I and 50 000 for sample II. The spectra were almost identical and were added together to improve signal-to-noise further. The resultant spectrum is shown in fig.2.

3. Results and discussion

The ^{113}Cd NMR spectrum of ^{113}Cd-induced rat liver metallothionein is shown in fig.2. Seven resonances, corresponding to 7 non-equivalent Cd binding sites, can be seen with large high-frequency (low-field) shifts of 670, 649, 640, 628, 618, 603 and 581 ppm relative to $Cd(H_2O)_6^{2+}$. Site 5 appears to have the major population, although peak areas cannot be interpreted with certainty since no NOE or spin-lattice relaxation checks were carried out. A typical deshielding sequence in inorganic Cd complexes is $Cd(SR)_2$, 318 ppm < $Cd(SR)_3$, 380 ppm < $Cd(SR)_4$, 520 ppm [6] where the CdS_3 and CdS_4 shifts refer to $CdS_3(OH)_3$ and $CdS_4/CdS_4(OH)$ (averaged) sites in $Cd_{10}(SCH_2CH_2OH)_{16}^{4+}$ and each sulphur is doubly or triply bridging. It would appear therefore that the chemical shifts of the ^{113}Cd resonances from metallothionein can only be accounted for by assuming at least CdS_4 coordina-

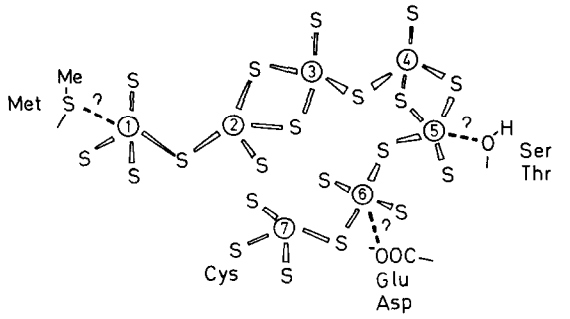

Fig.3. A possible arrangement of coordinated Zn^{2+} or Cd^{2+} ions in metallothionein, giving non-equivalent CdS_4 sites. For example, a Cd^{2+} ion in site 2 might be non-equivalent to one in site 3, if Zn^{2+} occupies site 4, due to a change in Cd(3)S bond lengths or transmission of an electronic effect.

tion. The additional low field shift of ∿ 100 ppm compared to the inorganic centres may arise because only one in four Cys S^- atoms needs to assume a bridging role in order to achieve CdS_4 coordination for 7 bound ions. However we must also consider the possibility that other side-chain donors, such as a terminal Met S, Asp or Glu COO^- (∿ 6 residues) and Ser or Thr OH (∿ 9 residues) increase the coordination of Cd^{2+} to 5 or 6. Alternatively, Zn^{2+} in a neighbouring site may induce an additional shift. Some of these possibilities are illustrated in fig.3.

The metallothionein used in this study had a bound Cd/Zn ratio of 1.7 (5 Cd^{2+} for every 3 Zn^{2+} ions) and Cd/Cu ratio of 39, and 91% of Cd in the liver after the 2 week exposure was bound to thionein. Such a small amount of Cu, presumably present as Cu^+, should not influence the NMR spectra (Cu^{2+} would give rise to paramagnetic shift and broadening effects). The broadness of the Cd resonances (> 300 Hz) suggests that there are Cd^{2+} inter-site exchange reactions taking place with an exchange rate comparable to the chemical shift differences or that sample heterogeneity, as noted for kidney metallothionein [1], is influencing the linewidths.

Cd^{2+} is usually a good isomorphous replacement for Zn^{2+} and our suggestion of CdS_4 centres in metallothionein fits neatly into the pattern which has emerged for Zn^{2+} sites in crystalline proteins and enzymes. Catalytic Zn^{2+} centres have 3 strongly-bound protein ligands (3 His in carbonic anydrase; 2 His, 1 Glu in carboxypeptidase A and thermolysin; and 2 Cys, 1 His in liver alcohol dehydrogenase) and the site 4 is available to the substrate through H_2O displacement. The structural Zn^{2+} ion in liver alcohol dehydrogenase, on the other hand, occupies a $Zn(Cys)_4$ site [7]. Although the electron micrograph of metallothionein in [8] was interpreted in terms of CdS_3 centres, there is considerable overlap of most of their neighbouring coordination triangles as would be expected if bridging sulphurs are present.

This work confirms the potential of ^{113}Cd NMR for studying the detailed coordination of Cd^{2+} in proteins and enzymes. The sensitivity of detection of ^{113}Cd ($I = 1/2$, 12.3% natural abundance) is readily brought into the mM range by isotopic enrichment, and the resonances observed here are well-shifted from those of carbonic anhydrase (228 ppm, CdN_2O_2) [9,10] or alkaline phosphatase (170 ppm) [11]. It should now be possible to study Cd^{2+} incorporation into thionein under a variety of physiological conditions, and also routes for de-metallation.

Acknowledgements

We thank Dr M. Webb for stimulating discussions and Dr T. Connors for encouragement, and we are grateful to RTZ Services Ltd. for a grant to purchase enriched Cd isotopes. This work was partly supported from The Norwegian Research Council for Science and the Humanities.

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