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88 MHz ^{113}Cd-n.m.r. studies of native rat liver metallothioneins

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Well-resolved ^{113}Cd-n.m.r. spectra of ^{113}Cd-induced rat liver metallothioneins 1 and 2 are obtainable even at 88 MHz (9.4 T). The line-widths of resonances are not dominated by chemical-shift-anisotropy relaxation. The increased spectral dispersion will significantly aid the study of the native (Cd- and Zn-containing) metallothioneins.

Metallothioneins are low-molecular-weight proteins (M_r approx. 6000–7000) that contain about 30% cysteine (20 residues) and seven to nine metal ions/molecule (Nordberg & Kägi, 1979). They were originally isolated from horse renal cortex by Margoshes & Vallee (1957), but have subsequently been found in a variety of tissues from a number of different species. Most mammalian metallothioneins are separable by ion-exchange chromatography into isometallothioneins, designated metallothioneins 1 and 2, which differ in their overall negative charges and amino acid compositions.

The arrangement of metal-binding sites within the protein is probably of functional importance. The Cys-Xaa-Cys and Cys-Xaa-Yaa-Cys sequences that occur in the amino acid sequence are highly conserved among animal species. Metallothionein has never been crystallized, and thus spectroscopic techniques are used for probing metal-binding sites. These have included electronic absorption, magnetic circular dichroism and e.s.r. (Vašák, 1980; Vašák & Kägi, 1981) and e.x.a.f.s. (extended X-ray-absorption fine-structure spectroscopy) (Garner *et al.*, 1982). However, the most informative technique still appears to be ^{113}Cd n.m.r. The isotope has a spin quantum number of $\frac{1}{2}$ and natural abundance of 12%. By injecting rats with 95%-enriched $^{113}Cd^{2+}$ we were able to isolate ^{113}Cd-enriched rat liver metallothionein, which gave rise to ^{113}Cd-n.m.r. spectra with reasonable signal-to-noise ratios (Sadler *et al.*, 1978). The resonances suggested that Cd was bound as $Cd(S\text{-}Cys)_4$ in several non-equivalent sites, with extensive thiolate sulphur bridging between Cd^{2+} ions. Similar spectra were reported by other workers (Suzuki & Maitani, 1978; Otvos & Armitage, 1979).

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The existence of Cd clusters in metallothionein was demonstrated conclusively by Otvos & Armitage (1979, 1980, 1982), who, with the aid of selective ^{113}Cd decouplings, were able to analyse the ^{113}Cd–^{113}Cd two-bond couplings. They have proposed a model in which seven Cd^{2+} ions are arranged in two separate clusters, cluster A containing four metal ions and cluster B three. Curiously, they found that the ^{113}Cd-n.m.r. spectra of homogeneous ^{113}Cd-labelled metallothioneins 1 and 2, in which Zn had been replaced *in vitro* by ^{113}Cd, were identical, whereas those of the native Zn-containing Zn,Cd-metallothioneins 1 and 2 differ significantly. Because the latter give complicated ^{113}Cd-n.m.r. spectra with many overlapping resonances, we have explored the possibility of obtaining ^{113}Cd-n.m.r. spectra of metallothioneins at a higher magnetic field (9.4 T) to improve dispersion of signals. There are no previous reports of attempts to observe n.m.r. spectra of metallothioneins at fields higher than 4.7 T, perhaps because it is widely believed that relaxation of the heavy ^{113}Cd nucleus via chemical-shift anisotropy would broaden signals beyond detection. Our finding of significant improvement in dispersion at higher field, with little increase in line-broadening, aids the study of the native proteins.

Materials and methods

^{113}Cd-labelled metallothioneins

Two preparations (I and II) of the crude metallothionein were made and resolved into the ^{113}Cd,Zn-metallothioneins 1 and 2 as follows.

Solutions for injection were prepared from ^{113}CdO (95% ^{113}Cd-enriched) as described by Sadler *et al.*

(1978). Female Porton–Wistar rats (200–250g body wt.) were injected subcutaneously at 48h intervals with three doses of 1.5mg of $^{113}Cd^{2+}$/kg, and then with three doses of 3.0mg of ^{113}Cd/kg. One month after the last dose the animals were decapitated, and their livers were removed, frozen in liquid N_2 and stored at −20°C. A liver cytosol (equivalent to 170g of tissue) was prepared and chromatographed at 4°C on a Sephadex G-75 column (80cm × 9cm) as described by Cain & Holt (1979). Fractions of volume 14ml were collected and were analysed for Cd by atomic absorption to locate the metallothionein ($V_e/V_0 = 1.8$–$2.0:1$). To minimize contamination, the leading and trailing edges of the metallothionein peak were discarded. The remaining fractions were pooled and applied to a DEAE-cellulose (Whatman DE-52) column (40cm × 1.5cm), pre-equilibrated with 10mM-ammonium formate buffer, pH 8.0, at 5°C. The ^{113}Cd,Zn-metallothioneins 1 and 2 were eluted with a linear gradient of 10–200mM-ammonium formate buffer, pH 8.0 (400ml), at a flow rate of 30ml/h. Fractions of volume 3ml were collected, and 0.3ml portions were analysed for Cd.

Elution profiles similar to those described previously (Fig. 4 of Cain & Holt, 1979) were obtained for both preparations I and II of the crude metallothionein. In each preparation, ^{113}Cd,Zn-metallothionein 2 was eluted as a sharp symmetrical peak. The pooled fractions of this peak were analysed for metals before being freeze-dried. In contrast, ^{113}Cd,Zn-metallothionein 1 was eluted as a more diffuse and apparently heterogeneous peak. For comparative purposes all of the fractions within the area of the ^{113}Cd,Zn-metallothionein-1 peak of preparation I were combined and freeze-dried, whereas only the central portion of this peak from preparation II was isolated. The Cd:Zn:Cu proportions (normalized to 7.0 metal ions) of the metallothioneins from the two preparations were: metallothionein 1, 4.46:2.35:0.19 (preparation I) and 4.12:2.62:0.06 (preparation II); metallothionein 2, 4.72:2.06:0.22 (preparation I) and 4.61:2.27:0.12 (preparation II).

N.m.r. measurements

^{113}Cd-n.m.r. spectra at 88.76MHz were recorded at 300K on a Bruker WH400 spectrometer with a broad-band probe and 10mm tubes. Spectra are the result of 40000–70000 transients, 35 μs (80° pulse), 32000 data points, frequency width 62.5kHz, acquisition time 0.33s. Proton irradiation was gated off for 1s before the observation pulse to suppress nuclear Overhauser effects.

^{113}Cd chemical shifts are quoted relative to $Cd(ClO_4)_2$ as reference ($\delta = 0$ p.p.m.). In practice, 0.5M-$CdSO_4$ and 2M-$Cd(NO_3)_2$ in 20% ^2H_2O/80% ^1H_2O were used as external references and were assumed to have shifts of +3 and +29p.p.m. respectively.

Samples for n.m.r. spectroscopy were prepared from the freeze-dried metallothionein 1 of preparation I by dissolving 70mg in 2.1ml of ^2H_2O. The resultant pH* (meter reading) was 4.05, and this was immediately adjusted to 8.3 with NaO^2H solution. For metallothionein 2, 43mg was dissolved in 1.5ml of ^2H_2O and the pH* was adjusted from 4.1 to 8.2. These solutions of approx. 4.7mM-metallothionein 1 and 4.1mM-metallothionein 2 were saturated with N_2 to minimize any air oxidation. Similar solutions of approx. 4.0mM-metallothionein 1 and 4.5mM-metallothionein 2 were obtained from preparation II. Solutions of metallothionein 1 were slightly cloudy, but those of metallothionein 2 were clear.

Results and discussion

The 88MHz ^{113}Cd-n.m.r. spectra of rat liver ^{113}Cd,Zn-metallothioneins 1 and 2 are shown in Figs. 1 and 2. When compared with spectra of other ^{113}Cd,Zn-metallothioneins at lower field (e.g. 44MHz; see Fig. 13 in Otvos & Armitage, 1982) it is apparent that there is a greatly increased dispersion of most resonances with little increase in line broadening. This was unexpected, since it seemed likely that large contributions to ^{113}Cd relaxation from the chemical-shift-anisotropy mechanism might broaden the resonances (Kidd & Goodfellow, 1978). The contribution from chemical-shift-anisotropy (CSA) relaxation to the line widths, $\Delta\nu_{\frac{1}{2}}$, increases with the anisotropy ($\Delta\sigma$), the molecular weight of the species (since the rotational correlation time increases) and square of the applied magnetic field, B_0^2, (Webb, 1978):

$$\Delta\nu_{\frac{1}{2}}(\mathrm{CSA}) \propto B_0^2 \cdot \Delta\sigma^2 \cdot \left(\frac{6\tau_c}{1+\omega^2\tau_c^2} + 8\tau_c\right)$$

It seems likely that the absence of large contributions from chemical-shift-anisotropy relaxation is related to the high symmetry of the Cd co-ordination sphere, most probably being tetrahedral Cd(S-CyS)$_4$. A co-ordination number of 4 was determined for Zn-metallothionein by extended X-ray-absorption fine-structure spectroscopy (Garner *et al.*, 1982) with a single Zn–S distance of 0.229nm (2.29 Å). The metal sites in Co(II)-substituted horse metallothionein are also closely tetrahedral (Vašák, 1980).

Relaxation of ^{113}Cd need not be dominated by chemical-shift anisotropy even for Cd^{2+} in octahedral co-ordination sites. Forsén & Lindman (1981) found that the T_1 values for $(Cd^{2+})_2$ carp parvalbumin did not change on increasing the field from 2.35 to 6.0T. Very-high-field ^{113}Cd-n.m.r.

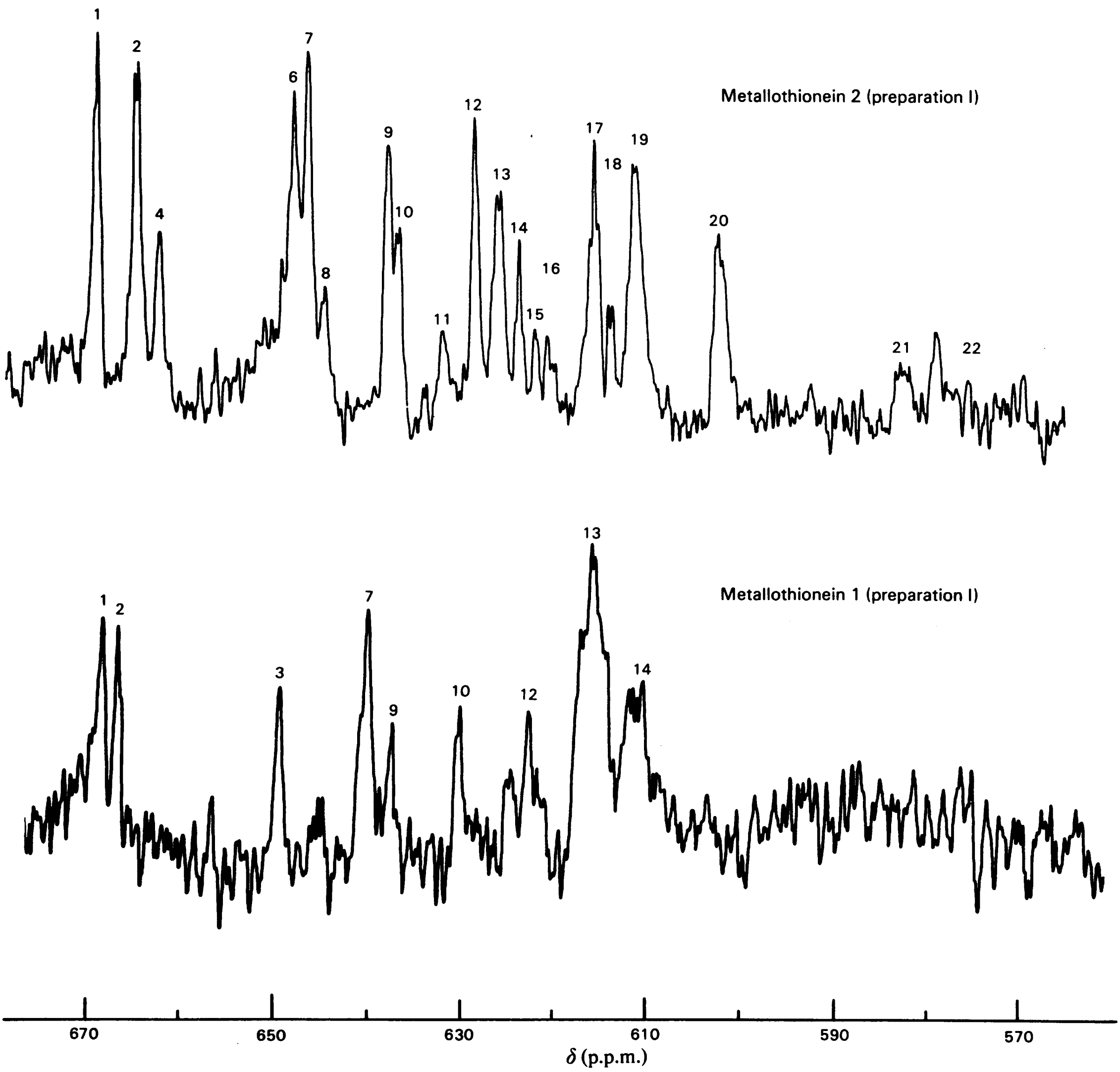

Fig. 1. *Comparison of the 88 MHz* $\{^1H\}$–^{113}*Cd-n.m.r. spectra of rat liver* ^{113}*Cd,Zn-metallothioneins 1 and 2 from preparation I*

Digital line-broadenings of 25 Hz have been applied. The peak numberings are based on those observed here and in the second preparation (Fig. 2).

studies may therefore be profitable for a wide range of Cd-containing proteins.

Comparison of Figs. 1 and 2 shows the good reproducibility from one preparation to the other. A notable difference between preparations I and II for metallothionein 2 is the absence of peaks 21 (582 p.p.m.) and 22 (579 p.p.m.) from the n.m.r. spectrum of preparation II (see Fig. 2). Although not observed for metallothionein 1, it is possible that these are related to the higher copper content of preparation I. Resonances between 590 and 603 p.p.m. were observed for ^{113}Cd-substituted calf liver metallothioneins, which have a high Cu^+ content, by Briggs & Armitage (1982).

Our samples of metallothionein 2 give rise to about 22 resolved ^{113}Cd resonances at high field, whereas metallothionein 1 gives rise to only 14 resonances. The signal-to-noise ratios are unfortunately not high enough to allow a confident assignment of ^{113}Cd–^{113}Cd couplings; however, by

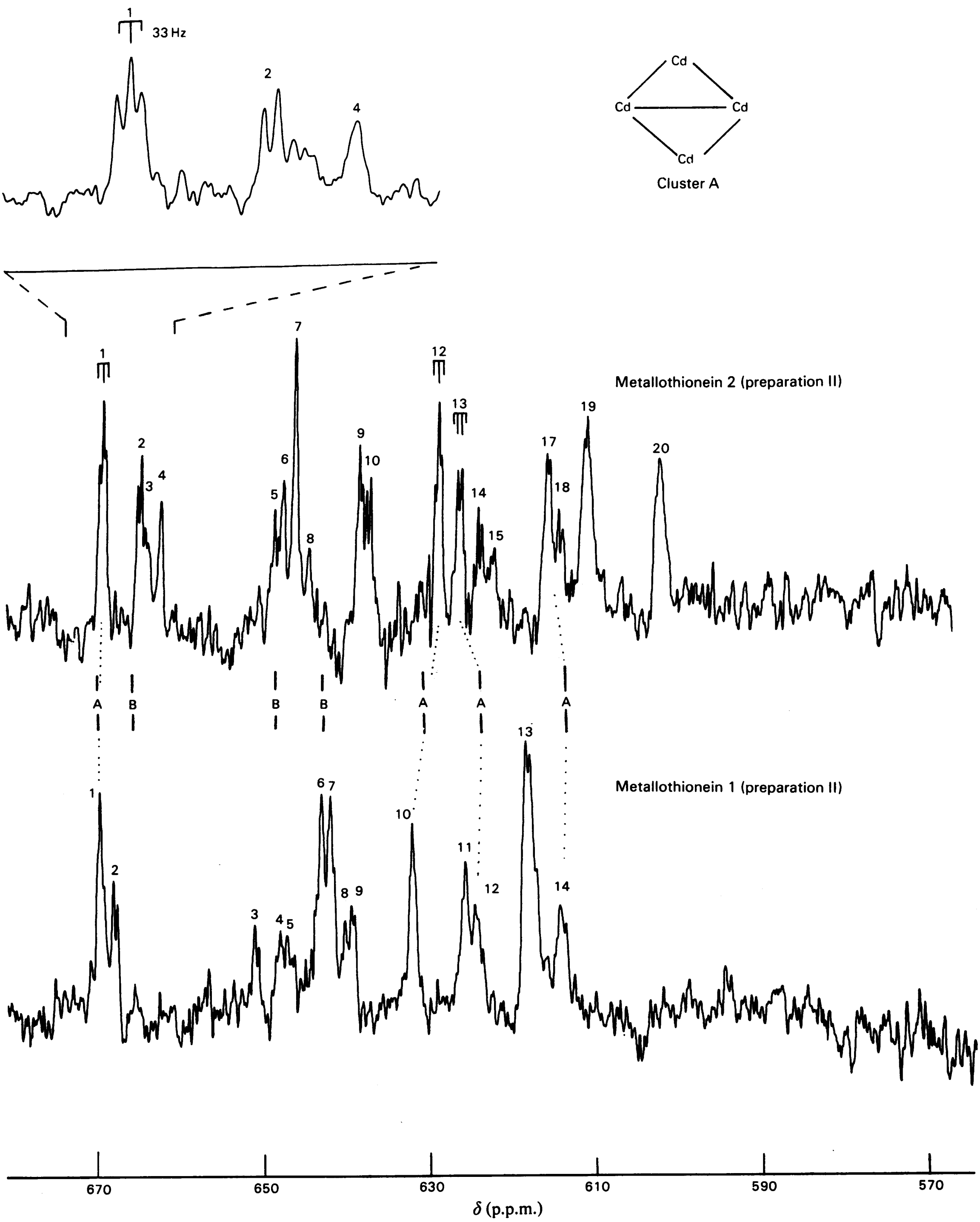

Fig. 2. *Comparison of the 88 MHz $\{^1H\}$–^{113}Cd-n.m.r. spectra from preparation II of rat liver ^{113}Cd,Zn-metallothioneins 1 and 2*

Digital line-broadenings of 18 Hz have been applied. The inset shows some of the resolved ^{113}Cd–^{113}Cd couplings in the high-frequency region of the spectrum of metallothionein 2, and a possible assignment of resonances to the Cd_4 cluster (shown without bridging sulphur atoms). The letters A and B in the centre of the Figure refer to the chemical shifts of ^{113}Cd in the Cd_4 (cluster A) and Cd_3 clusters proposed by Otvos & Armitage (1980) for rabbit liver metallothionein.

comparing the different preparations and examining spectra with various digital line broadenings, it was clear that resonances 1 and 12 for metallothionein 2 and 1 and 10 for metallothionein 1 were triplets with ^{113}Cd–^{113}Cd two-bond couplings of approx. 33 Hz. The shift of resonance 1 (669 p.p.m.) is strikingly similar to those of ^{113}Cd-substituted and native rabbit (670 p.p.m.) metallothioneins 1 and 2. It is possible that this resonance is due to a Cd^{2+} ion that occupies a key site in metallothionein, perhaps involving the sequence Cys-Cys-Ser-Cys-Cys, residues 33–37, which may provide a nucleation site for protein folding.

The smaller number of resonances observed for rat liver metallothionein 1 compared with metallothionein 2 suggests less heterogeneity in the distribution of Cd, Zn and Cu among the metal-binding sites. Particularly noticeable is the absence of the intense high-field resonance 20 (601 p.p.m.) of metallothionein 2 from the metallothionein-1 spectrum (Fig. 2). A resonance close to this, approx. 604 p.p.m., was observed for both the native Zn-containing ^{113}Cd,Zn-metallothioneins 1 and 2 of rabbit liver (Otvos & Armitage, 1982), but this disappeared when the proteins were fully substituted with ^{113}Cd. This suggests that metallothionein 2 has more mixed Cd–Zn clusters than has metallothionein 1.

A further comparison between the spectra of rat liver metallothioneins 1 and 2 suggests a correspondence between resonances 1, 12, 13 and 17 (or 18) of metallothionein 2 and 1, 10, 12 (or 11) and 14 of metallothionein 1. These appear to correspond to resonances 1, 9, 10 and 14 (two triplets and two doublets respectively) in the spectrum of rabbit metallothionein 2 (Otvos & Armitage, 1982). This tends to support the strong conservation of a cluster. It is noteworthy that the pattern of resonances, triplet, doublet, doublet, singlet, 1, 2, 3 and 4, of metallothionein 2 (see expansion, Fig. 2) is that which would be expected for a doubly bridged ^{113}Cd in cluster A (Fig. 2) with progressive Zn^{2+} substitution at the other sites. This would imply that rat liver metallothionein 1 contains more Cd-only and Zn-only clusters than does metallothionein 2, presumably because of a change in the relative binding constants for the different sites in the cluster. This may be important in relation to proposed functional differences for different metallothioneins (for a review see Webb & Cain, 1982). A refinement of these arguments should be possible when cadmium decoupling is performed at high field.

It seems likely that ^{113}Cd-n.m.r. chemical shifts are very sensitive to small changes in Cd^{2+}–Cys bond lengths and Cys–Cd–Cys bond angles. These could be transmitted to Cd^{2+} via Zn^{2+}, or Cu^{+} when the copper content is high.

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