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Cadmium Binding to Metal-free Metallothionein: a Correlation of UV, CD and ^{113}Cd NMR Data and a ^{113}Cd NMR Characterization of the Binding Sites in the Reconstituted Protein

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^{113}Cd nmr, circular dichroism and UV absorption spectra have been measured for rat liver ^{113}Cd, Zn-MT formed in vivo *following induction with $^{113}CdCl_2$. These spectra of the native metallothionein are compared with corresponding sets of data for the same sample but first acidified to pH 1.6 and then brought back to pH 7.6. It is shown that at pH 1.6 the cadmium is dissociated from the protein binding sites. The ^{113}Cd nmr spectrum of the sample returned to pH 7.6 demonstrates that when metal-free metallothioneins bind cadmium* in vitro *the cadmium ions occupy sites that are very similar, or may be even identical, to those occupied* in vivo. *Taken together, the nmr and CD results represent the first demonstration that metal-free metallothionein can form a metallated protein* in vitro *in which the geometry of the metal binding sites and the overall protein conformation are almost exactly the same as those found for the native metallated protein.*

Introduction

Metallothionein (MT), a low molecular weight, sulhydryl-rich protein containing the B group metal ions Cd, Zn, Cu and Hg has been isolated from a wide variety of mammalian species [1, 2], as well as from plants and microorganisms [2–4]. High levels of MT can be induced in the livers and kidneys of animals by the injection of the appropriate B group metals [2, 5]. All the sulfhydryl groups of the cysteine residues are considered to be involved in binding the metal ions, with an approximately tetrahedral geometry of sulfur around each metal [6]. A range of spectroscopic techniques have been used to study the coordination stereochemistry of the metals in the Cd, Zn-MT protein [7–24]. In the ^{113}Cd nmr spectra of ^{113}Cd-saturated rabbit liver metallothionein eight separate signals were observed [21, 22] with chemical shifts in the region where the CdS_4 units of model cadmium thiolates, with and without bridging by thiolate sulfur atoms, give ^{113}Cd resonances [25–27]. The characteristic ^{113}Cd–^{113}Cd scalar coupling led to a determination of the spatial arrangement of the seven cadmium ions that the protein contains [21, 22]. It is now clear that for mammalian metallothionein at least, two types of polynuclear metal clusters are present, one with three metal atoms and the other with four atoms [21–23].

One of the outstanding questions concerning the *in vivo* synthesis of metallated metallothioneins is whether metal-free metallothionein exists in the cell prior to metallation, or whether zinc- or copper-metallothionein are the precursors to the cadmium-containing protein. When solutions of Cd, Zn-MT are exposed to high proton concentrations the characteristic shoulder at 250 nm in the absorption disappears [7] and the CD and magnetic circular dichroism (MCD) envelopes collapse [9]. These spectral effects have been associated with the formation of the metal-free metallothionein as a result of protonation of the metal-coordinating thiolates [7, 9].

In this paper we report ^{113}Cd nmr and optical spectra for native rate liver Cd, Zn-MT in which the ^{113}Cd was bound *in vivo* following injections of $^{113}CdCl_2$. Spectra were recorded for the native protein and then for the same solution following acidification to low pH, and finally for the protein following reneutralization. These latter data represent the first ^{113}Cd nmr data obtained for a metallothionein in which the protein has been completely reconstituted with its complement of metal ions. This allows a comparison to be made between the structural information given by the absorption, CD and metal nmr experiments. In addition, we describe an attempt to measure the ^{199}Hg nmr spectrum of ^{199}Hg-MT which had been prepared by saturation of Cd, Zn-MT with $^{199}HgCl_2$.

Experimental

Protein Preparations

^{113}CdO (96.3% ^{113}Cd, from Oak Ridge National Laboratory) was converted to $^{113}CdCl_2 \cdot 2\frac{1}{2}H_2O$

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using HCl, and a solution prepared with a concentration of 2.8 mg Cd/mL. Male Sprague Dawley rats received a total of 9 injections (2.8 mg Cd/kg body wt.) over a period of 25 days and were sacrificed 24 hr after the last injection. The protein was purified by molecular filtration on Sephadex G-75 and the appropriate fractions freeze dried [10]. The freeze dried protein (420 mg) was then dissolved in a 1.5 mL 10% D_2O solution. The cadmium concentration of this solution was about 6.3 m*M*. The pH of the solution was adjusted by addition of aliquots of concentrated HCl or NaOH. ^{199}HgO (85.3% ^{199}Hg, from Merck Sharp and Dohme Canada Ltd.) was converted to $HgCl_2$ by evaporation to dryness with concentrated HCl. Horse metallothionein, obtained in the Department of Toxicological Chemistry (Lodz, Poland), was isolated from horse kidneys and purified as previously reported [28]. The metallothionein fraction was freeze dried and purified by preparative polyacrylamide gel electrophoresis [29]. The two isolated isoforms [MT1 and MT2] of horse metallothionein were desalted by gel filtration. Preparation of mercury metallothionein was achieved by dissolving 24 mg of both isoforms in 1.5 mL of 10% D_2O solution followed by the addition of $^{199}HgCl_2$ (2.8 mg) to this solution; the ^{199}Hg nmr spectrum was measured three hours later. A second ^{199}Hg nmr spectrum was measured after a further four hours, and after addition of a further 2.8 mg of $^{199}HgCl_2$.

Spectroscopic Measurements

UV absorption spectra were measured using a Cary 219 spectrophotometer. CD spectra were recorded with a Jasco CD/ORD-5 with Sproul SS-20 specifications. The 88.7 MHz ^{113}Cd nmr spectra at ambient probe temperature were obtained with broad band proton decoupling using a Bruker WH-400 nmr spectrometer. The spectrum of the native protein in a 10 mm od nmr tube was measured with 13600 90° (39 μs) pulses at 2.5 s^{-1} and a spectra width of 20 kHz, and that of the reconstituted protein with 11700 90° pulses at 8 s^{-1} and a spectral width of 62.5 kHz. The spectrum of the acidified protein required 20 45° pulses at 3 min^{-1} with a spectral width of 50 kHz. The spectra were referenced to 4 *M* $Cd(NO_3)_2$ (aq) by sample interchange and converted to 0.1 *M* $Cd(ClO_4)_2$ as reference using δ (0.1 *M* $Cd(ClO_4)_2$) = δ (4 *M* $Cd(NO_3)_2$) − 65 ppm; chemical shifts to lower shielding than the reference were taken as positive. The spectra shown in Fig. 2 contain 20 Hz line broadening. A few of the ^{113}Cd nmr spectra of free cadmium were measured using an XL-100 spectrometer system operating at 22.2 MHz. No proton decoupling was used and typically *ca.* 18000 30° (24 μs) pulses at 17 min^{-1} with a spectral window of 10 kHz gave an adequate signal; noise for a 0.08 M sample of 'cold' $CdCl_2$ in a 12 mm od sample tube. Attempts to measure the 298 K 71.5 MHz ^{199}Hg nmr spectrum of ^{199}Hg-MT from horse liver (see above) were also made using the WH-400 spectrometer. Repetitive 90° (55 μs) pulsing for periods of several hours using spectral windows covering the range deshielded by 1300–2700 ppm from the resonance of 0.1 *M* $Hg(ClO_4)_2$ in 5% $HClO_4$ (the region where soluble thiolatomercurates resonate [30]), gave no discernable signals.

Results and Discussion

^{113}Cd Nmr Spectra of Native Cd,Zn-MT and the Reconstituted Protein

Figure 1 shows the UV and CD spectra of native Cd, Zn-MT at pH 7.9 and the reconstituted protein at pH 7.6. It has been shown previously [7, 9] that the metal ions in Cd,Zn-metallothionein are displaced from the binding sites at low pH. Following neutralization to pH 7.6, the sulfur to cadmium charge transfer absorption near 250 nm returns to approximately the same intensity as in the native protein, which we take as an indication that the Cd–S bonds have reformed. At the same time, a return of the 260 nm CD band is observed, though this new band has a slightly lower intensity than that observed for the native protein, Fig. 1A. The reappearance of the CD intensity suggests that the cadmium goes back into binding sites that have a similar geometry to the original sites; however, the small decrease in intensity is thought to be the result of the inability of the zinc to rebind to the protein after the pH cycle [9].

Although the reappearance of intensity in the 250 nm shoulder in the absorption spectrum recorded following neutralization, Fig. 1B indicates charge-transfer activity and thus, in this case, that the cadmium has been bound to the thiolate groups in the protein, the magnitude of the intensity does not provide specific details above the geometry of the binding site; also the lack of resolution in this band means that band centre and width measurements cannot be used with certainty as indicators of changes in coordination. Magnetic circular dichroism spectroscopy is sensitive to coordination geometry but is insensitive to the protein conformation adopted as a result of the coordination of the cadmium by the thiolate groups on the cysteine residues. MCD spectra of the native protein simply show a derivative envelope that arises from the near-tetrahedral geometry of the cadmium sites [14, 26]. CD spectra measured for a solution that had been neutralized after acidification, Fig. 1B, provides evidence that the asymmetric conformation of the protein around the metal sites is the same or very similar following remetallation, because the CD intensity arises from mixing of the chirality in the coordinating cysteines and the protein into the sulfur → cadmium charge transfer state through the extrinsic Cotton effect.

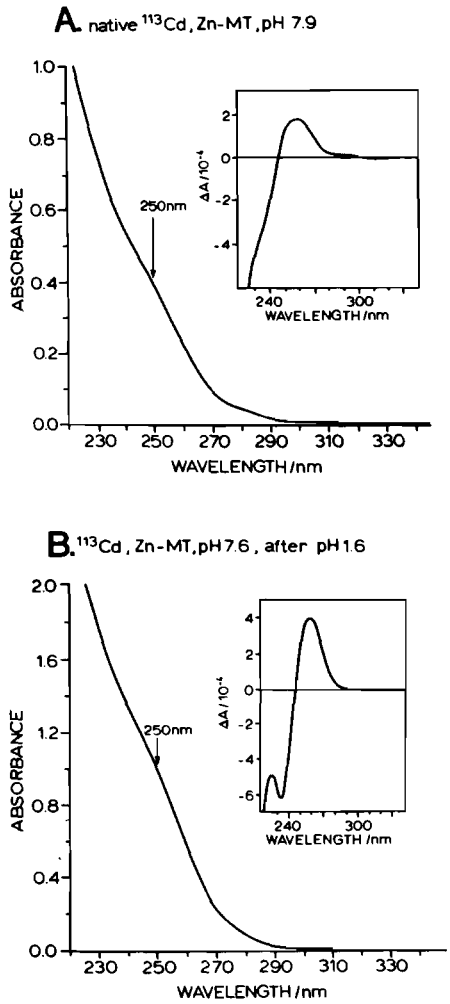

Fig. 1. Absorption and circular dichroism spectra of ^{113}Cd, Zn-metallothionein. (A) Spectra recorded at pH 7.9; the solution was made by diluting the solution used for the ^{113}Cd nmr experiment. (B) Spectra recorded at pH 7.6 after the solution used for the pH 7.9 ^{113}Cd nmr spectra had been acidified to pH 1.6, and then neutralized by the addition of aliquots of NaOH; the solution was made by diluting the solution used for the ^{113}Cd nmr experiment. Note, the absorbance values in (A) and (B) are different because two separate solutions were used.

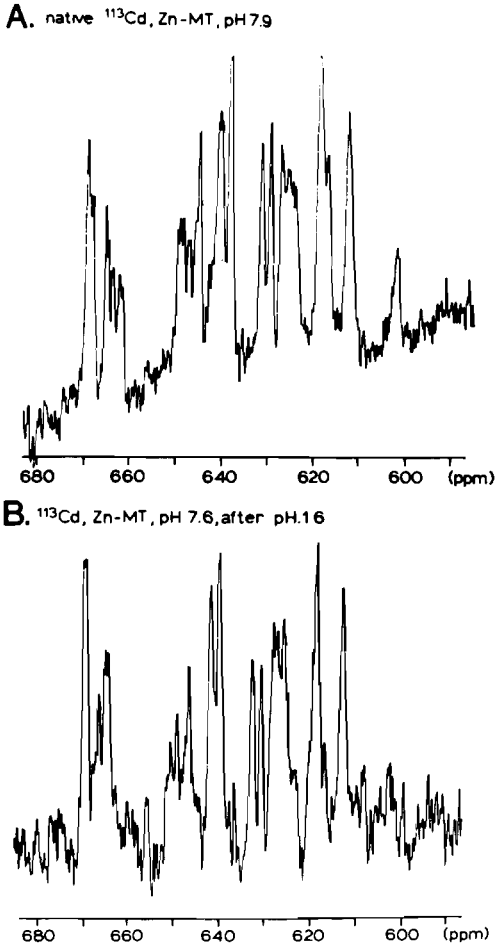

Fig. 2. ^{113}Cd nmr spectra of ^{113}Cd,Zn-metallothionein. (A) The native protein at pH 7.9. (B) The spectrum was recorded at pH 7.6 after the solution used for (A) had been acidified to pH 1.6, then reneutralized to pH 7.6 with aliquots of NaOH.

These data suggest that metal coordination is the same in the reconstituted protein as in the native protein, but the optical techniques do not provide information about the relative positions of the seven metal ions within the protein. Thus, although ^{113}Cd nmr studies of rabbit, calf and crab metallothioneins [21–24] have indicated the presence of a trinuclear and a tetranuclear thiolate cluster, analysis of the optical data cannot yet show which of the clusters is occupied, or give information on the distributions of different metal ions between the two clusters (for example, when both cadmium and zinc are present), or even indicate whether the arrangement of the bridging thiolates is the same as in the native protein. In contrast, the ^{113}Cd nmr spectrum of a species with more than one cadmium is sensitive to the proximity of the cadmium ions and this sensitivity can lead to a unique mapping of the distribution of the metal ions in three dimensional space. This map should, of course, be complementary to the result given by the CD experiment.

The 88.7 MHz ^{113}Cd nmr spectra of native rat liver ^{113}Cd,Zn-MT and the reconstituted protein are shown in Fig. 2. The distinct features in the spectrum of the native protein are the nine areas of resonance centred at *ca.* 671, 665, 648, 639, 631, 626, 618, 612 and 602 ppm relative to 0.1 *M* $Cd(ClO_4)_2$. These chemical shifts are very similar to those observed for native Cd,Zn-MT from rabbit liver [21, 22] which suggests that the binding sites in proteins from rat

and rabbit liver are essentially the same. Multiplet character is evident in many of the resonances in Fig. 2A. This fine structure has been shown [21, 22] to result from $^{113}Cd-^{113}Cd$ scalar coupling in polynuclear metal clusters. When the pH of this solution was lowered to 1.6 with aliquots of concentrated HCl, a signal due to 'free' Cd was observed with a chemical shift of 186 ppm from 0.1 *M* $Cd(ClO_4)_2$; this shows that complete dissociation of the metal ions from the protein binding sites has taken place. The absorption and CD spectra at pH 1.6 did not show any intensity at 255 nm, indicating that the sulfur → cadmium charge transfer band was not present.

Following subsequent neutralization to pH 7.6 with aliquots of concentrated NaOH the proton decoupled ^{113}Cd nmr spectrum shown in Fig. 2B is observed for the reconstituted protein. Overall, the spectrum of the reconstituted protein is remarkably similar to that of the native protein (Fig. 2A), suggesting that upon neutralization the cadmium ions do return to binding sites that are certainly very similar, and possibly identical, to those of the native protein. (In fact the resemblance between the spectra of the native and reconstituted proteins could be even closer than Figs. 2A and B suggest, as no attempt was made to keep the ionic strengths constant in the two solutions and the different ionic strengths alone will produce slight changes between the spectra.) The only major difference between the nmr spectra of the native and reconstituted proteins is the absence of the resonance at 602 ppm in the spectrum of the latter. The same effect was observed in rabbit metallothionein where *in vitro* displacement of the zinc by added cadmium to form the cadmium-saturated protein caused the disappearance of a resonance at 602 ppm [22].

Exchange of Free Cadmium and Cadmium Bound in the Reconstituted Cd-MT

The ^{113}Cd nmr spectra of the protein show that the cadmium turnover at any of the sites is slow on the nmr timescale: slower than *ca.* 30 s^{-1} based on the measurements of $J(^{113}Cd-^{113}Cd)$ reported in Refs. 21 and 22. Exchange between free and bound cadmium probably also occurs at this rate. For practical purposes it is of interest to put a lower limit on the rate of free to bound cadmium exchange. For this purpose excess isotopically natural $CdCl_2$ was added to the reconstituted ^{113}Cd-MT at pH 6.6 (approximately 13.5 mol natural Cd/mol bound ^{113}Cd). From the ^{113}Cd nmr signal intensity in the region of free cadmium, compared with a standard natural $CdCl_2$ sample, it was clear that complete scrambling of the isotopes between free and bound reservoirs occurred in a time much less than the time of measurement (17.6 hours in the 22.3 MHz ^{113}Cd nmr spectrum). Thereafter the intensity of the free cadmium signal (due to ^{113}Cd) was not affected by acidification, confirming that complete scrambling had occurred at pH 6.6.

^{199}Hg Nmr Experiments

In addition to zinc- and cadmium-containing metallothionein, metallothionein containing other metals is also known. Mercury-metallothionein can be obtained by both *in vivo* and *in vitro* methods [31–33]. The slow exchange Hg nmr spectrum of mercury metallothionein would be a key piece of information in a comparison of the metal binding sites of cadmium and mercury metallothionein. In an attempt to obtain such a spectrum a sample of ^{199}Hg-MT was prepared by completely displacing the zinc from a sample of horse kidney Cd,Zn-MT, the displacement being followed by UV spectroscopy. This sample gave no ^{199}Hg nmr signals in the region expected for mercury thiolates [30]. We tentatively concluded that the lability of the bound mercury between sites precludes the measurement of the desired spectrum at ambient probe temperature. Mercury thiolates are known to be highly labile [30].

Conclusions

Absorption, circular dichroism and ^{113}Cd nmr spectra recorded for native rat liver ^{113}Cd,Zn-metallothionein at pH 7.9 and at pH 7.6 following acidification to pH 1.6, demonstrate conclusively that the cadmium ions which bind to metal-free metallothionein *in vitro* do so at sites that closely resemble the sites occupied by the cadmium when bound *in vivo*. These data indicate that both the protein conformation and the two thiolate bridged clusters of cadmium ions that have been observed in the native protein are re-established when metal-free metallothionein binds cadmium *in vitro*. The exchange between cadmium in these sites and free cadmium is facile, and within the 18 hours it takes to record the ^{113}Cd nmr spectrum complete scrambling between the two pools of cadmium occurs. Finally, mercury in Hg-MT is probably labile on the nmr timescale and no ^{199}Hg nmr spectrum could be observed in the region expected for mercury thiolate complexes.

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References

1 P. Pulido, J. H. R. Kagi and B. L. Vallee, *Biochemistry, 5,* 1768 (1966).
2 M. Nordberg and Y. Kojima, in 'Metallothionein', eds., J. H. R. Kagi and M. Nordberg, Birkhauser, Basel, Switzerland, (1979), pp. 41–116.
3 Y. Kojima and J. H. R. Kagi, *Science, 3,* 90 (1978).
4 W. E. Rauser and N. R. Curvetto, *Nature, 287,* 563 (1980).
5 M. Margoshes and B. L. Vallee, *J. Am. Chem. Soc., 79,* 4813 (1957).
6 M. Vasak, J. H. R. Kagi, B. Holmquist and B. L. Vallee, *Biochemistry, 20,* 6659 (1981).
7 J. H. R. Kagi and B. L. Vallee, *J. Biochem., 236,* 2435 (1961).
8 U. Weser, H. Rupp, F. Donay, F. Linneman, W. Voelter, W. Voetsch and C. Jung, *Eur. J. Biochem., 39,* 127 (1973).
9 A. Y. C. Law and M. J. Stillman, *Biochem. Res. Commun., 94,* 138 (1980).
10 M. J. Stillman, A. Y. C. Law, A. Turko, P. Pisters and P. A. W. Dean, unpublished.
11 H. Rupp and U. Weser, *Biochim. Biophys. Acta, 533,* 209 (1971).
12 H. Rupp, W. Voelter and U. Weser, *Z. Physiol. Chem., 356,* 755 (1975).
13 G. Sokolowski and U. Weser, *Physiol. Chem., 356,* 1715 (1978).
14 A. Y. C. Law and M. J. Stillman, *Biochem. Biophys. Res. Commun., 102,* 397 (1981).
15 M. Vasak, H. A. O. Hill, A. Galdes, J. H. R. Kagi, F. Bremner and G. W. Young, *Biochemistry, 19,* 416 (1980).
16 M. Vasak, *J. Am. Chem. Soc., 102,* 3953 (1980).
17 M. Vasak, J. H. R. Kagi and H. A. O. Hill, *Biochemistry, 20,* 2852 (1981).
18 M. Vasak and J. H. R. Kagi, *Proc. Natl. Acad. Sci. USA, 78,* 6709 (1981).
19 P. J. Sadler, A. Bakka and P. J. Beynon, *FEBS Lett., 94,* 315 (1978).
20 K. T. Suzuki and T. Maitani, *Experientia, 34,* 1449 (1978).
21 J. D. Otvos and I. M. Armitage, *J. Am. Chem. Soc., 101,* 7734 (1979).
22 J. D. Otvos and I. M. Armitage, *Proc. Natl. Acad. Sci. USA, 77,* 7094 (1980).
23 R. W. Briggs and I. M. Armitage, *J. Biol. Chem., 257,* 1259 (1982).
24 J. D. Otvos, R. W. Olafson and I. M. Armitage, *J. Biol. Chem., 257,* 2427 (1982).
25 R. A. Haberkorn, L. Que (Jr.), W. O. Gillum, R. H. Holm, C. S. Lui and R. C. Lord, *Inorg. Chem., 15,* 2408 (1976).
26 G. K. Carson, P. A. W. Dean and M. J. Stillman, *Inorg. Chim. Acta, 56,* 59 (1981).
27 P. Dubois Murphy, W. C. Stevens, T. T. P. Cheung, S. Lacelle, B. C. Gerstein and D. M. Kurtz (Jr.), *J. Am. Chem. Soc., 103,* 4400 (1981).
28 A. J. Zelazowski, J. K. Piotrowski, E. M. Mogilnicka, J. A. Szymanska and B. W. Kaszper, *Bromat. Chem. Toksykol., 11,* 51 (1978).
29 A. J. Zelazowski, J. A. Szymanska and H. W. Witas, *Prepar. Biochem., 10,* 495 (1980).
30 G. K. Carson and P. A. W. Dean, *Inorg. Chim. Acta, 66,* 157 (1982).
31 J. M. Wisniewska, B. Trojanowska, J. Piotrowski and M. Jakubowski, *Toxicol. Appl. Pharmacol., 16,* 754 (1970).
32 A. Y. C. Law, J. A. Szymanska and M. J. Stillman, unpublished.
33 A. J. Zelazowski and J. K. Piotrowski, *Biochim. Biophys. Acta, 625,* 89 (1980).