ELUCIDATION OF THE STRUCTURE OF METALLOTHIONEINS BY Cd-113 AND PROTON NMR

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I. INTRODUCTION

Metallothioneins (MTs) are a class of low molecular weight (~6100 dalton), cysteine-rich, heavy metal binding proteins. First isolated over 25 years ago from equine renal cortex (1), MTs have subsequently been found to be ubiquitously distributed in the animal and plant kingdoms and also in some procaryotes (2). Heavy metals have been shown to act as inducers at the transcriptional level *in vivo* (3) and mammalian MTs contain a maximum of 7 g-atoms of metal per mole of protein. MTs are known to bind several heavy metals, including zinc, copper, cadmium and mercury and the metal composition is found to reflect the tissue of origin, the stage of development and the exposure of the organism to heavy metals (4). These properties have lead to the postulate that MTs function in heavy metal detoxification by sequestering toxic metals (Cd and Hg) (5) in addition to playing an essential role in the metabolism and/or storage of essential metals such as Zn and Cu (6,7).

Mammalian MTs contain 61 amino acids, 20 of which are cysteines while another 18 are serines and lysines. All 20 cysteines are known to participate in metal ligation via mercaptide linkages and there is a total conservation of their positions in the amino acid sequences of all the mammalian MTs that have been sequenced (8). This suggests that specific metal-thiolate interactions are structurally important and have been conserved throughout evolution to preserve the functional viability of the protein. It is also perhaps noteworthy that while MTs from lower organisms (i.e. *Scylla serrata* and *Neurospora crassa*) possess shorter amino acid chains with a smaller number of cysteines, they appear to utilize similar metal-thiolate interactions.

Additional structural characterization of this protein has been hampered for reasons related to the properties of MTs. There are no good chromophores for optical spectroscopy due to the absence of aromatic residues and histidine (9). The metal-thiolate charge transfer bands in UV and CD spectra (10,11) are indicative of metal-sulfur ligation, however, because these bands overlap severely, assertions of tetrahedral coordination, based on these data (11) must be viewed with caution. Finally, the d^{10} metal ions commonly found in MTs, Cu^{+}, Zn^{2+} and Cd^{2+}, are all ESR silent.

The application of NMR to the study of MTs has largely corrected this paucity of structural information. ^{113}Cd NMR studies on isotopically enriched ^{113}Cd-MTs have provided direct information on the structure of the cadmium binding sites and their spacial relationship (12), and 500 MHz ^{1}H NMR studies have extended our knowledge of the tertiary structure of this protein (13).

This article, which is intended to abstract our presentation at the 8th ISMAR meeting, will highlight the structural information on metallothioneins which we have obtained from ^{113}Cd and ^{1}H NMR studies.

II. CADMIUM-113 NMR STUDIES

The suitability of Cd-113 NMR to the study of the solution structure and

dynamics of biological metal coordination sites has recently been reviewed (12). While the large Cd-113 chemical shift range (~850 ppm) observed for $^{113}Cd^{2+}$-substituted metalloproteins reflects its sensitivity to the nature, number and coordination geometry of the ligands, deleterious effects from chemical exchange averaging may complicate spectral interpretations. That this latter phenomenon is not operative in the solution ^{113}Cd NMR studies of MT is indicated by the correspondence of the chemical shift in the solid (Fig. 1A & B) to that observed in solution (Fig. 1C). However, the large CSA, molecular motions on a timescale of $\lesssim 10^{-5}$s, and

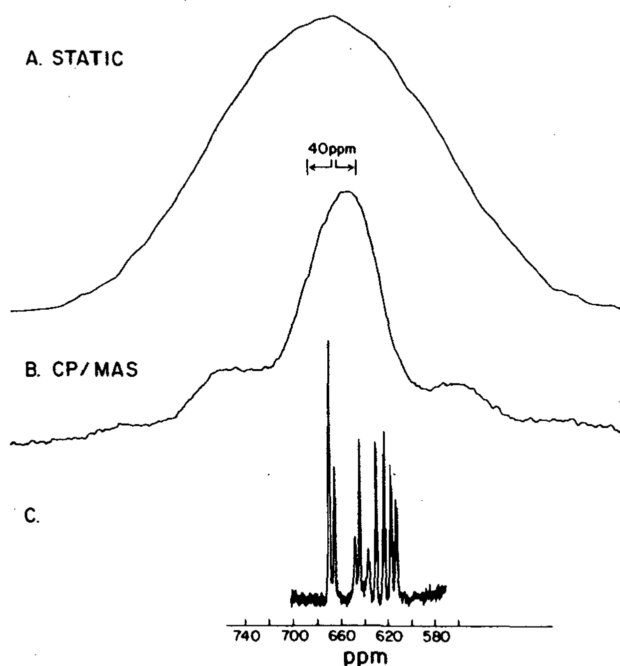

Figure 1. Comparison of the solution and solid state chemical shifts of the 44.4 MHz ^{113}Cd NMR spectra of rabbit liver ^{113}Cd-MT-1.(A) High power ^{1}H-decoupled CP spectrum, static.(B) High power ^{1}H-decoupled CP-MAS spectrum, spinning at ~4kHz. The ^{1}H pulse length was 7μsec, the contact time 4.5 msec and the repetition time 2.5 sec.(C) The ^{1}H-decoupled solution spectrum at pH 8.6 (~8mM) was accumulated using a pulse angle of 65° and a recycle time of 1.3 sec.

the multiple Cd^{2+} sites in MT precludes a further detailed structural analysis based on the solid state data. This was accomplished from the ^{113}Cd NMR spectral data in solution (Fig. 1C) where the resolution is such as to permit the observation of separate resonances for each of the metal binding sites in MT.

The proton-decoupled ^{113}Cd NMR spectra of aqueous solutions of ^{113}Cd-labeled MTs, isolated from rabbit, human, and calf livers, and from Scylla serrata hepatopancreas, are shown in Figure 2B-E. The chemical shifts of all the ^{113}Cd resonances (between 600-670 ppm) are consistent with tetrahedral coordination to cysteinyl sulfurs (12). Furthermore, the presence of ^{113}Cd-^{113}Cd scalar coupling, which gives rise to the fine structure of each of the Cd resonances, provided the first definitive evidence that the metals were clustered together rather than bound as isolated mercaptide complexes. The magnitude of the observed spin couplings (19-50 Hz) is suggestive of a two bond interaction, indicating that adjacent metals are linked by bridging thiolate ligands (12).

A detailed analysis of the data from ^{113}Cd-^{113}Cd homonuclear decoupling experiments indicated that the metals are located in two separate polynuclear clusters (12). In mammalian MTs, the 7 g-atoms of metal are located in a 3- and a 4-metal cluster. The 3-metal cluster corresponds to the cadmium resonances labeled 2,3 and 4, while the 4-metal cluster corresponds to the remaining resonances labeled 1,5,6 and 7 (Figure 2). A detailed discussion of these assignments and the origin of the duplication of the resonances from some of the sites has been presented elsewhere (13). In the invertebrate (Scylla serrata) the 6 g-atoms of metal are located in two 3-metal clusters.

The proposed cluster structures shown in Figure 2A are consistent with the ^{113}Cd NMR data and the participation of all 20 cysteines in mammalian MTs (18 Cys in crab MT) in metal

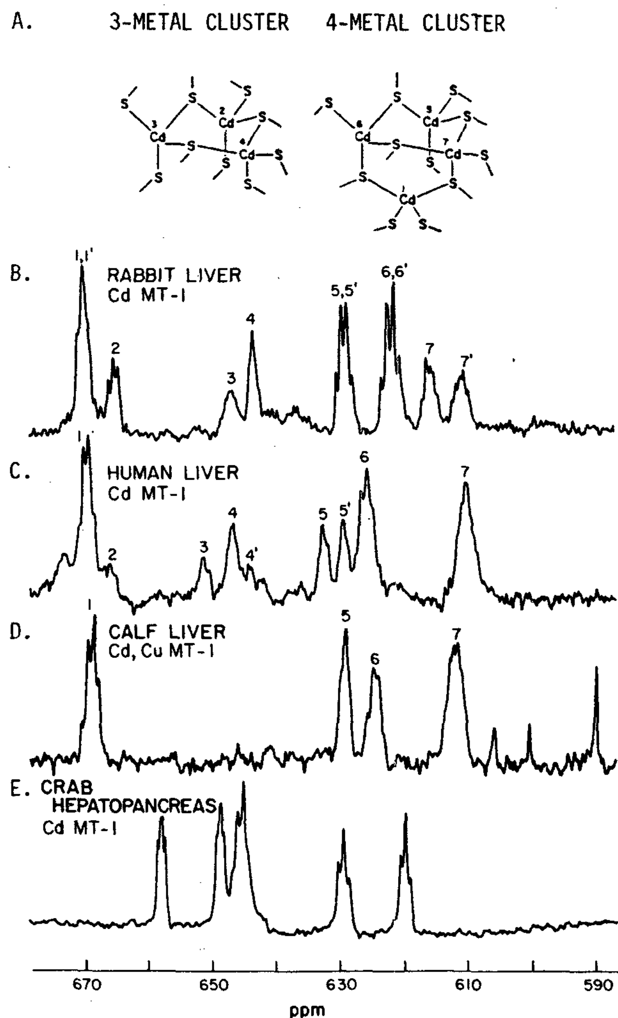

Figure 2. (A) The proposed structures of the 3-metal and 4-metal clusters of mammalian metallothioneins. (B-E) A comparison of proton-decoupled ^{113}Cd NMR spectra at 44.4 MHz of ^{113}Cd reconstituted mammalian metallothioneins and a ^{113}Cd induced invertebrate MT.

ligation. The 3-metal cluster forms a cyclohexane-like six-membered ring requiring 9 cysteine thiolate ligands, $Cd_3(Cys)_9$; while the 4-metal cluster forms a bicyclo[3:1:3] structure requiring 11 cysteine thiolate ligands, $Cd_4(Cys)_{11}$.

These same ^{113}Cd NMR spectra have also provided considerable insight into the differential metal binding affinities of the two clusters in mammalian MTs. For example, the lack of resonances from the 3-metal cluster in the spectrum of calf liver ^{113}Cd,Cu-MT-1 (Fig. 2D) reflects the preference of Cu^+ for the 3-metal cluster. Further studies indicated that the 3-metal cluster preferentially binds $Cu^+ > Zn^{2+} > Cd^{2+}$, while the reverse order of affinities applies in the 4-metal cluster.

The disposition of the two metal clusters in two distinct structural domains in MTs was provided unequivocally by the ^{113}Cd NMR spectrum of a proteolytically cleaved fragment of rat liver MT-1. The 32-residue fragment had an amino acid composition which corresponded to residues 30-61 of the MT, contained 11 cysteines, bound 4 Cd^{2+} ions and displayed a ^{113}Cd NMR spectrum which corresponded to the resonances assigned to the 4-metal cluster of the intact protein (14). This clearly established the independence of the metal clusters in the protein. The carboxyl terminal portion forms the domain of the 4-metal cluster while the amino terminal portion forms the domain of the 3-metal cluster domain.

III. PROTON NMR STUDIES

Proton NMR studies of MTs from several sources (calf, human, rat, rabbit, crab and Neurospora crassa) have provided additional information on the disposition of the metal clusters in the tertiary structure of the protein (13, 15).

A representative one-dimensional 500 MHz ^1H NMR spectrum of crab MT-1 is shown in the top portion of Figure 3. Although several resonances could be assigned by one-dimensional techniques, overlapping resonances from the large number of repetitive residues have necessitated the application of two dimensional techniques [2D J-resolved, spin-echo correlated spectroscopy and 2D correlated spectroscopy (COSY)] to definitively assign a large number of ^1H resonances (15). As an example, a

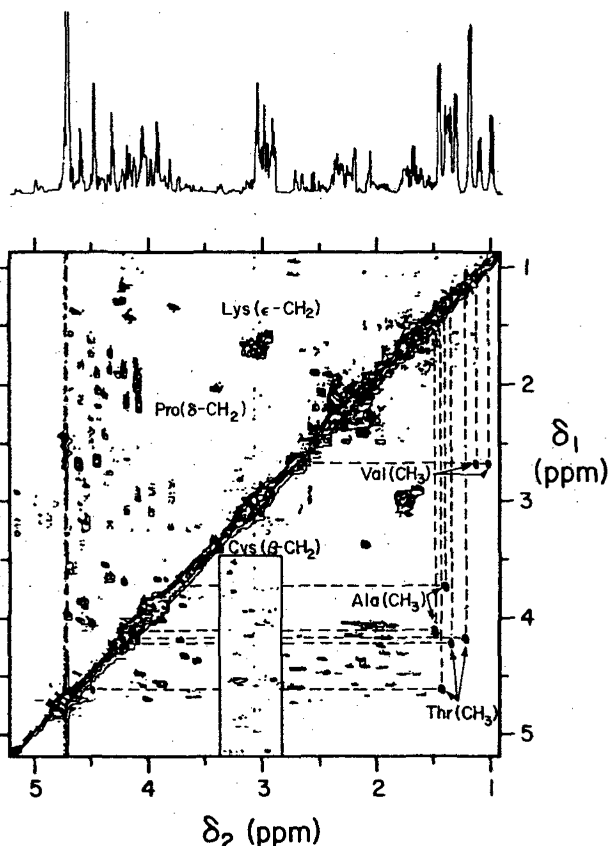

Figure 3. A contour plot of the 500 MHz 2D ^1H shift-correlated (COSY) data set of crab MT-1 (10mM) in ^2H_2O at 30°C.

contour plot of a 2D COSY data set on crab MT-1 is shown in the lower portion of Figure 3. The cross peaks in the contour plot correspond to the frequencies of the protons that are scalar coupled. The scalar connectivities between the methyl protons and the α-CH(Ala) and β-CH(Val,Thr) protons are illustrated by a dashed line in the figure connecting the cross peaks to the two coupled proton frequencies located on the diagonal. A particularly striking illustration of the resolution obtained by spreading the data into two dimensions is the complete separation of the Cys-(β-CH_2) protons from the Lys(ε-CH_2) protons.

Additional three-dimensional structural information has been obtained from ^1H-^1H nuclear Overhauser enhancement (NOE) difference spectra. Analysis of these spectra has enabled the spatial proximity of several residues to be established (13,15).

IV. CONCLUSION

Cadmium-113 and proton NMR have been key in the structural characterization of metallothioneins. Based on an analysis of these data, it has been possible to construct a model of the three-dimensional structure of the mammalian protein which is consistent with all the physicochemical data presently available. This model will almost certainly require a certain amount of refinement when the X-ray structure, currently in progress in the laboratory of C.D. Stout, becomes available. Nevertheless, we are confident that the structural refinement will be relatively modest and in no way negate our continued use of the solution derived model to elucidate the biological function(s) of MTs.

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