^{113}Cd NMR Studies on Metal–Thiolate Cluster Formation in Rabbit Cd(II)–Metallothionein: Evidence for a pH Dependence†

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ABSTRACT: The formation of two metal–thiolate clusters in rabbit liver metallothionein 2 (MT) has been examined by ^{113}Cd NMR spectroscopy at pH 7.2 and 8.6. The chemical shifts of the ^{113}Cd resonances developing in the course of apoMT titration with ^{113}Cd(II) ions have been compared with those of fully metal occupied $^{113}Cd_7$-MT. At pH 7.2 and at low metal occupancy (<4), a cooperative formation of the four-metal cluster (cluster A) occurs. Further addition of ^{113}Cd(II) ions generates all the resonances of the three-metal cluster (cluster B) in succession, suggesting cooperative metal binding to this cluster also. In contrast, similar studies at pH 8.6, at low metal occupancy (<4), reveal a broad NMR signal centered at 688 ppm. This observation indicates that an entirely different protein structure exists. When exactly 4 equiv of ^{113}Cd(II) are bound to apoMT, the ^{113}Cd NMR spectrum changes to the characteristic spectrum of cluster A. Further addition of ^{113}Cd(II) ions again leads to the cooperative formation of cluster B. These results stress the determining role of the cluster A domain on the overall protein fold. The observed pH dependence of the cluster formation in MT can be rationalized by the different degree of deprotonation of the cysteine residues ($pK_a \sim 8.9$), i.e., by the difference in the Gibbs free energy required to bind Cd(II) ions to the thiolate ligands at both pH values.

Metallothioneins (MTs)[1] constitute a class of low molecular weight cysteine- and metal-rich proteins widely distributed in nature. Although no specific biological function has yet been assigned to these proteins, it is generally believed that they play an important role in metal metabolism (Zn, Cu) and in heavy metal detoxification (Cd, Hg) (Kägi & Nordberg, 1979).

All mammalian MTs contain a single polypeptide chain with a total of 61 or 62 amino acid residues. Twenty of these residues are cysteine, which bind seven bivalent metal ions such as Zn(II) and/or Cd(II) (Kägi et al., 1984). Spectroscopic and chemical studies have established the existence of two metal–thiolate clusters containing three (cluster B) and four (cluster A) metal ions, respectively, (Otvos & Armitage, 1980; Winge & Miklossy, 1982) which are tetrahedrally coordinated by four thiolate ligands (Vašák, 1980; Vašák et al., 1981). The 3D solution structure of rabbit liver Cd_7-MT using 2D NMR (Frey et al., 1985; Braun et al., 1986) and the 3D X-ray structure of crystalline rat liver (Zn_2, Cd_5)-MT (Furey et al., 1986) have been determined. Whereas the X-ray structure and the 2D NMR solution structure differ in the assignments of a number of the sequence-specific cysteine–metal connectivities, the overall mode of metal binding within these clusters was found to be the same in both investigations (Wagner et al., 1987).

Although much knowledge has been gathered so far concerning the thermodynamic stability of both clusters, cluster B being considerably less stable than cluster A (Winge & Miklossy, 1982; Nielson & Winge, 1983), the mechanism of cluster formation and breakdown are still poorly understood. Thus, by monitoring the intensity of the Co(II) EPR signal of Co(II)-MT as a function of increasing Co(II) to apoMT ratios at pH 8.6, it was concluded that approximately the first four equiv of Co(II) are incorporated into separated sites prior to cluster formation (Vašák & Kägi, 1981). In contrast, from measurements of the Cd(II) to protein fragment ratios obtained upon the enzymatic digestion of the partially metal occupied protein at pH 7.5, it was inferred that each cluster is formed in a cooperative manner (Nielson & Winge, 1983). However, it should be noted that the latter experiments entailed a considerable amount of chemical separation. In order to support their conclusion, the same group (Byrd & Winge, 1986) performed ion-exchange chromatographic studies at pH 7.0 on apoMT to which 1–4 mol equiv of Cd(II) ions was added in the absence of the protease. The chromatographic profiles showed an increasing recovery of a charged species containing four metal ions apparently confined to cluster A.

To clarify the discrepancy between the previous results and the results obtained with the Co(II)-MT derivative, direct ^{113}Cd NMR studies were carried out to investigate the formation of the metal–thiolate clusters in MT. Moreover, since the recent titration studies of metal-free protein with Cd(II) ions at elevated pH (8.6) followed by circular dichroism spectroscopy (Willner et al., 1987) and by differential chemical modification of cysteine residues (Bernhard et al., 1986) have also indicated noncooperative cluster formation, the influence of pH upon this process was investigated. The results presented

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[1] Abbreviations: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; DTNB, 2,2′-dinitro-5,5′-dithiodibenzoic acid; Tris, tris(hydroxymethyl)aminomethane; MT, isoform metallothionein 2; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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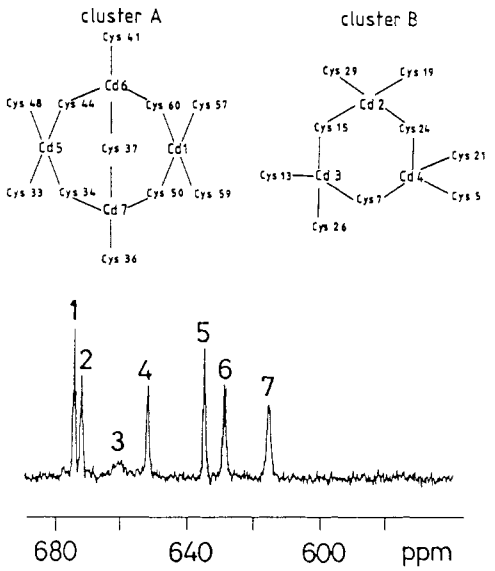

FIGURE 1: Schematic representation of the two cluster structures in $^{113}Cd_7$-MT. The numbers at the cysteines refer to positions in the amino acid sequence (top) (Frey et al., 1985). ^{113}Cd NMR spectrum of $^{113}Cd_7$-MT (bottom) at 25 °C; the numbering of the resonances corresponds to the position in the cluster (Otvos & Armitage, 1980; Frey et al., 1985). The spectrum remains virtually unchanged on going from pH 7.2 to pH 8.6 (Vašák et al., 1985).

clearly demonstrate a pH dependence of cluster formation in this protein.

Materials and Methods

Rabbit liver MT used in the present studies was purified as described (Kimura et al., 1979; Kägi et al., 1974). All MT preparations were characterized by amino acid analysis (Durrum 500) and by metal analysis using atomic absorption spectroscopy (Instrumentation Laboratory, Model IL 157). The protein concentration was determined spectrophotometrically by measuring the absorbance of the apoprotein at 220 nm in 0.01 M HCl (ϵ_{220} = 48200 M^{-1} cm^{-1}) (Bühler & Kägi, 1979). For reconstitution, all solutions used in the preparation of ^{113}Cd-MT were degassed on a vacuum line prior to use, and all manipulations were performed in an argon-purged glovebox (Vašák et al., 1985). ApoMT in 0.01 M HCl was mixed with $^{113}CdSO_4$ (^{113}Cd, >95% enriched; Harwell, England), yielding the desired metal to protein ratio. In a subsequent step the solution mixture was adjusted to pH 7.2 or 8.6 by the addition of 0.5 M Tris base (Trisma from Sigma). The metal to protein ratios were determined independently with a small aliquot of the sample. Prior to metal reconstitution, the presence of 20 cysteine residues in the apoprotein and in the partially metal occupied MT was confirmed with Ellman's reagent (DTNB) in 10 mM potassium phosphate buffer, 2 M guanidinium chloride, and 20 mM EDTA, pH 7.5 (ϵ_{412} = 13600 M^{-1} cm^{-1}; McGilvray & Morris, 1971).

^{113}Cd NMR spectra were recorded at 88.8 MHz on a Bruker AM-400 spectrometer. Aqueous solutions of MT (ca. 2–3 mM), containing 10% ^2H_2O to provide the field-frequency lock, were placed in 10-mm NMR tubes and sealed under reduced argon atmosphere. Typical acquisition parameters were as follows: 60° pulse; acquisition time 0.2 s (with ^1H broad-band decoupling); and relaxation delay 1.0 s (without ^1H decoupling). Unless otherwise stated, 60000 transients were acquired. A line-broadening function of 20 Hz was applied prior to Fourier transformation. Chemical shifts are reported in parts per million to high frequency of the ^{113}Cd resonance of 0.1 M $Cd(ClO_4)_2$ in ^2H_2O.

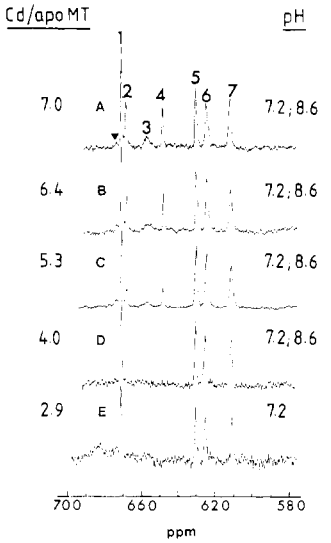

FIGURE 2: ^{113}Cd NMR spectra of ^{113}Cd-MT as a function of ^{113}Cd to apoMT ratio obtained at pH 7.2 and 8.6 (25 °C), except for E. Since spectra A–D were virtually unchanged at both pH values, only spectra at pH 7.2 are shown. As to the pH dependence of spectrum E, refer to Figure 3. The numbering of resonances in spectrum A refers to the position in the cluster (see Figure 1).

Results and Discussion

Figure 1 illustrates the ^{113}Cd NMR spectrum of fully metal occupied $^{113}Cd_7$-MT produced in vitro from the metal-free form of rabbit liver MT (Vašák et al., 1987). As shown previously, the observed ^{113}Cd resonances designated 1 and 5–7 originate from the four-metal cluster (cluster A) whereas resonances 2–4 originate from the three-metal cluster (cluster B) (Otvos & Armitage, 1980; Frey et al., 1985; Otvos et al., 1985).

Figure 2 shows the ^{113}Cd NMR spectra of Cd-MT as a function of increasing Cd(II) to apoMT ratios obtained at pH 7.2 and 8.6, with the exception of Figure 2E (see below). At pH 7.2, on the basis of the identity of the chemical shifts observed in the spectrum of the partially metal occupied protein ($^{113}Cd_{2.9}$-MT) (Figure 2E) with ^{113}Cd signals 1 and 5–7 of fully metal occupied $^{113}Cd_7$-MT (Figure 2A), it is concluded that the four-metal cluster is formed first and that the nature of the metal binding is cooperative. It may be noted that in the spectrum of $^{113}Cd_{2.9}$-MT an additional rather broad ^{113}Cd NMR signal is visible at 684 ppm, which was not observed for $^{113}Cd_7$-MT. Upon the addition of a fourth equivalent of ^{113}Cd to apoMT this broad ^{113}Cd signal disappeared and only the spectral pattern characteristic of the four-metal cluster was seen (Figure 2D). Further titration with 5.3 and 6.4 equiv of ^{113}Cd (spectra C and B, respectively, of Figure 2) causes the simultaneous appearance of a set of new ^{113}Cd resonances of increasing intensity at chemical shifts identical with those designated 2–4 (cluster B) in $^{113}Cd_7$-MT (Figure 2A). The integrated intensities of the three signals are equal at each titration step. This behavior is exemplary of a cooperative metal binding into the three-metal cluster when more than 4 equiv of ^{113}Cd are added to apoMT. In this work and in all ^{113}Cd NMR studies performed so far on the fully metal occupied mammalian $^{113}Cd_7$-MTs the ^{113}Cd signals of cluster B have always been found to be lower in intensity (ca. 20%) than the cluster A resonances (Otvos & Armitage, 1980; Vašák et al., 1985; Frey et al., 1985; Otvos et al., 1985). It was this effect that prompted the detailed ^{113}Cd NMR studies of rabbit liver $^{113}Cd_7$-MT, leading to the suggestion of conformational flexibility, thereby allowing dynamic processes within the cluster structures (Vašák et al., 1985). Direct evidence for

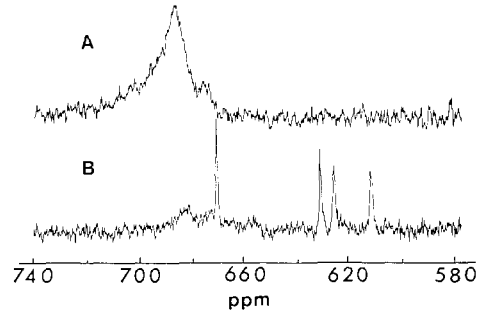

FIGURE 3: Comparison of ^{113}Cd NMR spectra of $^{113}Cd_{2.9}$-MT at pH 8.6 (A) (120000 transients) and 7.2 (B), at 25 °C (for details see text).

dynamic processes within cluster B has been provided by ^{113}Cd NMR saturation-transfer experiments, which have indicated intramolecular metal exchange (Otvos et al., 1987). Recently, the same experiments have been performed on an equimolar mixture of two rabbit isometallothioneins in which the chemical shifts of the cluster B resonances were slightly different due, presumably, to the specific substitution of amino acid residues other than cysteine. The results suggest that both intramolecular metal exchange and intermolecular metal exchange are taking place (J. D. Otvos, personal communication). The absence of such NMR-detectable processes within cluster A may account for the observed differences in intensity.

At pH 8.6 the ^{113}Cd NMR titration pattern of ^{113}Cd-MT differs significantly for low metal occupancy from that at pH 7.2. Thus when the pH of the $^{113}Cd_{2.9}$-MT sample was increased from 7.2 to 8.6, only a broad NMR feature centered at 688 ppm was seen in the region between 540 and 960 ppm (Figure 3A). The low-frequency shoulder at 673 ppm occurs at identical chemical shift with a small resonance present in the majority of our spectra (see arrow in Figure 2A) and may have a common origin. Prior to the NMR measurement, the fully metal saturated $^{113}Cd_7$-MT sample was passed through a Sephadex G-75 column to ensure size homogeneity, and since all cysteine residues in MT samples containing substoichiometrical amount of metal were accessible to DTNB modification (see Material and Methods), the presence of polymeric species, brought about by oxidation of cysteine thiolates, as a cause for the 673 ppm resonance is unlikely. The origin of this minor resonance is not yet known.

The broad ^{113}Cd NMR feature may be due to overlapping resonances, chemical shift anisotropy, chemical exchange, or a combination thereof. In order to investigate chemical exchange, the ^{113}Cd NMR spectrum of $^{113}Cd_{2.9}$-MT at pH 8.6 was taken at 1 °C. There was little change in its appearance when compared to the spectrum at 25 °C (Figure 3A). However, chemical exchange, on the basis of this experiment alone, cannot be ruled out. Although we are unable to account for the origin of the broad NMR features emerging with $^{113}Cd_{2.9}$-MT at pH 8.6, the results clearly indicate that, under these conditions, an entirely different protein structure exists.

In Figure 3 the ^{113}Cd NMR spectra of $^{113}Cd_{2.9}$-MT at pH values of 7.2 and 8.6 are compared. It is possible that the ^{113}Cd signal at 684 ppm, at neutral pH, is related to the broad features seen at high pH. This may be an indication that this form of MT also exists at pH 7.2. The chemical shifts of the broad ^{113}Cd signals at 684 ppm (pH 7.2) and 688 ppm (pH 8.6) imply that if an exchange process is involved, then it may be taking place between a cluster structure and separate tetrahedral tetrathiolate sites. Isolated $[^{113}CdS_4]^{2-}$ complexes are expected to have chemical shifts in the 700–800 ppm region, e.g., ^{113}Cd bound to four cysteine residues in horse liver alcohol dehydrogenase occurred at 751 ppm (Bobsein & Mayers, 1980). Although no indication of such a ^{113}Cd signal(s) was revealed (see above), the existence of such isolated $[MS_4]^{2-}$ metal binding sites at low metal occupancy has been demonstrated previously in titration studies of apoMT with Co(II) ions at pH 8.6 followed by EPR and electronic absorption in which an antiferromagnetic coupling, i.e., a transition from $[Co(II)S_4]^{2-}$ complexes to the cluster structure, occurred at a Co(II) to apoMT ratio greater than 4 (Vašák & Kägi, 1981). Recently, at pH 8.0, a similar transition from separate $[CdS_4]^{2-}$ complexes to the cluster structure at more than 3 equiv of Cd(II) added has been suggested on the basis of the changes developing in the electronic absorption and the circular dichroism spectra as a function of the increasing Cd(II) to apoMT ratio (Willner et al., 1987). Thus, the absence of resonances from cluster A in the spectrum of $^{113}Cd_{2.9}$-MT at pH 8.6 (Figure 3A) could be explained by the presence of separate high-affinity metal binding sites on the metal-free protein whose metal binding strength is considerably enhanced at elevated pH. It may be noted that the structural change(s) parallels (parallel) the increase in cysteine deprotonation (apparent $pK_a \sim 8.9$; Gilg, 1985). Overall, these results suggest the existence of two different cluster formation pathways, below the metal to protein ratio of 4, depending on the pH value and, consequently, a different starting polypeptide fold. In addition, the reappearance of the cluster A resonances at pH 8.6, upon the binding of the fourth equivalent of ^{113}Cd to the protein, indicates that a considerable rearrangement of the metal–cysteine bindings is taking place in favor of the apparently more stable cluster A structure.

Above 4 equiv of ^{113}Cd no pH effect is observed (Figure 2B,C)[2] It should also be noted that, in contrast to the low metal occupancy spectra (Figure 3), there is no indication of the broad downfield-shifted resonance evident. Hence, it appears that the presence of a protein population lacking cluster A is a prerequisite for the occurrence of the broad ^{113}Cd NMR profile. The virtual absence of a pH effect when the three-metal cluster is formed emphasizes the dominating role of the C-terminal four-metal cluster (A) on the overall polypeptide folding.

The next question that arises is whether the established pH dependence of the cluster formation is a property of the Cd(II)-MT derivative only or whether it is a general feature of this protein. For this purpose the pH dependence upon cluster formation in Co(II)-MT was examined. Preliminary Co(II) EPR studies performed on the sample of partially metal occupied $Co(II)_3$-MT at pH 7.2 and 8.6 showed considerable antiferromagnetic coupling occuring in the former case only (Good & Vašák, unpublished results). Since the antiferromagnetic coupling is brought about by the formation of a cluster structure (Vašák & Kägi, 1981), the observed pH effect with the Co(II)-MT derivative parallels the changes in the ^{113}Cd NMR spectra of $^{113}Cd_{2.9}$-MT at both pH values. That the spectroscopic changes at low Co(II) and Cd(II) occupancy are not due to the competition with the deprotonated buffer form employed (Tris, $pK_a = 8.3$) was ruled out by similar experiments in Hepes buffer. Moreover, on reversing the pH, the aforementioned behavior was found to be fully reversible.

[2] Assuming full metal saturation for cluster A, the overall intensity of the cluster B resonances with 5.3 and 6.4 equiv of Cd(II) is slightly lower (ca. 10% at pH 7.2 and ca. 15% at pH 8.6) than expected from the analytically determined Cd(II) to apoMT ratio. No additional resonances in the ^{113}Cd NMR spectra (Figure 2B, C) are apparent, and so this effect could be due to the presence of other minor conformations or to the errors associated with the integration of broad peaks, e.g., peak 3.

Thus the pH dependence of the filling up process appears to be an inherent property of MT molecules.

In conclusion, the results described here clearly demonstrate that the formation of metal–thiolate clusters in MT is sequential and cooperative at physiological pH, with the C-terminal cluster (A) being formed first. At elevated pH and below a metal to protein ratio of 4, an apparently entirely different protein structure is formed. Once the four-metal cluster becomes fully metal saturated, the subsequent addition of ^{113}Cd ions generates cluster B in a cooperative and pH-independent manner. At neutral pH and at low metal occupancy (<4) the cooperative binding of the metal ions into cluster A would suggest that molecules containing the fully metal occupied cluster A and apoMT are concomitantly present. The cooperative metal binding into cluster B when more than 4 equiv of Cd(II) is added to apoMT at both pH values would be consistent with the presence of MT forms containing the cluster A and the fully metal saturated Cd_7-MT. The established pH dependence of cluster formation in MT can be rationalized by the difference in the Gibbs free energy required to bind the Cd(II) ions to the predominantly protonated cysteine thiolates at neutral pH compared with an elevated pH, where their partial deprotonation occurs. In the light of the present studies a number of seemingly controversial literature reports dealing with the cluster formation in this protein, e.g., the presence of individual metal binding sites prior to cluster formation (Vašák & Kägi, 1981; Bernhard et al., 1986; Willner et al., 1987) as opposed to the cooperative buildup process (Nielson& Winge, 1983; Byrd & Winge, 1986), are understandable, providing that the pH values employed in these studies are considered.

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