Studies on the Assembly and Stability of the Metal-Thiolate Clusters of Metallothionein in Dimethyl Sulfoxide*

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Metallothioneins (MTs)1 are members of a class of proteins ubiquitous in nature that are characterized by their unusual amino acid composition and structure. These proteins are rich in cysteine residues and bind d10 metal ions such as Zn(II), Cd(II), and Cu(II), and Hg(II) with high affinity. Since the biosynthesis of MT is induced by these metals and by certain hormones, it is believed that they play a crucial role in the physiological handling of essential trace metals such as zinc and copper and in the detoxification of nonessential metals such as cadmium and mercury (1, 2).

All mammalian MTs (M, 6,000-7,000) contain a single polypeptide chain of 61 or 62 amino acids, 20 of which are cysteine residues involved in the binding of seven divalent zinc and/or cadmium metal ions (3). The isoostructural replacement of the naturally occurring metal ions by Co(II) (4, 5) and 111Cd(II) (6), followed by their spectroscopic characterization, has established the existence of two adamantine-like clusters containing three [M(4)(Cys-S)6]17− and four [M(4)(Cys-S)13]21− metal ions and possessing tetrahedral tetrahedrate metal coordination. The determination of the three-dimensional structure of metallothionein in crystals by x-ray diffraction (7) and in solution by two-dimensional NMR (8–10) confirmed the spectroscopic cluster model. However, both three-dimensional models showed marked differences regarding the assignment of the sequence specific cysteine-metal coordination bonds. The correctness of the NMR model has been confirmed by the recent reinvestigation of the crystal structure (11).

An understanding of the biological function of MT as a metal storage protein is closely related to the cluster stability, as well as with their formation and degradation. Although the pathway of cluster formation in MT has been studied (5, 12, 13), the picture is as yet incomplete. We have carried out solvent perturbation studies on MT in an effort to learn more about the factors stabilizing the three-dimensional structure and the mechanism of cluster formation. Dimethyl sulfoxide (Me2SO) was chosen because it is known to perturb the hydrogen bonding network in proteins. In the course of our spectroscopic studies, i.e. UV-visible-NIR, CD, MCD, and EPR spectroscopy on Co-MT, we have shown that the protein preserves its structural integrity in 100% Me2SO and that, most remarkably, it can refold in this solvent in discrete steps similar to those observed in aqueous solution. The structural features of the protein governing this behavior are discussed.

MATERIALS AND METHODS

Preparation of apoMT—Rabbit liver MT used in this study was isolated and purified as described previously (14, 15). Protein concentration was determined spectrophotometrically by measuring the absorbance at 220 nm in 0.1 M HCl (ε220 = 47 300 M−1 cm−1) (16). ApoMT was prepared by gel filtration using a Sephadex G-50 column eluted with 10 mM HCl. To confirm the availability of all 20 of the cysteine thiols, a small portion of the lyophilized apoMT was dissolved in 0.1 M HCl, and the protein and thiol concentrations were determined (16, 17) with typical values of 19–21 being obtained for the thiol/apoMT ratio. In the spectroscopic studies, MT-1 and MT-2 were used. Both isoforms showed the same spectral behavior.

Reconstitution of apoMT with Cobalt—All solutions used were degassed on a vacuum line prior to use, and manipulations were carried out in an argon-purged glove box. ApoMT was dissolved in degassed Me2SO, and the protein and thiol concentrations was established by determining the thiol concentration (17) of a small aliquot of the sample. MT concentrations of 0.18 and 0.8 mM were used for the absorption and EPR studies, respectively. For the reconstitution, all 20 cysteine residues and the three carboxylic groups of the protein were deprotonated by Et3N (24 eq), followed by the addition of a solution of CoCl2 (Fluka) in Me2SO to give the desired Co(II)/protein ratio. To examine whether the seven primary amine groups of the lysine residues in apoMT are protonated prior to reconstitution, 1H NMR studies were performed. For this purpose, a 1 mM apoMT solution in d6 Me2SO was prepared, and the 1H NMR spectra, in the presence and absence of 10 mol of HCl, were recorded. The close similarity of the aliphatic regions in both spectra confirmed that the lysine residues were in fact already protonated.

The metal-to-protein stoichiometries were checked at the end of the measurements using a small portion of the sample. For this

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1 The abbreviations used are: MT, metallothionein; Me2SO, di-methyl sulfoxide; Et, ethyl; Ph, phenyl; MCD, magnetic circular dichroism; NIR, near-infrared.
purpose, a portion of the reconstituted metalloprotein, still in Me$_2$SO, was placed on a Speed-Vac overnight and then on a high vacuum pump for 4 h. The residue was dissolved in 0.1 M HCl, the protein concentration was determined as described above, and the metal concentration was determined by atomic absorption spectroscopy. Me$_2$SO (Fluka, Buchs, Switzerland) and d$_6$-Me$_2$SO (Dr. Glaser, Co. Basel, Switzerland) were distilled over CaH$_2$ (Fluka) in vacuo and stored under argon. Et$_3$N (Fluka) was used without further purification.

**Physical Measurements**—UV, visible, and NMR spectra were recorded on a Perkin-Elmer Hitachi 340 spectrometer. Metal concentration was determined using an Instrumentation Laboratory Video 12 atomic absorption spectrometer. The CD and MCD spectra were recorded on a Jasco 500 spectropolarimeter interfaced with an Epson-QX PC. A field strength of 1.5 tesla was applied during the MCD measurements. The units for the absorption (ε), CD (Δε), and MCD spectra (ΔM) are M$^{-1}$ cm$^{-1}$, M$^{-1}$ cm$^{-1}$, and M$^{-1}$ cm$^{-1}$ tesla$^{-1}$, respectively, and were calculated based on protein concentration.

A Bruker ESP-300 spectrometer was used to obtain the x band EPR spectra. To determine the relative spin concentrations (18), the size of the EPR signals of the MT (0.5 mM) samples in Me$_2$SO with varying Co(I1)-to-MT ratios were compared with that of Co$_7$-MT. The samples were placed in 3-mm quartz EPR tubes sealed under argon and stored in liquid nitrogen.

**Results and Discussion**

Comparison of Co$_7$-MT Structures in Aqueous Solution and in Me$_2$SO—In order to show that the reconstitution of MT with Co(I1) ions in Me$_2$SO does not irreversibly change or alter the protein structure, spectral features in Me$_2$SO were compared with those obtained in aqueous solution. For this purpose, the absorption spectrum of Co$_7$-MT was recorded in water (Tris buffer) and, upon the solvent removal by lyophilization, recorded again in Me$_2$SO. The unaltered absorption profiles of the high energy region in both Me$_2$SO (Fig. 1, trace f) and water (19), originating from S–Co(I1) charge-transfer transitions, indicate that the metal-thiolate coordinations are preserved. The intensity and position of absorption features in the visible and near-infrared regions, characteristic of the tetrahedral tetrasulfate metal coordination (19), were also very similar. The only differences is that the lower energy separation of the resolved spin-orbit coupling components of the spin allowed $v_1(A_2 ightarrow T_1(P))$ transition. In water, the maxima and/or shoulders occurred at 600, 690, and 748 nm (19), whereas in Me$_2$SO (Fig. 1, trace f), they occurred at 620, 682, and 742 nm. The decreased energy separation in the latter solvent is consistent with the smaller departure from the $T_d$ symmetry. The observed spectral changes between water and Me$_2$SO were fully reversible regardless of the starting solvent used in the preparation of Co$_7$-MT. Note that in recent low temperature MCD studies of Co$_7$-MT, the presence of the Co$_{4+}$ and Co$_{4+}$-clusters having ground state values of S = 3/2 and S = 0, respectively (20), has been clearly established.

Cluster Formation in Co(II)-MT—The stepwise incorporation of Co(II) ions into metal-free protein in Me$_2$SO was monitored by UV-visible-NIR, CD, MCD, and EPR spectroscopy. Because of the close similarity of the spectroscopic features in Me$_2$SO and water, and since a rather detailed interpretation has been already given (5, 19), we will restrict our discussion to the features unique to the individual filling steps and differences regarding the data in water.

The UV-visible-NIR spectra are shown in Fig. 1. The intensity increase of the visible $v_1(A_2 ightarrow T_1(P))$ and the near-IR $v_1(A_2 ightarrow T_1(F))$ ligand-field transitions clearly indicate that the $T_d$ type of symmetry is preserved up to a Co/apoMT ratio of 6. The blue shift of the latter transitions when the 7th cobalt eq is bound and the increased binding capacity of MT above 7 cobalt eq, not seen in the previous studies in water (5), is discussed below. As has been noted, based on the comparison of absorption properties of the crystallographically defined inorganic mononuclear [Co(SPh)$_3$]$_2^-$ (21) and [Co(SEt)$_3$]$_2^-$ (22) complexes and the adamantane-type [Co$_6$(SPh)$_6$]$^{2-}$ (21) and [Co$_6$(SET)$_6$]$^{15-}$ (22) cluster models, the emergence of a cluster structure is characterized by a red shift of the low energy S → Co(II) charge-transfer transition and an increased absorption at about 500 nm. In Co-MT this red shift, due to the participation of bridging thiolates on metal binding, clearly developed upon the binding of more than 3 cobalt eq to the protein (Fig. 1). Convincing evidence for cluster formation is provided by corresponding changes in the EPR spectra. The EPR signals of Co-MT (Fig. 2) show features characteristic of a rhombically distorted high spin Co(II) complex (S = 3/2). The almost linear increase in the relative spin concentration up to a Co(II)/protein ratio of about 3 (Fig. 3) compares well with the previously reported EPR (5) and magnetic susceptibility (12) studies of this process in water, where the presence of mononuclear Co(II) complexes at an early stage of metal filling have been confirmed. By analogy, we suggest that also in our case no important magnetic interaction occurs. In addition, on going

![Fig. 1. Electronic absorption spectra (UV-visible-NIR) of Co-MT in 100% Me$_2$SO as a function of increasing Co/apoMT ratios.](image)

![Fig. 2. X band EPR spectra of Co-MT in 100% Me$_2$SO.](image)
The first cluster structure develops at about 4 cobalt eq. Concomitantly, the low energy electronic absorption and MCD band of the \( \nu_3 \) transition (Figs. 1 and 5) undergo a 10-nm red shift to 752 nm. In the corresponding CD spectrum (Fig. 5, top) a generation of rather strong oppositely signed CD bands with a cross-over point at 750 nm is seen. The splitting of the low energy component of the \( \nu_3 \) transition may suggest an excitonic type of contribution to the CD features of the Co\(_4\)-MT complex. The latter CD features are abolished with 7 cobalt eq. Both EPR and CD features suggest the generation of an intermediate 3-metal cluster form, i.e. \([\text{Co}_3\text{S}_9\text{I}_3^-]\) developing in the 4-metal cluster domain and a mononuclear \([\text{CoS}_4]^{2-}\) complex in the 3-metal cluster domain. The reasons for this conclusion are as follows: (a) if the Co\(_4\)-MT cluster would be formed, then the developed CD bands should be preserved also with 7 cobalt eq and (b) in view of the \( S = 0 \) ground state of the Co\(_4\) cluster (20), no appreciable EPR signal should be detected. The formation of such an intermediate cluster form at this titration step has been suggested from the previous \(^1\)H NMR and magnetic susceptibility measurements of Co-MT in water (12). Overall, the first four titration steps in Me\(_2\)SO and in water are identical. The subsequent binding of the next Co(II) equivalent yields the Co\(_7\)-MT complex, which shows a substantially increased relative spin concentration (Fig. 3) and the CD \( d-d \) transition profile in which the features of the intermediate Co\(_3\)-MT cluster are still observed (Fig. 5, top). These results are consistent with the presence of an intermediate Co\(_3\) cluster and two noninteracting tetrahedral tetrathiolate complexes in the 3-metal cluster domain, whereas in water the formation of the Co\(_4\) cluster together with one noninteracting tetrahedral tetrathiolate complex has been suggested (12). Further addition of another \( 2 \) eq of cobalt generates Co\(_7\)-MT characterized by a substantially diminished EPR intensity and a blue shift of the low energy component of the \( \nu_3 \) transition in the absorption and MCD spectra. Detailed studies between 6 and

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**FIG. 3.** Relative spin concentrations of Co-MT as a function of the Co(II)/apoMT ratios 1.5, 2.6, 3.1, 4.1, 5.2, 6.4, 7.4, and 8.7. Relative spin concentrations were obtained from the double integration of the EPR spectra of Co-MT and were normalized relative to the signal magnitude of the first titration point containing 1.5 Co(II)/apoMT. For conditions, see Fig. 2.

**FIG. 4.** High energy region of the CD (top panels) and MCD (bottom panels) spectra of Co-MT in 100% Me\(_2\)SO as a function of the Co(II)/apoMT ratio. Ratios are as in Fig. 1.

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from the mononuclear complexes to a cluster structure, marked EPR changes in the respective principal g-values from 6.10, 3.05, and 2.0 to 5.70, 3.00, and 1.90, respectively, are discernable (Fig. 2). The corresponding room temperature MCD studies show a linear increase in intensity of the four MCD bands of the \( \nu_3 \) transition, originating mainly from the paramagnetic C-term (23–26), with the first 2 cobalt eq (Fig. 4, bottom) followed by a small increase up to a Co(II)/protein ratio of 3 and only minor changes in subsequent titration steps when cluster structure(s) are formed; this is most likely due to the partial loss of the paramagnetism (C-term) (Figs. 4 and 5, bottom). From the inspection of the changes in relative spin concentration as a function of Co(II) added (Fig. 3), it appears that the cluster formation in Co-MT is stepwise.
plexes (21). In light of the present studies, the preservation of the metal-free protein is capable of refolding upon metal addition in steps similar to those found in water. To our knowledge, the majority of enzyme-catalyzed reactions in nonaqueous media, where the well defined Hg$_7$-MT complex (3, 27) is provided by the 28 metal-thiolate coordination bonds, is clearly attributed to conformational changes of the enzymes (32). How- ever, it is noted that in the recent studies on the Cd-induced dimerization of Cd$_2$-MT, the dimeric Cd-MT form contains two additional, more weakly bound Cd(II) ions/monomeric subunit.

As more cobalt is added (giving metal/protein ratios of up to approximately 20) a new absorption band at 615 nm and an isobestic point at 696 nm develop (data not shown). No structural information concerning this species is currently available. It may be noted, however, that in the titration studies of the apoprotein with Hg(II) ions in water, besides the well defined Hg$_7$-MT complex (3, 27), an increased metal binding has also been reported (28).

Protein structure—The present results show that Co-MT dissolved in 100% Me$_2$SO not only preserve its structural integrity, but more important, the metal-free protein is capable of refolding upon metal addition in steps similar to those found in water. To our knowledge, the majority of protein structures are strongly perturbed under these conditions (29–31). This fact is supported also by the recent studies of enzyme-catalyzed reactions in nonaqueous media, where the absence of enzymatic activity in 100% Me$_2$SO has been attributed to conformational changes of the enzymes (32).

The secondary structure elements of Cd-MT are limited to two short stretches of 3$_1$,- helix and a number of "half-turns" (33); it may be argued that once the MT structure containing seven metals is formed, the major contribution to its stability is provided by the 28 metal-thiolate coordination bonds. However, it should be noted that the thiolate bridges in inorganic models with adamantane-type structures, present also in MT, are disrupted in Me$_2$SO, giving rise to monometallic complexes (21). In light of the present studies, the preservation of the structural integrity of Co-MT upon the transition from water to Me$_2$SO suggests an important contribution of non-covalent interactions within the protein structure to the thermodynamic stability of this form.

However, a different starting situation exists in the filling up process of the apoprotein with cobalt. In this case, the phenomenon of "pH memory" of proteins (34) is clearly absent. The similarity between the cluster formation in Co-MT with the first 4 cobalt eq in water (12) and in Me$_2$SO, i.e. starting from different distributions of initial folding states, suggests a thermodynamically controlled folding process characterized by a multistate folding mechanism (35). The presence of thermodynamically stable intermediates further implies that short range sequence-specific interactions within the protein structure govern the individual stages of folding (35). Currently, we favor the anion-cation hydrogen bonding interactions, between the negatively charged metal-thiolate complexes and/or carboxylic acid groups, and the positively charged amine group of the seven evolutionarily conserved lysine residues as the main factor contributing to the stability of these forms. The stabilizing effect of the lysine residues on the cluster structures in Cd- and Zn-$\text{_{7}}$-MT has already been shown (36). In contrast to the first four titration steps, the last three steps in the formation of Co$_7$-MT in water (12) and in Me$_2$SO differ. The reversed order of the cluster completion may indicate the involvement of a kinetically controlled folding process (35). Further studies on the relevant structural features leading to the formation and stabilization of the metal-thiolate clusters in this protein are currently in progress.

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