Mn,Cd-metallothionein-2: A room temperature magnetic protein

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Abstract

Naturally occurring metallothionein (MT) is a metal binding protein, which binds to seven Zn^{2+} through 20 conserved cysteines and forms two metal binding clusters with a Zinc-Blende structure. We demonstrate that the MT, when substituting the Zn^{2+} ions by Mn^{2+} and Cd^{2+}, exhibits magnetic hysteresis loop observable by SQUID from 10 to 330 K. The magnetic moment may have originated from the bridging effect of the sulfur atoms between the metal ions that leads to the alignment of the electron spins of the Mn^{2+} ions inside the clusters. The protein backbone may restrain the net spin moment of Mn^{2+} ions from thermal fluctuation. The modified magnetic-metallothionein is a novel approach to creating molecular magnets with operating temperatures up to 330 K.
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Metallothionein (MT) is a metal binding protein that can bind seven divalent transition metals avidly via its 20 cysteines (Cys) [1]. These Cys' form two metal binding clusters located at the carboxyl (α-domain) and amino (β-domain) terminals of MT [2]. The two clusters were identified as α-cluster $(M_4S_{11})^{3-}$, and the β-cluster $(M_3S_9)^{3-}$ (Fig. 1) [3–5], where M denotes metal ions (Zn^{2+}, Cd^{2+}, or others), according to X-ray crystallographic and solution NMR studies [3]. MT also binds to metal ions via metal-sulfur linkages [2]. As shown in Fig. 1, the $(M_3S_9)^{3-}$ and $(M_4S_{11})^{3-}$ have the same Zinc-Blende structure which is similar to those of "diluted magnetic semiconductor (DMS)" compounds [6].

In general, semiconductors are not magnetic. However, a diluted magnetic semiconductor exhibits magnetic properties by doping with Mn and Cd or other metal ions in certain ratio. The structures ((Mn,Cd)S) of these doped semiconductors are in Zinc-Blende structure. Meanwhile these semiconductors possess magnetic property only in low temperatures, such as 4–10 K [6]. The magnetic properties may be the result of the d-sp^3 orbital hybridization and the alignment of the electron spins. The bridging sulfur atoms may also contribute to the alignment of the spins of the Mn^{2+} ions. Thus, we suspect that the entire MT, containing Mn^{2+} and Cd^{2+} (i.e., Mn,Cd-MT), may become a "magnetic protein."

Recently, the single molecule magnets (SMMs) have raised notable attention [7]. However, the Curie temperature of these molecules has to be as low as 2–4 K [7–9] to avoid the thermal fluctuation among the electron spin within the molecules [10]. To be of practical utilization, it is highly desirable to create a room temperature molecular
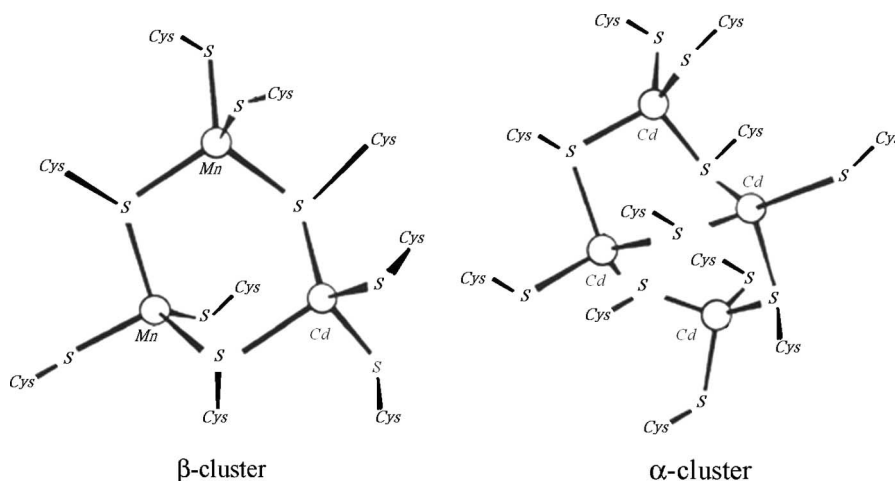
Fig. 1. Metal binding clusters of metallothionein which modified from X-ray crystal structure [2]. Where the circles denote metal ions Zn^{2+}, Cd^{2+} or Mn^{2+}. Each metal ion was linked with protein via metal-thiol bonds.

magnet [11]. With this intention in mind, we constructed and studied a new metal binding protein, metallothionein-2 (MT-2), which sustained characteristic magnetic hysteresis loop from 10 to 330 K. The protein backbone may restrain the net spin moment of Mn^{2+} ions so as to overcome minor thermal fluctuation. The magnetic-metallothionein (mMT) presented may reveal a possible approach to create high temperature molecular magnet.

Materials and methods

Materials and buffers. Native purified rabbit liver metallothionein was purchased from Sigma (St. Louis, MO). It contained six Cd^{2+} and one Zn^{2+} in each MT molecule. All other chemicals were obtained from Merck (Rahway, NJ). The denaturing/unfolding buffer (in pH 11) contained 4.5 M urea with 10 mM Tris base, 0.1 M dithiothreitol (DTT), 0.1% mannitol, and 0.5 mM Pefabloc. There were six folding buffers employed in this study. Their compositions are summarized in Table 1. Thus, the folding buffers dialyzed away the urea and mannitol in the denaturing/unfolding buffer. In the meantime, the pH value was lowered from the basic to the neutral range.

Synthesis of Mn,Cd-MT-2. A total of 5 mg of MT-2 (Sigma, St. Louis) was dissolved in 5 mL buffer solution at pH 11 to strip all metal ions. The free metal ions were removed by dialysis against the same buffer solution [12]. The denatured MT-2 was refolded by the quasi-static-procedure, developed in our laboratory [12], but with a minor modification. In addition to the basic constituents of buffer solutions [12], 1 mM Mn^{2+} and 1 mM Cd^{2+} were added in refolding buffers during the dialysis process (Steps 1–5). The excess Mn^{2+} and Cd^{2+} in the solution were removed in the last step (Step 6). Thus, the MT-2 was folded back to its native-like form [12] without excess metal ions in solution. The detailed steps were shown in the following:

Step 1: the unfolded MT (U) in the denaturing/unfolding buffer was dialyzed against folding buffer 1 for 72 h to dilute the urea concentration from 4.5 to 2 M.
Step 2: M_2 was obtained by dialyzing M_1 against folding buffer 2 for 24 h to dilute the urea concentration to 1 M.
Step 3: M_3, an intermediate without denaturant (urea) in solution, was then obtained by dialyzing M_2 against folding buffer 3 for 24 h.
Step 4: M_3 was further dialyzed against folding buffer 4 for 24 h, and the pH changed from 11 to 8.8 to produce M_4.
Step 5: the chemical chaperon mannitol was removed by dialyzing M_4 against the M_5 buffer for 8 h to yield M_5.
Step 6: finally, M_5 was dialyzed against the native buffer for 8 h to yield Mn, Cd-MT-2.

All Mn^{2+} substituted MT-2 (designated as Mn-MT-2) was also synthesized and refolded as a control. The procedures were the same as those for Mn,Cd-MT-2, except only Mn^{2+} was added into the dialysis buffer solutions.

Atomic absorption, spectroscopic, and dynamic light scattering measurements. The metal content was measured through the graphite furnace atomic absorption spectrophotometer (Z-8200, Hitachi, Japan) at 20 °C. UV spectra were recorded on a J550 (JESCO, Japan) UV-vis spectrophotometer. The size of refolded Mn,Cd-MT-2 was measured by dynamic light scattering. The light scattering apparatus was equipped with an argon ion laser-light source (4880 A) (Ion Laser Tech., UT). The scattered light was collected at 90° by a goniometer from Brookhaven Instruments (BIC, Holtsville, NY). The chamber temperature was controlled by a water circulator.

Measurement of magnetization. The magnetic moment of Mn,Cd-MT-2 was measured on solution and lyophilized powder sample, weighed 1.8 mg, by a commercial SQUID magnetometer (Quantum Design, San Diego) in a sealed capsule from 10 to 330 K. By applying a cyclic external magnetic field between 3 T and −3 T, a clear magnetic hysteresis cycle was observed (see Results and discussion).

Table 1
Chemical compositions of refolding buffers

	Tris base (mM)	pH	Urea (M)	DTT (mM)	Mannitol (%)	Pefabloc (μM)	Cd^{2+}/Mn^{2+} (mM)
Folding buffer 1	10	11	2	0.1	0.1	0.5	1
Folding buffer 2	10	11	1	0.1	0.1	0.5	1
Folding buffer 3	10	11		0.1	0.1	0.5	1
Folding buffer 4	10	8.8		0.1	0.1	0.5	1
Folding buffer 5	10	8.8		0.1		0.5	1
Folding buffer 6 (the native buffer)	10	8.8		0.1		0.5	

Results and discussion

Mn,Cd-MT-2 has been refolded via over-critical reaction path

As mentioned previously, the Mn,Cd-MT-2 and all Mn ions replaced MT-2 (Mn-MT-2) were obtained by replacing the Cd and Zn from the naturally occurring MT-2 (Sigma, St. Louis). First, the native MT-2 was denatured by stripping all metal ions in basic solution (pH 11) and then refolded back to active state by over-critical process [12] with Mn^{2+} (1 mM) and Cd^{2+} (1 mM, but no Cd^{2+} added for Mn-MT-2). We used the dialysis to avoid the surplus Mn^{2+} and Cd^{2+} in Mn,Cd-MT-2 solution. The content of metal ions in Mn,Cd-MT-2 was determined by atomic absorption spectroscopy and the analysis indicated that there are five Cd^{2+} and two Mn^{2+} ions.

According to the UV absorption spectra, Mn,Cd-MT-2 are similar to the native MT-2 (Fig. 2). We found that the metal-cluster absorption shoulder around 250 nm was slightly lower than that of the native purified MT. This is consistent with the Cd contents of Mn,Cd-MT-2 which is slightly lower than those of the native purified MT-2 (five Cd for Mn,Cd-MT-2, six Cd for purified MT-2). Meanwhile the dynamic light scattering analysis indicated that the particle size of the refolded MT molecule is identical to that of the native purified MT and the effective diameter of these molecules is 2.89 nm.

These led to the results that Mn,Cd-MT-2 was folded into their native-like conformation. Recall that the binding sites of Cd^{2+} and Mn^{2+} in the clusters are fixed due to the preferential selection feature of the metal ions [5]. Hence, we propose that two Mn ions are located in the β-domain of Mn,Cd-MT-2, i.e., the β metal binding cluster can be expressed as $(Mn_2CdS_9)^{3-}$ (Fig. 1) [5].

Magnetization measurement indicated that Mn,Cd-MT-2 is a magnetic protein

By applying a cyclic external magnetic field between 3 T and −3 T, the magnetic moments of Mn-MT-2, native MT-2 (all in a lyophilized powder), and Mn-acetate, $CdCl_2$ were measured on a commercial SQUID magnetometer (Quantum Design, San Diego) from 10 to 330 K. No hystersis loops (i.e., a straight line was observed) were obtained—meaning that there were no or minute detectable magnetic moments (data not shown). On the contrary, obvious hysteresis loops were observed for Mn,Cd-MT-2, at 10 and 325 K, after subtracting a linear diamagnetic background (Fig. 3). The magnitude of its magnetic moment was saturated at +/−0.2 T and the value of magnetic moment is about 306 emu/mol (emu, electron magnetic unit). The remanence was about 5% of the saturation and the coercive field was around 44 Oe (Oersteds) at 325 K. By comparing Mn-MT-2, native MT-2 (all in a lyophilized powder), and Mn-acetate with Mn,Cd-MT-2, we concluded that the magnetization was due to the Mn,Cd-MT-2 molecule after we ruled out the possibility of any contaminations. According to the literature, the average induced magnetization for a cluster smaller than a typical magnetic domain is equal to $(Nng_e\mu_B)^2B/(3kT)$ [13], where N and n denote the total number and the effective number of unpaired electrons of magnetic atoms in the cluster, respectively; g_e is the g factor of electrons ($g_e = 2$); μ_B, B, k, and T are the Bohr magneton, external magnetic field, Boltzmann constant, and temperature in Kelvin, respectively. We estimated accordingly that the spin alignment is around 93%. Meanwhile the thermally stable conformation of MT provides a strong restraining environment for the metal binding cluster, which in turn forces the electron spins of the magnetic ion, Mn, to line up irrespective of the ambient temperature (10 or 325 K). Therefore, the magnetic hysteresis loops at
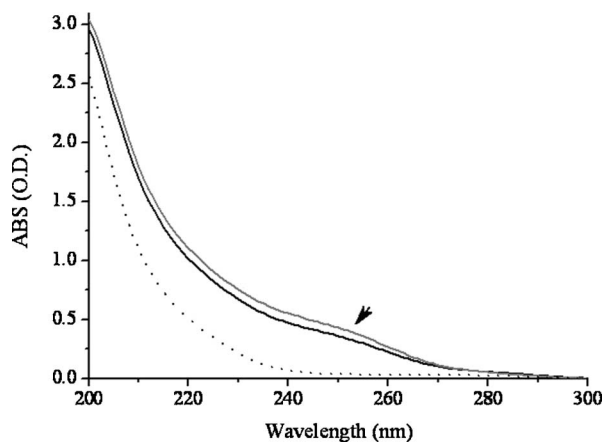
Fig. 2. UV absorption spectra of Mn,Cd-MT-2 (black line), native purified MT-2 (gray line), and apo-MT (black dot line). The arrow denotes the metal-thiol absorption region around 250 nm.
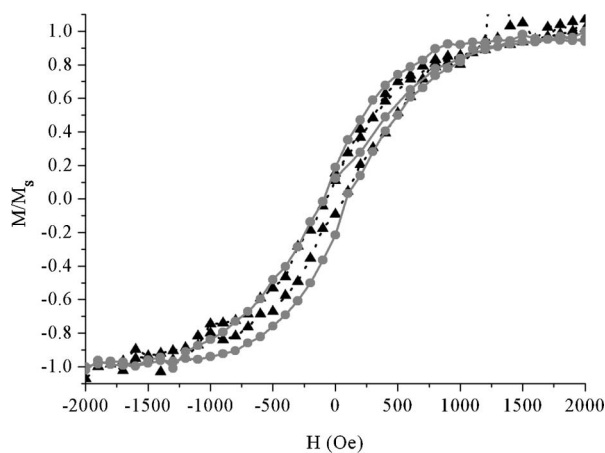
Fig. 3. Magnetization hysteresis loops of Mn,Cd-MT-2 at 325 K (the black line and symbol) and 10 K (gray line and symbol). The error bar of each data point is smaller than the symbol size. Where M_s denotes the magnetization at saturation field.

different temperatures are similar (Fig. 3). This indicates clearly that the protein is a magnetic molecule from 10 to 330 K. However, we also found that the magnetic hysteresis curve cannot be observed in solution form Mn,Cd-MT-2 (data not shown).

As we know that the magnetic hysteresis phenomenon is a collective effect, a single magnetic moment from a single molecule is too weak to work against a much stronger external magnetic field and cannot be observed, such as the case when these molecules are in solution. However, when micro-clusters form in solids, the inter-domain interactions among clusters may be large enough to be observed (Fig. 4A). However, the magnetic MT molecules, in lyophilized powder form, may form a micro-cluster and the surface potential interactions among these micro-clusters may interact with the external magnetic field and exhibit a magnetic hysteresis curve.

Protein micro-environment protects net electron spin of molecules from thermal fluctuation

The double exchange [14,15] like mechanisms of spin correlation between magnetic ions [16] may be responsible for the case of bridging ligands (i.e., sulfur atom, S). As indicated in Fig. 1, the valance bonding electrons of the bridging Cys may hop between bonded metal ions, such as Mn^{2+} and Cd^{2+}; whereas the Cd^{2+} in β metal cluster is rather important in restraining the orientation of electron spins of the bridging sulfurs and in aligning the spins of Mn^{2+} in the metal binding clusters. Therefore, this electron hopping effect may turn the Mn,Cd-MT-2 into a magnetic molecule (Fig. 4B). However, the protein backbone surrounding the β metal cluster may provide a strong restraining effect to overcome the thermal fluctuations
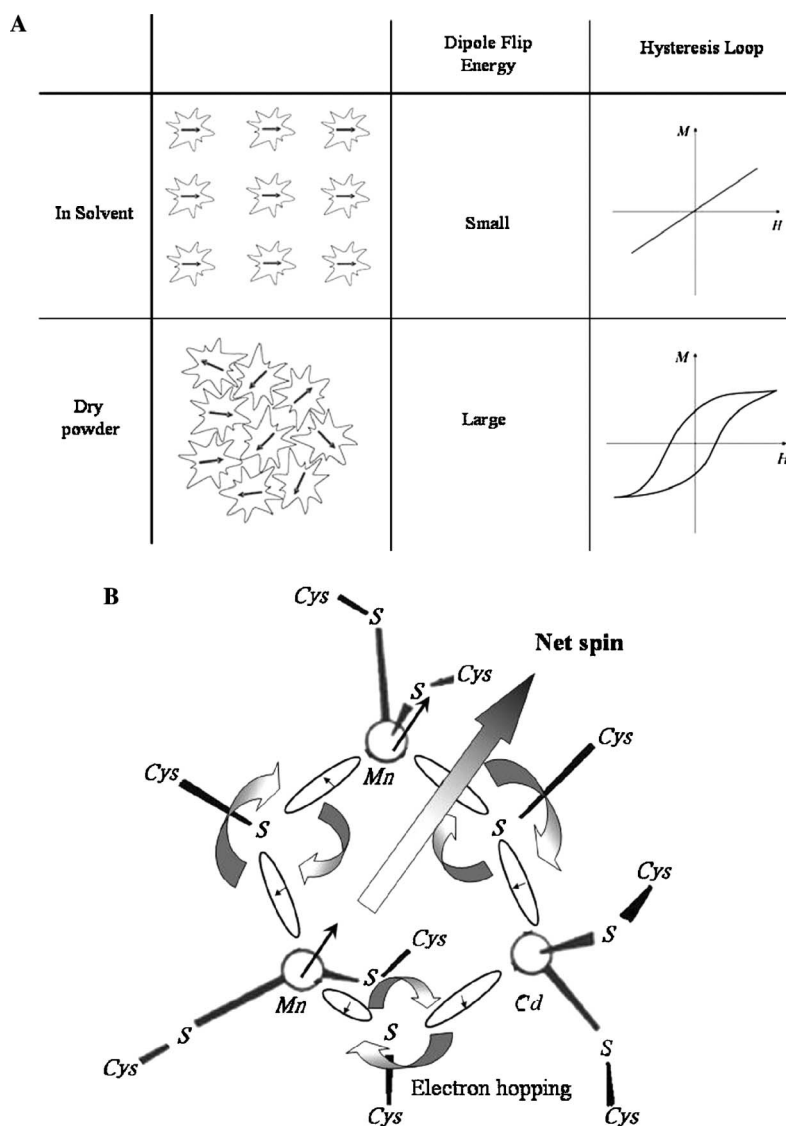

Fig. 4. Magnetization models of Mn,Cd-MT-2 molecules. (A) Magnetic hysteresis model in solution and powder forms. The star-like shapes denote the surface potential of protein molecule, in solution, or micro-cluster, in powder form. The arrows denote the net spin dipole of each molecule or micro-cluster. (B) A propose electron spin model, guided and modified by double-exchange model [16], of Mn^{2+} in β metal binding cluster of Mn,Cd-MT-2.

from the environment. Therefore, the magnetization can be observed in room temperature. However, geometrical symmetry of the spin arrangement in Mn-MT-2 may cause partial or complete cancellation of detectable magnetization. These results also indicated that the threshold temperature of molecular magnet may rise to room temperature if the proper prosthetic environment, such as protein backbone, can be linked to the thermal fluctuation of the temperature.

In summary, we have successfully constructed a molecular magnet, Mn,Cd-MT-2, that is stable from 10 to 330 K. The observed magnetic moment can be explained by the highly ordered alignment of $(Mn_2CdS_9)^{3-}$ clusters embedded in the β-domain in which sulfur atoms serve as a key bridging ligand. The discovery of magnetic-metallothionein (mMT) may allude to new schemes in constructing a completely different category of molecular magnets.

Acknowledgments

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