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The microcalorimetry study on the complexation of lead ion with metallothionein

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Abstract

Isothermal titration calorimetry was firstly applied to investigate the metal-binding properties of metallothionein. Thermodynamic data of the replacement of metallothionein (Zn_7MT) and the reconstitution of thionein (apoMT) with Pb(II) ion at pH 4.70 have been examined. The UV and CD spectrometries were also used to identify the products. The product of the reaction of Pb^{2+} with Zn_7MT (reaction 1), Pb_7MT, has a similar structure with Zn_7MT, while a new lead–MT complex, Pb_7MT', is formed in the case of reconstitution of apoMT (reaction 2). The thermodynamic parameters of the reactions for MT binding each mole of lead(II), $\Delta H_1 = -25.2$ kJ mol^{-1}, $\Delta G_1 = -28.7$ kJ mol^{-1}, $K_1 = 1.3 \times 10^5$, $\Delta S_1 = 11.6$ J mol^{-1} K^{-1} and $\Delta H_2 = -17.8$ kJ mol^{-1}, $\Delta G_2 = -32.4$ kJ mol^{-1}, $K_2 = 5.3 \times 10^5$, $\Delta S_2 = 48.8$ J mol^{-1} K^{-1}, demonstrate that both reactions are spontaneous, exothermic, and entropy-increasing processes. The contributions of relative interactions in both reactions to enthalpy and entropy changes are discussed.

Keywords: Metallothionein; Lead(II); Isothermal titration calorimetry; UV spectra; Circular dichroism

1. Introduction

Metallothioneins (MTs) [1] are a class of low molecular mass proteins characterized by rich sulphydryls and metal content. Mammalian MTs contain 61–62 amino acids of which 20 are cystein residues, therefore have high abilities to bind d^{10} metal ions. Usually, the binding number of divalent metal ions in MTs is 7, and 12 for monovalent metal ions. The structure of Cd_5Zn_2MT, Cd_7MT have been well established by X-ray [2] diffraction and 2D-NMR [3] techniques, that all seven divalent metal ions are tetrahedrally coordinated with four sulphydryls provided by cysteine residues, and two metal-thiolated clusters, $M_4S_{11}$and M_3S_9, are present in α domain and β domain, respectively. The high metal content and the unique structure of MT imply that they play a central role in metal-related cell-biological processes. Suggestions for their functions have included storage and transfer of the essential metals zinc and copper [4], detoxification of heavy metals [5], and as a radical scavenger [6].

Lead is widely recognized as a toxic substance and an environmental pollutant, which has identifiable toxic effects to the hematopoietic system and the central nervous system. Lead is known to inhibit δ-aminolevulinic acid dehydratase (ALAD)'s activity [7]. ALAD is a cytosolic enzyme that catalyzes the

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second step in heme biosynthesis and is activated by zinc, lead can displace zinc in ALAD and therefore inhibits its activity. But the inhibition is reversed in the presence of renal lead-binding protein (PbBP), a kind of zinc-containing, lead-binding MT [8]. Since the biosynthesis of MTs is induced by lead(II) [9], and MT is a major cytosolic binding site for lead(II) [10], therefore, MT may play an important role in regulating the intracellular toxicity of lead [11,12].

The complexation of lead ion to MT has been investigated by monitoring UV and CD spectra changes previously [13,14], it is found that two species, Pb_7MT and Pb_7MT', are formed at alkali (or neutral) and weak acid conditions, respectively. Especially, the new MT species Pb_7MT', which charaterized by multiple UV absorption bands and strong CD Cotton effects is very interesting, because it may have a novel MT structure motif different from general MT containing other divalent metal ions. In order to better understand the lead-binding properties of MT and the differences between Pb_7MT and Pb_7MT', isothermal titration calorimetry (ITC) is firstly used to measure the thermodynamic data of the reactions of MT and apoMT (metal-free metallothionein) with Pb(II) under same pH conditions, UV and CD spectroscopic methods are also applied to identify the products.

2. Experimental

2.1. Materials

Rabbit liver Zn_7MT2 was purchased from Wei-Ming Biological Engineering Company of Peking University. ApoMT2 was prepared by removing metal ion of Zn_7MT2 on a Sephadex G-25 column equilibrated with 0.01 mol/l $HClO_4$ solution. The concentration of MT was evaluated from its absorbance in 0.01 mol/l $HClO_4$ at 220 nm (ε_{220}=47 300 mol^{-1} l cm^{-1}) [15]. The protein sulphydryl content was measured by Ellman assay (ε_{412}=13 600 mol^{-1} l cm^{-1}) [16]. All other chemicals were analytical grade or better, and twice distilled water was used.

2.2. Methods

ITC is a computerized titration calorimeter with high sensitivity, rapid calorimetric response and fast thermal equilibration [17]. The heat signal is a nearly universal property of binding reactions. So ITC is applicable to the system of MT or apoMT binding with metal ions. ITC is also the only technique which allows simultaneous determination of all binding parameters including the reaction equilibrium constant K, the stoichiometric number n of ligand to biomacromolecule, the enthalpy and entropy changes of biological interactions.

The microcalorimetral titrations of Zn_7MT and apoMT with Pb(II) as lead acetate were carried out at pH 4.70 and T=293.15 K using a CSC Model 4200 ITC (Calorimetry Sciences). To avoid the sample oxidation, the solutions of apoMT (pH 2.0) in 0.01 mol/l $HClO_4$, lead acetate and the buffer of $HClO_4$–NaAc (pH 5.30) were all deaerated by purging with high pure N_2 for 10 min. Equal volumes of apo-MT and the buffer were mixed to adjust pH value of apoMT from 2.00 to 4.70 in a N_2-purged glovebox, then it was quickly transferred into the titration cell and sealed. All the Pb(II)-binding experiments were repeated at least three times.

For an isothermal titration, automated sequence of 25 injections, each of 4 μl, spaced at 400 s intervals, were carried out using a 100 μl injection syringe while stirring at 300 rpm. The reaction cell contained 1 ml Zn_7MT or apoMT solution. The reference cell contained 1 ml buffer solution (pH 4.70). The concentrations of Pb^{2+} in the syringe and protein in the cell were listed in Tables 1 and 2. The quantity of 4 μl Pb^{2+} was about 0.5 equiv. of MT or apoMT in the reaction cell.

Data points were collected every 4 s with no subsequent filtering by software ITC DATA COLLECTION. All of thermodynamic data arise from softwares ITC-DATA and BINDWORK supplied with the instrument, the dilution heats of $Pb(Ac)_2$ have been subtracted from the data of titration experiment using the *subtract blank* command under the *analysis* menu of ITC-DATA software. According to the previous results [13,14], the binding model of set of independent binding sites is applied to data analysis.

After each experiment, UV and CD spectra of reactants and products were recorded on a UV-240 spectrophotometer (Shimadzu, Japan) and a J-500C automatic recording spectropolarimeter (Japan Spectroscopic).

Table 1
Thermodynamic data for replacement of Zn_7MT by Pb^{2+} in $HClO_4$–NaAc buffer, pH 4.70, T=293.15 K

No.	1	2	3	Average	RMS error	S.D. (%)
$C_{Zn7MT}\times10^5$ (mol l^{-1})	9.10	6.83	4.55			
$C_{Pb(II)}\times10^2$ (mol l^{-1})	1.150	1.150	0.5750			
Equiv. of (Pb(II)/Zn_7MT)	12.6	16.9	12.6			
n	7.47	6.79	7.27	7.18	0.29	4.0
$K\times10^{-5}$	1.0	1.3	1.7	1.3	0.3	23.0
ΔH (kJ mol^{-1})	−25.3	−26.0	−24.4	−25.2	0.7	2.6
ΔG (kJ mol^{-1})	−28.0	−28.6	−29.4	−28.7	0.6	2.0
ΔS (J mol^{-1} K^{-1})	9.23	8.52	17.0	11.6	3.9	33.0

3. Results

3.1. Replacement of Zn_7MT by Pb^{2+}

The raw data of the heatflow during automatic titration of Pb^{2+} into Zn_7MT solution is shown in Fig. 1A. According to the heat effects of each titration provided by ITC-DATA analysis, the cumulative heat Q of replacement versus the molar ratio (equiv.) of Pb^{2+} to Zn_7MT is plotted in Fig. 1B, and the replacement molar number (n) 7 of Zn^{2+} by Pb^{2+} per MT molecule is determined. This binding number is the same with that provided by software, and substantiates our previous results [14]. From the similarity of UV and CD spectra with those reported previously [13], it is defined that Pb_7MT was the final product (Fig. 2A and B).

Thermodynamic data for each molar equiv. of Pb^{2+} bound to MT are summarized in Table 1.

3.2. Reconstitution of apoMT with Pb^{2+}

Fig. 3A and B show the raw data obtained from isothermal titration calorimetry and the plot of cumulative heat versus the molar ratio of Pb^{2+} to apoMT. The binding number 7 of Pb^{2+} per reconstituted MT molecule, obtained both from Fig. 3B and from software, is consistent with previous result, too. As shown in Fig. 4A and B, the unique UV and CD spectra indicate that Pb_7MT' is formed.

Thermodynamic data of the reconstitution of apoMT with Pb^{2+} is exhibited in Table 2.

4. Discussion

The plots of cumulative heat Q vs. molar ratio of Pb^{2+} to Zn_7MT or apoMT is a smooth curve and linear until 7 equiv. of Pb^{2+} added to reaction cells, suggest-

Table 2
Thermodynamic data for reconstitution of apoMT with Pb^{2+} in $HClO_4$–NaAc buffer, pH 4.70, T=293.15 K

No.	1	2	3	Average	RMS error	S.D. (%)
$C_{apoMT}\times10^5$ (mol l^{-1})	0.955	1.55	2.03			
$C_{Pb(II)}\times10^3$ (mol l^{-1})	1.150	1.621	2.875			
Equiv. of (Pb(II)/apoMT)	12.0	10.6	14.2			
n	6.66	7.30	6.69	6.88	0.29	4.3
$K\times10^{-5}$	5.5	5.8	4.7	5.3	0.5	8.8
ΔH (kJ mol^{-1})	−17.6	−18.0	−17.8	−17.8	0.2	1.0
ΔG (kJ mol^{-1})	−33.2	−32.3	−31.8	−32.4	0.6	1.8
ΔS (kJ mol^{-1} K^{-1})	49.8	48.9	47.6	48.8	0.9	1.9

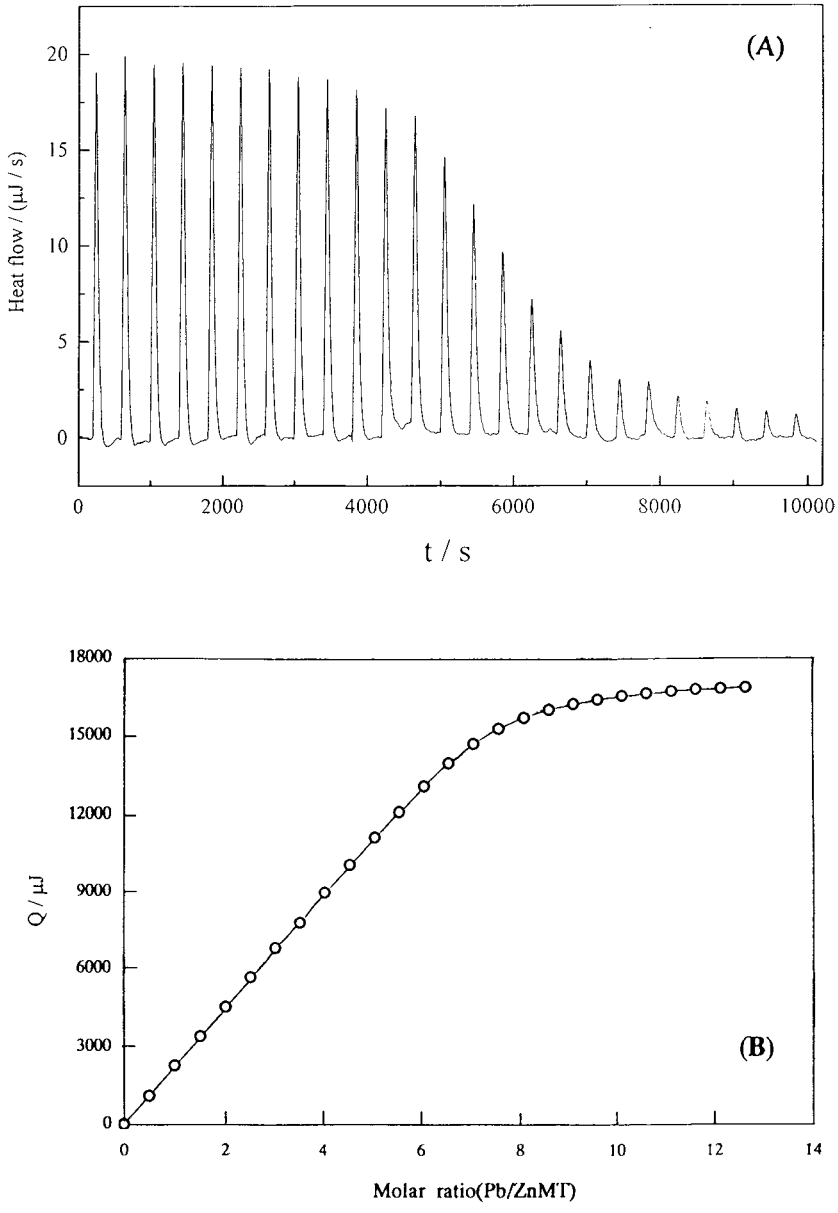

Fig. 1. ITC of $Pb(Ac)_2$ to Zn_7MT, pH 4.70, T=293.15 K. (A) ITC raw data for 25 automatic injections, each of 4 μl, of $Pb(Ac)_2$ solution into the sample cell containing 1 ml Zn_7MT solution. The concentration of $Pb(Ac)_2$ solution in the injection syringe was 1.150×10^{-2} mol l^{-1}. The concentration of Zn_7MT solution was 9.10×10^{-5} mol l^{-1}. (B) The plot of cumulative heat Q (corresponding to raw data of Fig. 1A) vs. molar ratio of $Pb(Ac)_2$ to Zn_7MT.

ing that lead-binding number is 7 both in replacement of Zn_7MT and reconstitution of apoMT with Pb^{2+}. But the great differences between their UV and CD spectra indicate that the replacement product has different conformation from reconstitution product. According to the same characters of their UV and CD spectra with those reported previously [13,14], it is easy to deduce that Pb_7MT and Pb_7MT' are the products of replacement and reconstitution, respectively. So the reaction formulations are supposed as follows:

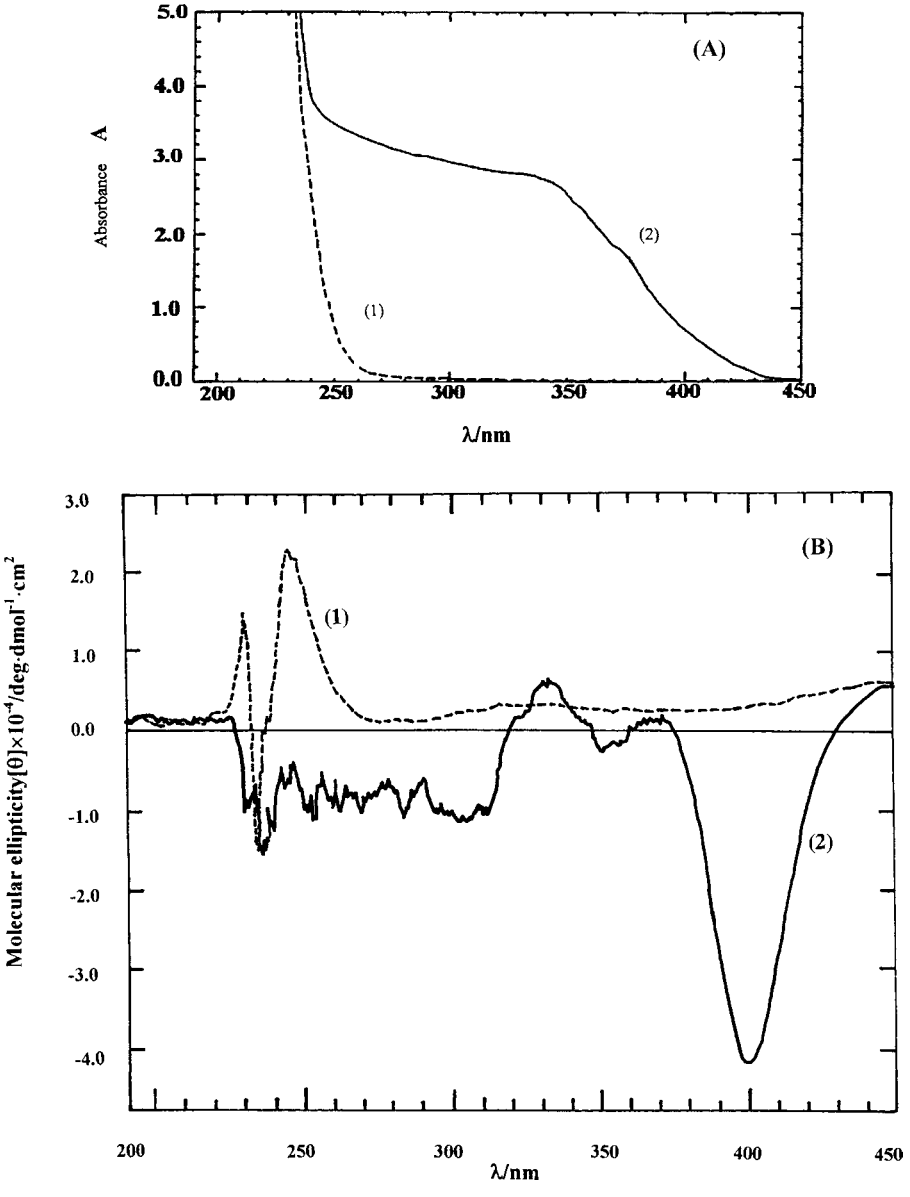

Fig. 2. (A) UV spectra of (1) Zn_7MT and (2) the substituted product Pb_7MT after the titration of Zn_7MT with $Pb(Ac)_2$. Experiment conditions were the same with Fig. 1. (B) CD spectra of (1) Zn_7MT and (2) the substituted product Pb_7MT after the titration of Zn_7MT with $Pb(Ac)_2$.

$$\text{Replacement}: \quad \tfrac{1}{7}\,Zn_7MT + Pb^{2+} \rightarrow \tfrac{1}{7}\,Pb_7MT + Zn^{2+} \quad (\text{pH } 4.70) \qquad (1)$$

$$\text{Reconstitution}: \quad \tfrac{1}{7}\,\text{apoMT} + Pb^{2+} \rightarrow \tfrac{1}{7}\,Pb_7MT' \quad (\text{pH } 4.70) \qquad (2)$$

On the base of the model of independent binding sites, the thermodynamic data were delivered by software BINDWORK and ITC-DATA.

Since the structure of Pb_7MT is similary to Zn_7MT, the factors that affect the thermodynamic data are mainly attributed to: (1) disruption of Zn–S bonds and formation of Pb–S bonds; (2) hydrated layer of lead

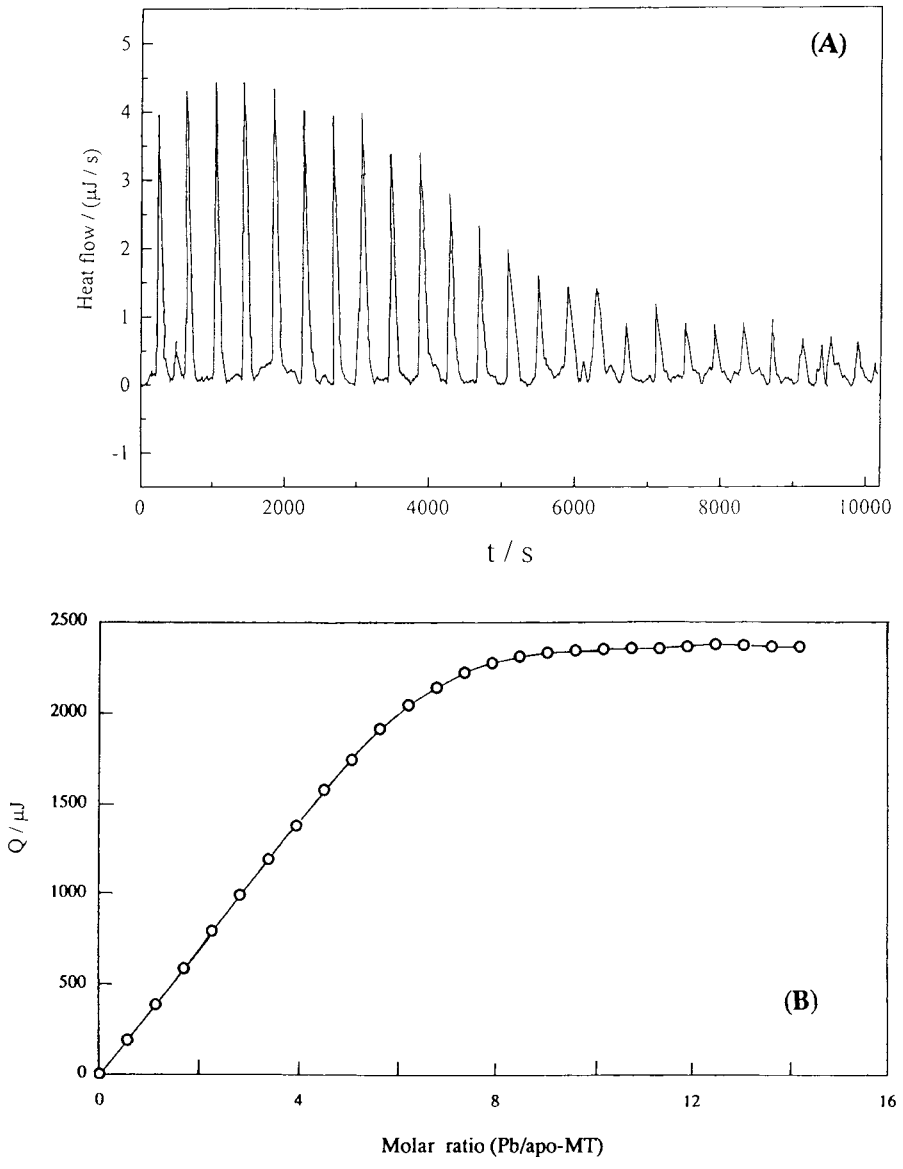

Fig. 3. ITC of $Pb(Ac)_2$ to apoMT, pH 4.70, $T = 293.15$ K. (A) ITC raw data for 25 automatic injections, each of 4 μl, of $Pb(Ac)_2$ solution into the sample cell containing 1 ml apoMT solution. The concentration of $Pb(Ac)_2$ solution in the injection syringe was 2.875×10^{-3} mol l^{-1}. The concentration of apoMT solution was 2.03×10^{-5} mol l^{-1}. (B) The plot of cumulative heat Q (corresponding to raw data of Fig. 2A) vs. molar ratio of $Pb(Ac)_2$ to apoMT.

ion broken and formation of hydrated layer of zinc ion; (3) changes of the volume of protein. The replacement of Zn^{2+} with Pb^{2+} follow the 1:1 model [14], therefore, the second factor is small enough to be omitted. After the replacement of Zn_7MT by Pb^{2+} ion, the original two-domain structure of MT might be kept, so that the ΔH_1 value is mainly attributed to the disruption of Zn–S bonds and the coordination of Pb–S. Because the latter releases more energy than that consumed by the former, this reaction is a energy releasing process ($\Delta H_1<0$). On the other hand, the radius of Pb^{2+}(1.21×10^{-10} m) is larger than that of

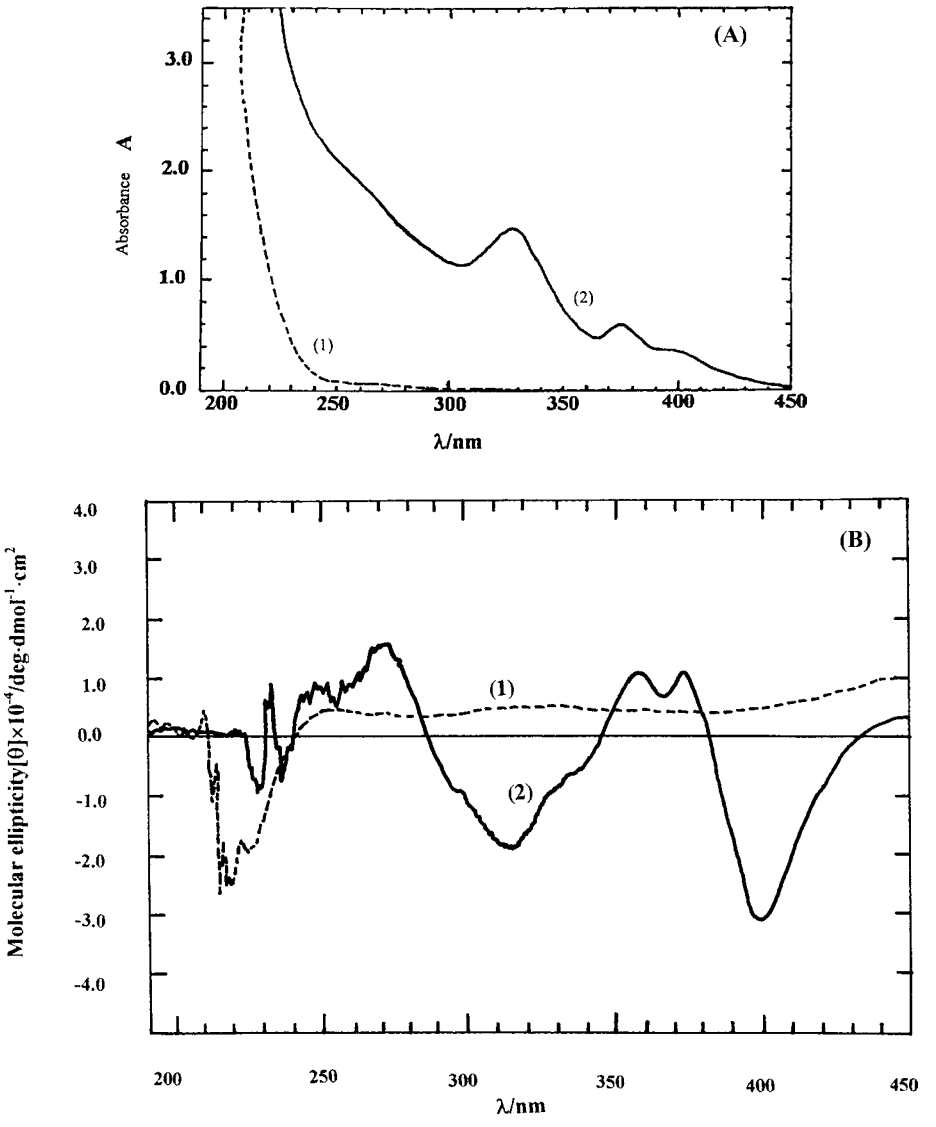

Fig. 4. (A) UV spectra of (1) apoMT and (2) the reconstituted product Pb_7MT' after the titration of apoMT with $Pb(Ac)_2$. Experiment conditions were the same with Fig. 3. (B) CD spectra of (1) apoMT and (2) the reconstituted product Pb_7MT' after the titration of apoMT with $Pb(Ac)_2$.

$Zn^{2+}(0.74\times10^{-10}$ m), the volume of MT molecule is expanded after replacement, which makes the entropy increasing.

In the case of reconstitution of apoMT with lead ion, factors determining the ΔH and ΔS values are more complex, two kinds of major interactions should be considered: (1) the coordination of thiolate ligands to lead ion and concomitant hydrated layer of lead ion broken; (2) folding of peptide backbone which was in random coil initially, including the disruption and reformation of hydrogen bonds, hydrophobic effect, Van der Waals interactions and so on. The $\Delta G_2<0$ and $\Delta S_2>0$ mean that the reconstitution of apoMT with Pb^{2+} is a entropy-increasing spontaneous process.

Although the lead-replacement and lead-reconstitution are all entropy-increasing processes, the entropy

change of reconstitution (ΔS_2=48.8 J mol^{-1} K^{-1}) is larger than that of replacement (ΔS_1=11.6 J mol^{-1} K^{-1}). Because the original structure of Zn_7MT is still retained when lead replacement takes place, the replacement has little effect on structure of solution system and free particle number in solution. In the case of reconstitution, the folding process of apoMT peptide backbone takes place concomitantly with the coordination of thiolate ligands to lead ion that makes the conformation of peptide from random to more order, the protons and water molecule bound to MT initially are released, simultaneously; the hydrated layer of lead ion is broken and releases free water molecule, all of those increase the sum of particle greatly. Therefore, the reconstitution reaction has a larger ΔS.

5. Conclusion

Thermodynamic data of replacement of Zn_7MT and reconstitution of apoMT with lead(II) ion at 293.15 K, pH 4.70 have been measured using ITC technique. From Gibbs free energy change ΔG and equilibrium constant K, and enthalpy and entropy changes, both reactions of metallothionein with lead(II) ion are spontaneous, exothermic and entropy-increasing processes. According to different characteristic UV and CD spectra, the product Pb_7MT of replacement of Zn_7MT with Pb^{2+} may keep the conformation of Zn_7MT, while Pb_7MT′ produced from the reconstitution of apoMT with Pb^{2+} at pH 4.70 may adopt a novel structure motif. Interestingly, the enthalpy change of replacement is larger than that of reconstitution of apoMT, otherwise, the values of entropy change are positive in both reactions and the ΔS of reconstitution of protein is greater than that of the replacement of Zn_7MT. Those data demonstrate that the process of reconstitution of protein from peptide in random coil is very complicated and involves multiple effects, including coordination, folding of molecular backbone, disruption and formation of hydrogen bond, the broken of hydrated layer of ions and hydrophobic interactions, Van der Waals interaction, etc. Although the contribution of those effects cannot be divided clearly up to date, the thermodynamic data in this study have made sense for structure information.

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