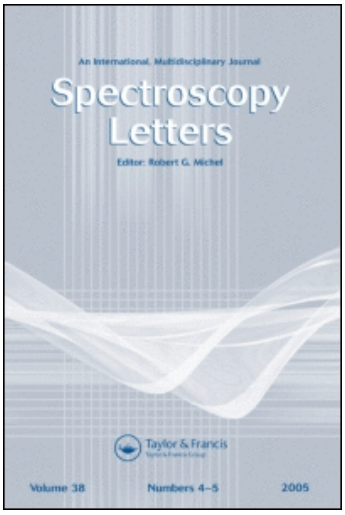

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STRUCTURE STUDIES OF PLATINOTHIONEINS BY USING EXTENDED X-RAY ABSORPTION FINE STRUCTURE SPECTROSCOPY

Key words: extended x-ray absorption fine structure spectroscopy; platinothionein; structure

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Abstract: The metal binding sites in the two Pt containing metallothioneins (Pt_7MT and $Pt_{14}MT$) were examined by means of Pt(II) L_3-edge extended X-ray absorption fine structure (EXAFS) spectroscopy. Comparisons between the phase and amplitude functions derived from the isolated shells to those of Pt···Pt, Pt-S and Pt-N model components showed that each platinum in Pt_7MT was coordinated by four sulfur atoms at a distance of 2.31±0.01 Å. Analysis of the outer shell data of platinum atom in Pt_7MT indicated backscattering platinum atom at approximate 4.29 Å. Strikingly different structural parameters had been obtained for the $Pt_{14}MT$ species, fitting of the first shell revealed that each platinum was coordinated by two sulfurs at the distance of 2.30±0.02 Å and two nitrogens at 2.02±0.02 Å. The results of the work provided the detailed information concerning the local environments of the coordinated Pt(II) in these two platinothioneins.

INTRODUCTION

Metallothioneins are metal binding proteins found in a variety of mammals [1], invertebrates [2], birds, fish and micro-organisms. These proteins are of low molecular weight (ca. 6500 daltons) and high cysteine content, containing 20 cysteines of a total of 61 amino acids [1]. The function of metallothioneins is not completely understood, although they are believed to play a general role in the metabolism of the essential metals and detoxification of heavy metals [3].

Platinum(II) complexes including cisplatin and carboplatin are used widely in treating a number of human cancers [4]. The kinetic and thermodymatic studies on the reactions cis-DDP and its derivatives with MT have been well performed [5-7]. But the detailed information on platinum atoms binding sites and coordination geometry in platinothioneins are still unclear. To shed more light on the composition and the coordination environment, a suitable method promised to be the extended X-ray absorption fine structure (EXAFS) spectroscopy. This technique has been used extensively to probe the structure of metal proteins and particular metallothioneins [8-10]. The unique advantage of this method is that the metal coordination environment can be directly obtained in the metal binding sites. Previous EXAFS studies [11-12] on copper metallothionein, Zn_7MT and Cd_7MT had provided excellent values for M-S bonds that complemented those values obtained by analysis of single crystal X-ray diffraction methods. Pattanaik *et al* [13] have characterized the metal binding sites as having a PtS_4 environment and two different Pt-S distances existed in Pt-containing metallothionein ($Pt_{10}MT$) previously. However, in their experiment the chloride was introduced in the reaction systems by using cis-DDP as a reactant, and it is difficult to distinguish Cl and S as neighbours in terms of their influence on an X-ray absorbtion spectrum. Their spectra can not be conclusive with regard to loss of the Cl ligands. In this paper, Pt_7MT and $Pt_{14}MT$ were prepared by reaction of cis-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ (cis-DAP) with apo-MT and Zn_7MT-II respectively . Thus the possibility of Cl coordinated to platinum does not exist. We measured the EXAFS of the two Pt-containing metallothioneins at room temperature and the

results of the coordinated Pt environment in these two different platinothioneins were discussed, too.

EXPERIMENTAL SECTION

Sample preparation. Rabbit liver Zn_7MT-II was isolated and purified according to literature method [14]. Apo-MT-II was prepared from Zn_7MT-II by passing the protein down a Sephadex G-25 gel column(1.6×40 cm), which had previously been equilibrated with a pH 2.0 perchloric acid. Prior to using, all the solution was degassed on a vacuum line and saturated with nitrogen gas. Preparation of Pt_7MT was carried out by addition of a small volume of 7-fold molar excess cis-DAP to apo-MT-II. The pH value was then adjusted rapidly to 7.4 by adding deaerated and saturated K_2HPO_4 solution. The reaction mixture was left standing for 72 hours at 25°C. Pt_{14}MT was prepared by adding 14-fold molar excess cis-DAP to Zn_7MT-II directly (final MT concentration was maintained at 10 μM) in 3.6mM potassium phosphate buffer solution, pH 7.4, under an anaerobic condition at 25°C. After incubation for 72 hours, both of the solutions were eluted over a Sephadex G-50 column (1.6×90 cm), with 3.6mM potassium phosphate buffer solution of pH 7.4 , respectively. The MT-containing fractions were concentrated and desalted by using an Amicon YM3 ultrafiltration membrane. The resulting products were then freeze-dried and stored at -20°C before use.

The contents of S and metal ions were determined by inductively coupled plasma (ICP) spectrometric method [15] performed on a JOBIN YVON JY 38S ICP spectrometer. The emission line is of S (181.978 nm), Pt (224.552 nm), and Zn (213.856 nm), respectively. The concentration of proteins were determined by the absorbance at 220 nm of apoMT at pH 2.0 (ε=47,300 $mol.L^{-1}.cm^{-1}$) [16-17]. During the experiment, all chemicals used were reagent grade or better and deionized water was used.

The EXAFS experiments were carried out in transmission mode on the beam 4W1B at Beijing Synchrotron Radiation Faculty (BSRF), with the storage

ring providing 2.2-GeV. The electron beam was 20~60mA. A Si (111) double crystal monochromator was used with the entrance slit set at 0.5 mm. The spectra were recorded from 11,360 eV to 12,600 eV. The energy scale at the Pt L_3-edge was calibrated with reference to the strong absorption peak of element platinum at 11,564 eV. Lyophilized samples were mounted in a sample cell of an aluminum sheet 2 mm thick and 2.5×3 cm^2 in cross-section, in which a window 4×12 mm^2 had been cut; Care was taken to make the sample thickness as uniform as possible. The two open ends of the aluminum holder were sealed with mylar film.

RESULTS AND DISCUSSION

Data analysis. Figure 1 shows a typical raw data set for Pt_7MT and $Pt_{14}MT$. The EXAFS interference function is defined as $\chi(k) = [\mu(E)-\mu_0(E)]/\mu_0(E)$, where E and k are the X-ray photon energy and the wavenumber of photoelectrons excited by the X-ray photon and $\mu_0(E)$ is the atomic absorption coefficient. The EXAFS spectra weighted k^3 of platinothionein are shown in Figure 2, which were extracted from the raw data. The pre-edge background was removed by the victoreen function and post-edge background was subtracted byathree section cubic spline fit. Then the data were normalized by the edge in transmission jump with extrapolation of a straight line fit above the edge. Through a Fourier transform, the contribution of each resolved shell in R-space, which corresponds to the absorber-scatterer distances, were back transformed with a Fourier filter and resolved into their phase and amplitude functions [18]. In this paper, these functions for Pt containing metallothionein were compared to those for a platinum-nitrogen model, bis-(dimethylglyoximato) platinum(II) [Pt-$(DMG)_2$], which has an average Pt-N distance of 1.94 Å[19], and a platinum-sulfur model, potassium tetrathiocyanatoplatinate(II), [$K_2Pt(SCN)_4$] with an average Pt-S distance of 2.32 Å[20]. To determine the Pt···Pt distance in the higher shell data, $K_2Pt(SCN)_4$ and a hexanuclear complex of platinum(II) with 2-aminoethanethiolate [$Pt_6(AET)_8$] [21]were used as models, which had absorber-scatterer distances of 3.68 Å and

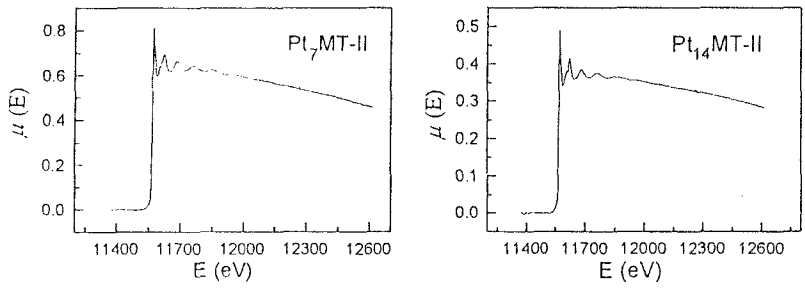

Figure 1. A typical Pt(II) L_3 absorption EXAFS data of Pt_7MT (left) and $Pt_{14}MT$ (right) set in the experiment

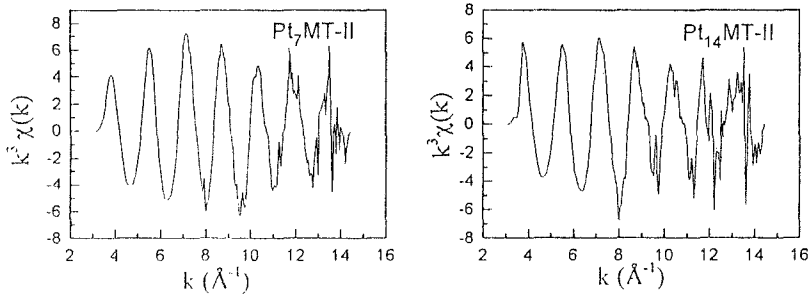

Figure 2. Background substracted, k^3-weighted, EXAFS modulations of Pt_7MT (left) and $Pt_{14}MT$ (right) obtained at 300 K.

4.29 Å, respectively. During the fitting , the EXAFS data of the models were adjusted to exactly the same lengths in the k-space as EXAFS of two platinum containing metallothionein samples.

To determine the metal-ligand distances, the data of two proteins were compared to those of model platinum complexes by fitting four parameters for each model to the sample data separately [22]. The four parameters(R, N, $\Delta\sigma^2$, ΔE_0) were obtained from a non-linear least squares fit of the Fourier filtered multishell data to EXAFS function, shown as follows:

$$\chi(k) = \sum_j \frac{N_j}{kR_j^2} s_0^2 s(k) f_j(k) e^{-2R_j/\lambda} e^{-k^2\sigma_j^2} \sin[2kR_j + \delta_j(k)]$$

Where R_j and N_j are respectively the mean internuclear distance between the central atom and the atoms of the jth neighbour shell and the coordination number of the jth shell; $S_0^2S(k)$ is a dimensionless function of k assigned to the reduction of the EXAFS signal due to multiple excitation effects; λ is the photoelectron mean free path; $f_j(k)$ is the jth atoms backscattering amplitude function; σ_j^2 is the mean-squared relative displacement in R_j, and $\delta_j(k)$ is an energy dependent phase shift in the photoelectron wave introduced by the molecular potential. k, magnitude of the photoelectron wavenumber, is given by:

$$k = [2m_e(E-E_0)]^{1/2}/\hbar$$

where m_e is the electron mass and E is the energy of the photoelectron. The relative Debye-Waller factor or thermal and lattice distortion parameter $\Delta\sigma^2$, which was highly correlated to N, is derived from amplitude function. The threshold energy ΔE_0 is expressed as differences between the values for the protein samples and those of the models [23]. The quality or "goodness of the fit " is indicated by the sum of residuals squared χ^2, calculated during the fitting procedure.

Pt_7MT. The background subtracted, k^3-weighted EXAFS is shown in Figure 2, The data for Pt_7MT, as well as for [Pt-$(DMG)_2$] and [$K_2Pt(SCN)_4$], are truncated to k = 14.5 Å^{-1} and the magnitude of Fourier-transformed of the k^3-multiplied data from Fig. 2 (left) is indicated in Figure 3 (left). Besides the most prominent peaks near 2.0 Å in the Fourier transform, another two smaller peaks are appeared at 3.4 Å and 4.0 Å, respectively. These signal peaks represent backscattering contribution of the first and outer shell atoms. The features in the transform below 1.0 Å are partially due to residual background in the k-space data and are not considered significant. Since they are well separated from the main peak.

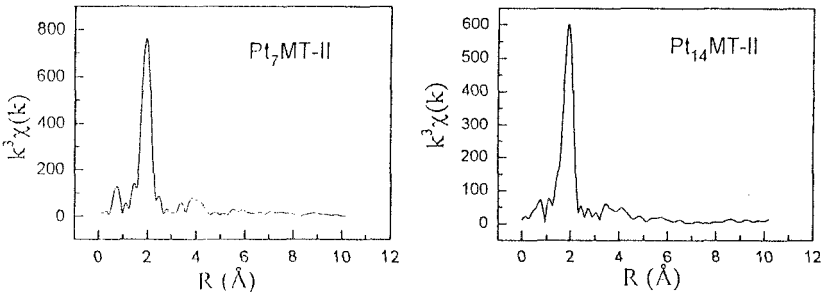

Figure 3. The magnitude of the Fourier transform of the k^3-weighted EXAFS data from **Figure** 2 (uncorrected for phase shift).

The excellent results can be obtained when fitting the main peak near 2.0 Å in **Fourier** transform with a single platinum thiolate coordination model as shown in Figure 4 (left). There are four sulfurs in the first coordination sphere of the platinum centers. The nearest neighbour bond length for Pt-S in Pt_7MT is 2.31 Å. Attempts to fit the filtered first shell data with Pt-N component or mixtures of (Pt-N and Pt-S) always give significant worse fits (indicated in **Table I**). To examine the possibility that unequal Pt-S bonds are present in Pt_7MT, the data are fitted to the four-sulfur model with different Pt-S bond distances. For all **solutions**, the introduction of unequal bond lengths do not give improvement over one-shell Pt-S fitting. So our data are in tendency to support the results that PtS_4 environment with a single Pt-S distance of 2.31 Å are in the Pt_7MT.

As the intensity and position of **the other two** bumps (3.4 Å and 4.0 Å, respectively) do not change under different testing transforms (different k range and k^n weighting). They may contain the contribution from outer shell backscattering atoms. Fit of the peak at 4.0 Å provide the satisfactory result by using Pt···Pt model for phase and amplitude standards. The result indicates that 1.16 Pt appears at 4.29 Å. The rest parameters χ^2, ΔE_0 and $\Delta\sigma^2$ in this case is 0.065, 0.04 eV, 0.2 $\times 10^{-3}$ (Å^2), respectively. As for the feature at 3.4 Å, it is difficult to interpret reliably for its weaker intensity. Perhaps it resulted from the

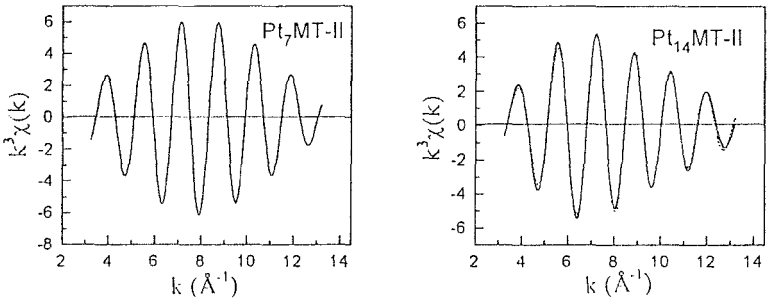

Figure 4. $k^3\chi(k)$ of the Fourier filtered first shell data (solid) and the fit (dash). Left: the fit using 4S model for Pt_7MT. Right: the fit using 2S2N model for $Pt_{14}MT$. The broken line shows the best fit.

Table I

Results of Nonlinear Least-Squares Curve-fitting for the first shell with S and N scatters in Pt_7MT

Coordination Mode	R_N (Å)	R_S (Å)	$\Delta\sigma_N^2\times10^{-3}$ (Å^2)	$\Delta\sigma_S^2\times10^{-3}$ (Å^2)	$\Delta E_{0,N}$ (eV)	$\Delta E_{0,S}$ (eV)	χ^2
4S		2.306		1.1		0.01	0.0235
3S1N	2.08	2.31	0.9	-0.2	0.05	-0.01	0.0701
2S2N	2.07	2.31	4.6	-1.1	0.05	-0.01	0.1215
1N3S	2.07	2.31	3.5	-3.8	0.00	0.00	0.2180
4N	2.13		-0.8		0.04		0.6566

contribution of remote S or Pt backscattering. The presence of resolved higher shells suggests that the absorbing metal is coordinated in a highly organized structure [24].

Overall, the EXAFS data confirmed that each platinum is coordinated by four cystein sulfurs in Pt_7MT. These data also demonstrate the similar metal-thiolate and metal-metal distances with the values obtained from the zinc

Table II

Results of Nonlinear Least-Squares Curve-fitting for the first shell with S and N scatters in $Pt_{14}MT$

Coordination Mode	R_N (Å)	R_S (Å)	$\Delta\sigma_N^2\times10^{-3}$ ($Å^2$)	$\Delta\sigma_S^2\times10^{-3}$ ($Å^2$)	$\Delta E_{0,N}$ (eV)	$\Delta E_{0,S}$ (eV)	χ^2
4S		2.283		3.0		0.04	0.1826
3S1N	2.04	2.291	0.7	0.8	0.04	0.04	0.1028
2S2N	2.02	2.295	1.6	-0.5	0.03	0.03	0.0471
1S3N	2.07	2.293	1.3	-0.7	0.15	0.15	0.3445
4N	2.10		0.9		0.0		0.6475

containing metallothioneins [25]. Considering the same stoichiometry of metal:sulhydryl as those in Zn_7MT-II and Cd_5Zn_2MT-II, it is reasonable to hypothesize that Pt_7MT should retain the two domains structure of Zn_7MT-II and Cd_5Zn_2MT-II. This hypothesis suggests that Pt(II) joins the protein in constructing two Pt-thiolate cluster.Although the details of how the additional requirement of planar coordination for each Pt(II) are accommodated by the protein structure still remain unclear.

$Pt_{14}MT$. Figure 2 (right) shows the k^3-multiplied, background subtracted EXAFS portion of the data. Figure 3 (right) indicates the magnitude of its Fourier transform. The Fourier transform also shows a dominant peak around 2.0 Å and some small bumps in the higher region which are real features confirmed by the same procedure described above. However, the intensity is too weak to be quantitatively analyzed. Again, the intensity below 1.0 Å is partially due to the residual background in k-space data.

Analysis of the first coordination sphere of $Pt_{14}MT$ is summarized in Table II. As compared with the results of the above Pt_7MT data, significant difference can be

found. Fitting of the first shell only with one Pt-S component or one Pt-N component always give worse results. The best fittings are obtained by a model in which platinum is coordinated by two sulfur atoms at the distance of 2.30 Å and two nitrogen atoms at 2.02 Å. These Pt-S and Pt-N distances are in good agreement with the values from Pt L_3-edge EXAFS spectrum of Pt_7MT described above and the X-ray diffraction of cis-$[Pt(NH_3)_2Cl_2]$ [26], respectively.

The EXAFS experiments for $Pt_{14}MT$ have confirmed that not only two sulfur atoms but also two nitrogen atoms are coordinated to each platinum center. These results imply that there is quite different structure between Pt_7MT and $Pt_{14}MT$. The details of the structure $Pt_{14}MT$ are not well known. But one point can be verified that the two-domain cluster structure, M_4S_{11} (α) and M_3S_9 (β) as that in M_7MT species (M=Zn(II) and Cd(II)) no longer exists.

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