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Comparative Raman study of four plant metallothionein isoforms: Insights into their Zn(II) clusters and protein conformations

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ABSTRACT

Four Metallothioneins (MTs) from soybean (*Glycine max*) were heterologously synthesized and comparatively analysed by Raman spectroscopy. The participation of protein donor groups (S-thiol and N-imidazol) in Zn(II) chelation, as well as the presence of secondary structure elements was comparatively analysed. Metal clusters with different geometry can be hypothesised for the four GmMTs: a cubane-like or an adamantane-like metal cluster in Zn-GmMT1, and dinuclear Zn–S clusters in Zn-GmMT2, Zn-GmMT3 and Zn-GmMT4. The latter have also a similar average Cys/Zn content, whereas a lower ratio is present in Zn-GmMT1. This is possible thanks to the involvement in metal coordination of a greater number of bridging Cys, as well as of some carboxylate groups. As regards secondary structure elements, a large content of β-turn segments is present in all four Zn-GmMTs, especially for isoforms 1 and 4. β-strands give a contribution to the folding of three GmMTs isoforms, and the highest percentage was found in Zn-GmMT2 (~45%). Conversely, the α-helix content is negligible in all the GmMTs except in Zn-GmMT3, where this peculiar feature coincides with the possible involvement of the two His residues in metal coordination. Conversely, His is predominantly free and present as tautomer I in Zn-GmMT4. In conclusion, this work illustrates the attractive potential of Raman spectroscopy, combined with other techniques, to be a very informative tool for evidencing structural differences among *in vivo* synthesized metal-MT complexes.

1. Introduction

Metallothioneins (MTs) are intracellular, low molecular weight, sulphur-rich proteins, characterized by a high content of Cys (close to a third of the total number of amino acids), and a high capacity for binding both biologically essential (Zn^{II} and Cu^{I}) and non-essential (Cd^{II}, Hg^{II} or Ag^{I}) d-block metal ions. They are widely distributed among the animal and plant kingdoms, although showing a high heterogeneity of sequences [1–3]. Primary structure of plant MTs differ from that of animal MTs by the presence of some aromatic residues and a peculiar amino acid sequence organization consisting of two or three short Cys-rich domains (containing from four to eight Cys residues each) linked by Cys-devoid regions (up to 50 residues), the so-called *linkers* or *spacers* [4]. The members of the plant MT family are characterized by great sequence diversity, requiring further division into four subfamilies based on the number and the distribution of the Cys residues [5]. Subfamilies p1, p2 and p3 show two Cys-rich domains separated by one Cys-free region, and they contain six Cys in their C-terminal Cys-rich region. Their Cys content diverges at the N-terminal Cys-rich domain, exhibiting six, eight and four Cys residues for the p1, p2 and p3 subfamilies, respectively. Contrastingly, p4 subfamily presents three Cys-rich regions that respectively contain six, six and five Cys residues, separated by two Cys-free regions which are considerably shorter than those of subfamilies p1–3.

The general structural feature that characterizes all MTs is the presence of metal-thiolate clusters, involving terminal and bridging cysteinyl thiolate groups. Recently, it has been shown that metal coordination to the MT polypeptides constitutes a more complex scenario than the simplistic consideration of metal-thiolate bonds contributed by the MT Cys residues. Particularly, the participation of other ligands in the metal coordination sphere of MTs has been shown for other amino acid side chains (His, Asp, Glu) [6–8] and for exogenous inorganic ligands, such as sulphide ions and chloride ions [9,10]. Despite the presence of His being rather unusual in most MT families, subfamily p3 soybean (*Glycine max*) GmMT (GmMT3 from now on) contains two His residues, one in the C-terminal Cys-rich domain and another one in

Abbreviations: CD, Circular Dichroism; ESI-TOF-MS, Electrospray ionization time-of-flight mass spectrometry; FWHM, Full width at half maximum; GC-FPD, Gas Chromatography-Flame Photometric Detection; GmMT, *Glycine max* metallothionein; ICP-AES, Inductively-Coupled Plasma Atomic-Emission Spectroscopy; MT, Metallothionein; M-MT, Metal-Metallothionein; QsMT, *Quercus suber* metallothionein; UV–vis, Ultraviolet–visible absorption.

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the spacer region (near the second Cys-rich domain), while one His residue is present in the spacer of GmMT4 isoform (Fig. 1 and Table 1). Interestingly, in a recent work [11], we showed that the central His of GmMT3 participates in Zn(II) binding, while the terminal His of GmMT3 and the single His of GmMT4 mainly do not.

For more than three decades, Raman spectroscopy has been used for protein investigation, allowing specific band assignments, signatures of secondary structure and Raman markers of side chain environments to be established [12,13]. Despite the well-known potentialities of this technique, to our knowledge it has been scarcely used in MT structural studies until now [14,15]. However, Raman spectroscopy is a powerful tool to unambiguously characterize two basic structural features in MTs: the participation of ligands (*i.e.* Cys, His, *etc.*) to the metal-coordination sphere and the presence of secondary structure elements.

In this work we present a comparative study of one member of each of the four subfamilies of the soybean (*G. max*) MT system (Fig. 1). GmMTs were recombinantly synthesized in *Escherichia coli*, in order to get physiologically-relevant Zn-GmMT complexes, which had been previously characterized by UV–vis, CD and ICP-AES spectroscopies and ESI-TOF mass spectrometry [11,16], and were analysed by Raman spectroscopy to shed light into their structural features. The use of the genetic engineering approach is an optimum way for obtaining significant amounts of highly pure MTs of the most diverse organisms that do not require any further manipulation prior to analysis, thus being representative of the *in vivo* folded species since they are formed into a cell environment [17,18]. Furthermore, the participation of peptidic donor groups (S-thiol and N-imidazole) in Zn(II) chelation, as well as the possible presence of secondary structure elements, were comparatively analysed for each of the four Zn-loaded GmMT isoforms by this vibrational spectroscopy. The results are discussed, together with other structural data previously obtained, in order to deepen further into the folding model of plant MTs.

2. Materials and methods

2.1. Synthesis and purification of the recombinant Zn(II)-GmMT complexes

The recombinant metal complexes were biosynthesised in 5-L cultures of *E. coli* BL21 cells supplemented with a final concentration of 300 μM $ZnCl_2$. Each of the pGEX-GmMT-1, -2, -3 and -4 expression plasmids were constructed as previously reported [16]. Expression was induced with 100 μM isopropyl β-D-thiogalactopyranoside and cultures were allowed to grow for further 3 h. Total protein extracts were prepared from these cells as previously described [17]. Metal complexes were recovered from GST-fusion constructs by batch-affinity chromatography using Glutathione-Sepharose 4B (GE Healthcare) and thrombin cleavage. After concentration using Centriprep Microcon 3 (Amicon), the metal complexes were finally separated from thrombin through FPLC gel filtration in a Superdex75 column (GE Healthcare) equilibrated with 50 mM Tris–HCl, pH 7.0. Selected fractions were kept at −80 °C until further use. As a consequence of the cloning procedure, the dipeptide Gly–Ser is added to the N-terminus of the corresponding MT polypeptides. This minor modification of the native forms was previously shown not to alter any of the MT metal-binding capacities [18].

2.2. Characterization of the Zn(II)-MT complexes

The S and Zn content of all Zn-MT preparations was analysed by means of inductively-coupled plasma atomic-emission spectroscopy (ICP-AES) in a Polyscan 61E (Thermo Jarrell Ash) spectrometer, measuring S at 182.040 nm and Zn at 213.856 nm. Samples were treated as previously reported [19], but were alternatively incubated in 1 M HNO_3 at 65 °C for 10 min prior to measurements in order to eliminate possible traces of acid-labile sulphide ions, as otherwise described [9]. Protein concentrations were calculated from the acidic ICP-AES sulphur measurement, assuming that all S atoms were contributed by the MT peptide.

Molecular mass determinations of Zn-MTs were performed by electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) on a Micro Tof-Q instrument (Bruker) interfaced with a Series 1100 HPLC Agilent pump, equipped with an autosampler, all of them controlled by the Compass Software. Calibration was attained with ESI-L Low Concentration Tuning Mix (Agilent Technologies). Samples containing Zn-MT complexes were analysed under the following conditions: 20 μL of protein solution injected through a PEEK (polyether heteroketone) tubing (1.5 m × 0.18 mm i.d.), at 40 $\mu L \cdot min^{-1}$; capillary counter-electrode voltage 5 kV; desolvation temperature 90–110 °C; dry gas 6 $L \cdot min^{-1}$; spectra collection range 800–2000 m/z. The carrier buffer was a 5:95 (*v*/*v*) mixture of acetonitrile:ammonium acetate/ammonia (15 mM, pH 7.0). For analysis of the apo-forms of all the recombinant MTs, 20 μL of the corresponding Zn-MT samples were injected under the same conditions described before but using a 5:95 mixture of acetonitrile:formic acid (pH 2.4) as liquid carrier, which caused the complete demetallation of the peptides. For each MT analysed, the apo-form molecular masses perfectly matched the theoretical values calculated from their amino acid sequences (Table S1 in Supplementary Information), which fully confirmed both the identity and the integrity of the recombinant proteins used in this study.

2.3. Raman spectroscopic analysis of the Zn(II)-MT complexes

To obtain samples suitable for the vibrational analysis avoiding the spectroscopic masking effect of the Tris–HCl buffer, a dialysis-lyophilization protocol was implemented prior to Raman measurements, analogously to what has already been done in previous studies of other MTs [7,14,20]. Approximately 1 mL of each of the recombinant Zn-GmMT preparations containing up to 1 mg of metal-protein complex were dialysed during 2 h against 200 mL of 5 mM Tris–HCl pH 7.2 followed by two cycles of 2 h dialysis against distilled water by using 6.3 mm-radius dialysis membranes (Medical International). Dialysed samples were lyophilized on a Modulo 4 K Freeze Dryer equipped with a RV8 Rotary Vane Pump (Edwards) and kept at −20 °C until further use. Raman spectra were obtained on lyophilised samples with a Bruker Multiram FT-Raman spectrometer, equipped with a cooled Ge-diode detector. The excitation source was a Nd^{3+}-YAG laser (1064 nm), the spectral resolution was 4 cm^{-1} and the total number of scans for each spectrum was 6000. The laser power on the sample was about 100 mW.

The curve fitting analysis was implemented using the OPUS/IR v5.0 programme, which uses the Levenberg–Marquardt Algorithm. A realistic identification of the peak component number and position was carried out by the fourth-derivative spectra, smoothed with a thirteen-

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GmMT1   GSMS-SCG---------CGSSCNCGSNCG-CNKYSFDLSYVEKTTTETLVLGVGPVKAQLEGAEMGVASENGGCNCGSSCTCDPCNCK- 77
GmMT2   GSMS-CCGGNCG-----CGSACKCGNGCGGCKMYP-DLSYTESTTTETLVMGVAPVKAQFESAEMGVPAENDGCKCGANCTCNPCTCK- 81
GmMT3   GSMSNTCG----------NCDCADKTSCTKGNSYG-VIVETEKSYIETVVMDVP----------AAEHDGKCKCGTNCTCTDCTCGH 66
GmMT4   GSMADTSGGDAVRPVVICDNKCGCTVPCTGGSTCRCTSVGMTTGGGDHVTCSCGEYCGCNPCSCPKTAASGTGCRCGTDCSCASCRT-- 87
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Fig. 1. Clustal alignment of the four soybean (*Glycine max*) GmMT isoforms studied in this work The shaded boxes indicate the Cys residues, and histidines are shown in bold. The plasmid derived Gly–Ser dipeptides are shown in italics.