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COMMUNICATION

Metal-dependent Glycosylation in Recombinant Metallothioneins

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We show for the first time glycosylation of recombinant metallothioneins (MTs) produced in *E. coli*. Interestingly, our results show that the glycosylation level of the recombinant MTs is inversely proportional to the degree of protein structuration, and reflects their different metal preferences.

Glycosylation is a crucial posttranslational process that affects stability, folding, and solubility of many eukaryotic proteins, and is involved in cell differentiation and growth, signal transduction, cell-cell and virus-cell interaction, and host immune responses.[1–3] Also, glycosylation of heterologously produced eukaryotic proteins is often essential for their biological activity. However, despite the increasing number of bacterial protein modifications reported,[4] no examples of recombinant glycoproteins produced in *Escherichia coli* have been reported so far. Our discovery of glycosylation in *E. coli* recombinant metallothioneins (MTs), and the essential role of metal ions in the glycosylation process become therefore issues of the upmost interest.

MTs are small (6–10 kDa), Cys-rich metalloproteins that naturally bind Zn^{2+}, Cd^{2+} and/or Cu^{+}.[5,6] Although their biological function is still a matter of debate,[7] their general role as major players in the intracellular homeostasis of physiological Zn^{2+} and Cu^{+} concentrations, and in controlling the toxicity of some harmful metal ions such as Cd^{2+} is generally accepted.[8]

Over decades, many researchers have devoted their efforts to chemically characterize MTs by mass spectrometry (ESI-MS) and spectroscopy of metal-protein complexes obtained from heterologously expressed MTs in *E. coli* BL21 strain cultures supplemented with Zn^{2+}, Cd^{2+} or Cu^{2+} ions.[9] During the synthesis of a batch of recombinant MTs, we identified some unexpected ESI-MS peaks that, after total removal of metals by acidification, showed masses increasing by multiples of 162 Da to that of the expected apo-form (Figure 1).

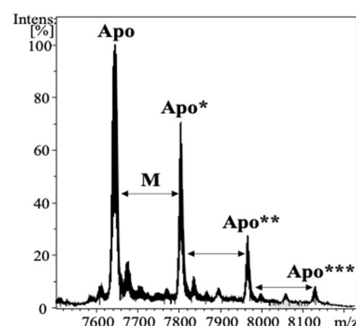

Figure 1. Deconvoluted acidic ESI-MS spectrum (pH 2.4) of *Lottia gigantea* MT1, LgiMT1,[10, §] showing de-metallated apo-MT and sequential peaks of decreasing intensity with additional M (M=162 Da) marked with asterisks (*).

After confirmation of the covalent binding of this 162 Da unit to the MTs and the absence of additional amino acids in their primary sequences, we envisaged the presence of hexose sugars bound to MTs. In order to check this possibility, enzyme-linked lectin assay (ELLA) experiments were carried out on a total of eleven MT samples (seven of which showed the unexpected MS peaks) produced in *E. coli* cultures supplemented with Zn^{2+} (4 MTs), Cd^{2+} (4 MTs), or Cu^{2+} (3 MTs) ions. Interestingly, the presence of galactose, mannose or fructose molecules was confirmed in the seven MT preparations showing the extra MS peaks (Table 1),[§§] clearly establishing that these recombinant MTs were glycosylated.

In order to verify that the MT glycosylation was a biological outcome rather than an experimental artefact occurring during metal-MT complexes' purification, the same experimental procedures were followed to purify complexes from *E. coli* cultures grown in MM9 minimal medium supplemented only with glycerol (0.4% v/v) and metal ions. Under these conditions, MTs were not glycosylated (Figure 2D, 2E and 2F), revealing that glycosylation was not caused by the experimental manipulation but occurred in the *E. coli* cells, during the MT production when the carbohydrates present in the Luria-Bertani (LB) medium were bound to the recombinantly expressed protein.

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Protein (Species)	Metal-binding behavior	Metal supplemented	Mannose	Galactose	Fructose	Ref
αNpoMT1 (*Nautilus pompilius*)	Cd-thionein	Cd	✗	✗	✗	*
LgiMT1 (*Lottia gigantea*)	Cd-thionein	Cd	✗	✗	✗	10
NpeMT1 (*Nerita peloronta*)	Zn-thionein	Cd	✗	✗	✗	11
FcaMT1 (*Falcidens caudatus*)	Zn-thionein	Cd	✗	✗	✗	*
NpeMT1 (*Nerita peloronta*)	Zn-thionein	Cu	✗	✓	✗	11
CaCuMT (*Cantareus aspersus*)	Cu-thionein	Cu	✓	✓	✓	12
mMT3 (*Mus musculus*)	Cu-thionein	Cu	✗	✓	✗	13
αNpoMT1 (*Nautilus pompilius*)	Cd-thionein	Zn	✓	✓	✗	*
LgiMT2 (*Lottia gigantea*)	Cd-thionein	Zn	✓	✓	✓	10
FcaMT1 (*Falcidens caudatus*)	Zn-thionein	Zn	✗	✓	✗	*
NpeMT1 (*Nerita peloronta*)	Zn-thionein	Zn	✓	✓	✓	11

Table 1. Enzyme-linked lectin assay (ELLA). Qualitative presence/absence (✓/✗) test of mannose, galactose and fructose performed on 11 selected samples of recombinant MTs displaying three different metal-binding preferences (Zn-, Cd- and Cu-thioneins) produced in three different metal-supplemented (i.e., Zn^{2+}, Cd^{2+}, Cu^{2+}) *E. coli* cultures. *Unpublished work

Noteworthy, whereas MTs produced in Zn^{2+} or Cu^{2+}-enriched media tested positive for carbohydrates, those produced in Cd^{2+} media were not glycosylated (Table 1). The inhibitory effect of Cd^{2+} ions was compatible with *E. coli* glycosyltransferases (GTs) and nucleotidyltransferases (NTs) catalyzing the MT glycosylation. It is known that bacterial GTs recognize activated carbohydrates –*e.g.* monosaccharides bound to a nucleotide unit with two phosphate groups (like UDP-glucose and UDP-galactose)–, providing chemical energy for driving the glycosylation reaction.[14] Carbohydrates are activated by the action of the *E. coli* NT, whose native structure is Zn-dependent (PDB entrance 1GUQ and 1GUP).[15] Due to their equivalent properties, Cd^{2+} ions would compete with Zn^{2+} ions inhibiting the activity of Zn-dependent NTs, and disabling thereby protein glycosylation. Although it would be desirable identifying the machinery catalyzing MTs glycosylation in *E.coli* BL21, the experiments required to achieve this purpose are certainly beyond the scope of this communication.

It is thought that protein structuration influences protein glycosylation by sterically limiting the access of enzymes and sugar nucleotides to the amino acid side chains in eukaryotes.[16] We predicted, therefore, that the higher the degree of structuration of the MTs (*i.e.*, rigid, and stable metal-MT complexes), the lower their glycosylation level. To prove our postulation, we took advantage of the fact that MTs uniquely achieve their full 3D structure and stability after complete metal-coordination. Thus, the recombinant production of the

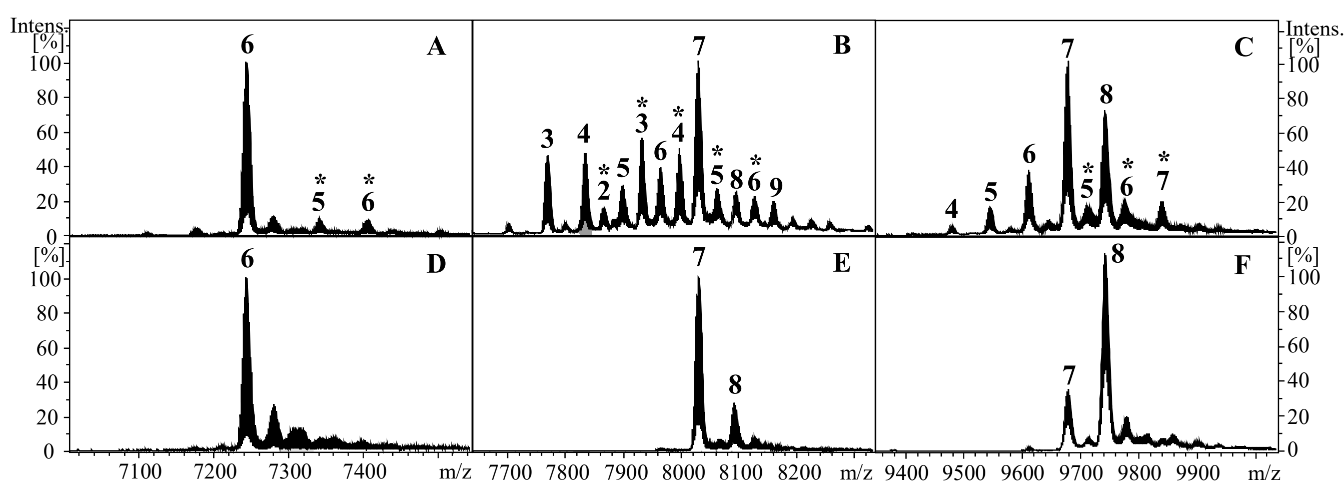

Figure 2. Deconvoluted ESI-MS spectra at pH 7.0 of Zn-thionein *Nerita peloronta* MT1, NpeMT1,[11] (A and D), Cd-thionein *Lottia gigantea* MT2, LgiMT2,[10] (B and E) and *Pomacea bridgesii* MT1, PbrMT1, with Cu-preference[18] (C and F) synthesized in LB medium (A, B and C) and in minimal medium with 0.4% glycerol (D, E and F) supplemented with Zn^{2+}. All metallated complexes shown in the figure uniquely contain Zn^{2+} ions and the metal-to protein stoichiometry of the Zn-MT complexes is indicated with Arabic numbers. In addition, glycosylated species are marked with as many asterisks (*) as hexoses are attached to the MT.

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same MT (we chose LgiMT2 as case study) was performed with different amounts of supplemented Zn^{2+}: 300 μM for a "conventional" synthesis, 100 μM for equaling the assumed physiological intracellular Zn^{2+} concentrations,[17] and nominal 0 μM to avoid any foreign metal ion surplus. Productions without additional Zn^{2+}-enrichment yielded under-metallated species containing a maximum of 4 Zn^{2+} ions per protein (Figure 3C), whereas both Zn^{2+}-supplemented cultures (100 μM and 300 μM of Zn^{2+}) rendered analogous mixtures of several species with a stoichiometry of up to 7 Zn^{2+} ions per protein (Figure 3A). Under-metallated species, which are MTs less tightly structured and more flexible, showed a glycosylation rate higher (up to 4 hexoses per protein) than those more structured and stable MTs obtained under sufficiently Zn^{2+}-supplemented conditions (compare Figures 3B &3D), supporting our initial prediction.

The tightness of the metal-MT complex structure depends on the amount of metal (*e.g.* 0 μM, 100 μM or 300 μM of supplemented Zn^{2+}) but also on whether the available metal is the preferred (cognate) metal ion of the MT. Thus, we also predicted that the glycosylation level of the recombinant MTs should be low or negligible if MTs were produced in the presence of their cognate metals since in this case they would give rise to a unique metallated species with a high degree of structuration. In contrast, facing non-cognate metal ions MTs should yield misfolded and more dynamic structures, mirroring the same loose aggregates as those observed in the MTs recovered from non-metal supplemented conditions.

In agreement with this prediction, we observed that MTs considered as genuine (or almost genuine) Zn-thioneins (high preference for Zn^{2+}) like NpeMT1 is, produced under Zn^{2+} supplementation conditions showed a negligible degree of glycosylation by ESI-MS (Figure 2A). Under the same Zn^{2+} supplementation conditions, the degree of glycosylation was higher for MTs with low Zn-thionein character. For instance, MTs with high Cd- or Cu-specificity (*i.e.* Cd- or Cu-thioneins) such as LgiMT2 or PbrMT1 respectively are, were notably glycosylated when produced in Zn^{2+}-supplemented media (Figure 2B and 2C). Significantly, these findings not only demonstrated that MT glycosylation in *E.coli* depends on the type and degree of metal loading –which determines the robustness degree of the 3D-structure and how enzymes and sugar nucleotides access to the peptide chain– but also suggested that glycosylation occurred after the MTs were completely synthetized and folded in the presence of the provided metal ions.

Similar results were observed in the preparations recovered from Cu^{2+}-supplemented cultures. Cu-thioneins (*e.g.* CaCuMT and mMT3, see Table 1), rendering stable well-structured Cu-MT complexes, showed a very low glycosylation degree by ESI-MS,[12,13] although the sugars in these proteins were still detected by ELLA (Table 1), whereas Zn- and Cd-thioneins were abundantly glycosylated under Cu^{2+}-surplus conditions (see Table 1 and results in ref. 11).These results have led us to propose that glycosylation levels on a recombinantly expressed MTs are, in fact, indicative of their metal specificity: the lower their glycosylation degree in Zn^{2+}- or Cu^{2+}-supplemented *E. coli* cultures, the higher their Zn-thionein or Cu-thionein character, respectively.

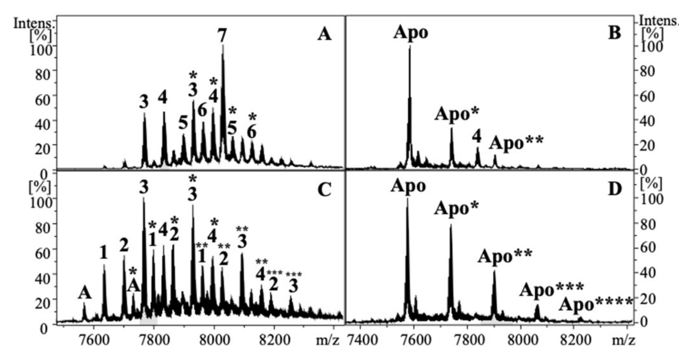

Figure 3. Deconvoluted ESI-MS spectra recorded at pH 7.0 (A and C) and pH 2.4 (B and D) of *Lottia gigantea* MT2, LgiMT2,[10] syntheses supplemented with 300 μM (or 100 μM) of $ZnCl_2$ (A and B), and without Zn^{2+}-supplementation (C and D). All metallated complexes shown in the figure uniquely contain Zn^{2+} ions and the metal-to-protein stoichiometry of the Zn-MT complexes is indicated with Arabic numbers. In panel C, "A" stands for apo-MT. In addition, glycosylated species are marked with as many asterisks (*) as hexoses are attached to the MT.

Finally, we want to notice that recombinant engineering techniques have made possible synthesizing metalloproteins loaded with non-cognate metal ions, sometimes rendering metal-protein complexes that we would not encounter in nature. These techniques have allowed the recombinant production of MTs bound to non-cognate metal ions that, as here showed, can be glycosylated by the bacterial glycosylation machinery. These glycosylated MTs might show somewhat altered metal-to-protein ratios (*e.g.* compare panels B and E, and C and F in Figure 2). Glycosylation, however, is low or absent when a MT is produced in the presence of its cognate metal (e.g. compare Figure 2A and 2D), and therefore we argue that glycosylation does not alter the conclusions achieved neither about the stability nor the metal-affinity when recombinant MTs have been characterized in the presence of their cognate metal, which in fact is probably the functionally relevant one.

In summary, we have shown that a fraction of the recombinant MTs produced in bacteria is glycosylated. Our analyses allowed us to conclude that (1) glycosylation occurs inside the *E. coli* cells probably catalyzed by the bacterial machinery, which, in turn, it is inhibited by the presence of Cd^{2+} ions; and (2) the level of glycosylation is inversely proportional to the degree of MT structuration, which would be determined by the amount and the affinity of MTs for the available metal ions. Overall, our results suggest that the glycosylation level of the metal-MT complexes recombinantly expressed in bacteria reflects the preference of the MT for the metal ion to which it is bound: the lower its glycosylation degree, the higher the preference for the coordinated metal. We consider that these results are relevant for the scientists dedicated to the recombinant production in bacteria of MTs or other metalloproteins, as well as for researchers interested in the production of glycoproteins with biotechnological or pharmaceutical applications.

Author contributions

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M.G.-R. contributed to all reported MTs chemical characterization and proposed glycosylation reactions after analysing unexpected results; A.G., and F.J.L-J. contributed to ELLA assays; R.A., and S.C. produced the recombinant MTs; V.P-M. and R.D. contributed to data compilation on protein glycosylation; O.P. contributed to the characterization of metalloproteins by ESI-MS; R.A., R.D., M.G.-R., O.P., J.M.D-V., and M.C. contributed to the study design, data analysis, and writing of the manuscript. All authors contributed to data analysis and writing relevant sections of the manuscript.

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Notes

§ All the reported MTs have been synthesized and characterized by using the methodology that this group has followed since 1997.[19] Therefore, the overexpression of recombinant MTs has been performed through the protease deficient strain *E. coli* BL21, in which the recombinant constructs of interest were introduced. The transformed host strains were grown overnight in LB or MM9 minimal medium supplemented with 100 mg·L^{-1} of ampicillin at 37 °C and, afterwards, inoculated to a x10 fresh medium volume. Upon reaching an A600 value of 1.0, the bacteria were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration) and allowed to grow for 30 min. Afterwards, the media were supplemented with $ZnCl_2$, $CdCl_2$ or $CuSO_4$ and continued growing for 2.5 h more. The generated biomass is harvested and washed with phosphate-buffered saline (PBS) solution (1.4 M NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4). After centrifugation at 7700 x *g* for 10 min, the pellet was resuspended in argon-bubbled ice-cold PBS-0.5% v/v β-mercaptoethanol. Cells were sonicated at 4 °C with 20 s pulses for 5 min and centrifuged at 12000 x *g* for 15 min. The produced GST-MT fusion protein was recovered from the supernatant and purified by batch affinity chromatography with Glutathione-Sepharose-4B (Pharmacia) at a volume ratio 1:10 matrix:sample. The recombinant fusion protein was cleaved with thrombin (10 units of thrombin (Pharmacia)/mg of fusion protein) through an overnight digestion at 23-25 °C. The GST portion remained bound to the gel matrix and, thus, the MT was eluted along with the solution. The eluate was 5-fold concentrated by means of a Centriprep Concentrator (Amicon) with a 3 kDa cutoff and purified by FPLC using a Superdex-75 (Pharmacia) exclusion column equilibrated with 50 mM Tris-HCl (pH 7.0). 1 mL of protein containing fractions were collected by their absorbance at 254 nm, chemically characterized as elsewhere reported,[10] and stored at -70 °C for further use.

§§ *Detection of glycoproteins by Enzyme-linked lectin assay (ELLA):* the experiments were carried out on MTs produced in Zn^{2+}-, Cd^{2+}- and Cu^{2+}-enriched media, respectively. MTs were fixed on an ELISA plate, and the presence of the hexose moieties was revealed by incubation with horseradish peroxidase-lectins conjugates. The lectins of choice were concanavalin A (ConA), *Ulex europaeus* agglutinin (UEA), and Peanut agglutinin (PNA), which specifically interact with mannose, fructose, and galactose, respectively. Each MT (final concentration 5 µg/mL) was adsorbed on ELISA plate wells by incubation in 200 µL (100 mM carbonate buffer, pH 9) at 4 °C overnight. Wells were washed with PBST (3 x 300 µL/3 min) and then incubated with 200 µL of horseradish peroxidase-lectin conjugates (HRP), ConA (3 µg/mL), UEA (3 µg/mL) and PNA (0.3 µg/mL) at 37 °C for 1h. After washing with PBST (3 x 300 µL/3 min), the presence of lectin was detected by the peroxidase activity by incubation at 37 °C with 200 µL of 3.7 mM 1,2-phenylenediamine dihydrochloride in citrate-phosphate buffer (100 mM, 50 mM, pH 5) with 0.05% (v/v) H_2O_2. Reaction was stopped with 50 µL of 4 M H_2SO_4. Quantification was carried out by measurement of the absorbance at 495 nm with a Sunrise absorbance reader (Tecan).

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