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Mass Spectrometry and Metallomics: stability and binding site location in the Cyt *c*-CDDP model system

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The bottom-up mass spectrometry approach is today a powerful tool to characterize the binding of metal-based drugs to proteins. Yet, the stability of metal-protein coordination bonds along the whole process may be critical. This led us to build up a general protocol to test metallodrug-protein adduct stability under the typical conditions of the filter-aided sample preparation (FASP)/bottom-up procedure, ranging from the analysis of solutions containing metal-protein adducts to tandem mass spectrometry experiments. More in detail, we identified nine critical situations, either solution or instrumental,[1-6] as a potential source of metal-protein bond impairment when using FASP operative conditions and a nano high performance liquid chromatography-nanoelectrospray ionization-LTQ-Orbitrap (nanoLC-nanoESI-LTQ-Orbitrap) mass spectrometer system equipped with a preconcentration/purification device. These are: 1) sample permanence in the ammonium bicarbonate buffer; 2) denaturation with urea; 3) reduction with dithiothreitol; 4) alkylation with iodoacetamide; 5) sample permanence in the loading mobile phase; 6) sample permanence in the elution mobile phase; 7) the nanoESI process; 8) the transfer of the adduct through ion transfer tube and tube lens; 9) collision induced dissociation in the ion trap. Accordingly, an ad hoc experimental protocol was developed and applied to the adducts formed between cytochrome *c* (Cyt *c*) and two different metallodrugs, i.e. cisplatin (*cis*-diamminedichloridoplatinum(II), CDDP) and RAPTA-C, a well known ruthenium(II)-arene compound [Ru(η*6*-*p*-cymene)Cl_2(pta)] (pta= 1,3,5-triaza-7-phosphaadamantane), used here as models. Notably, Cyt *c*-CDDP adducts were stable through all the above conditions while Cyt *c*-RAPTA-C adducts turned unstable in the ammonium bicarbonate buffer. This latter finding supports the need to perform a test-protocol of this kind when starting any extensive bottom-up MS investigation of protein-metallodrug systems.
Then, the FASP/bottom-up high resolution mass spectrometry (HR-MS) approach was applied to the Cyt *c*-CDDP model system, in order to test the above procedure. The binding site location was accomplished in an automated way by using Mascot search engine: the potential coordinating amino acid residues M, C, H, K, W, T, S, E, D and Y (S-, N- and O-donor) were included in the search files as modified residues with mass gains relative to the possible CDDP fragments Pt^{2+}, $[Pt(NH_3)]^{2+}$, $Pt[(NH_3)_2]^{2+}$ and $[Pt(NH_3)_2Cl]^{+}$ and considering the charge brought by each of them. The platinated peptides found with Mascot were also manually assessed to positively confirm the presence of the characteristic Pt isotopic profile in the HR-MS full scans of the precursor ions and in their MS/MS spectra.[5,7,8] The following ten binding sites were identified: T58, W59, K60, E61, E62, T63, M65, E66, Y67 and M80. Among them, E61, E62, T63, M65 and M80 have already been reported in literature[9-12] while T58, W59, K60, E66 and Y67 have been detected for the first time. Due to the small number of characteristic b and y fragments obtained, probably related to Pt binding on protein, all these binding sites result equally probable and no one of them can be excluded at this level of our investigation. Anyway, the FASP/bottom-up approach used here has demonstrated its ability to highlight the remarkable selectivity of Cyt *c*-CDDP binding since only two specific portions of the protein (T58, W59, K60, E61, E62, T63, M65, E66, Y67 in peptide 56-73 and M80 in peptide 80-86) resulted involved.

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