ESI mass spectrometry and X-ray diffraction studies of adducts between anticancer platinum drugs and hen egg white lysozyme

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The interactions of cisplatin and its analogues, transplatin, carboplatin and oxaliplatin, with hen egg white lysozyme were analysed through ESI mass spectrometry, and the resulting metallodrug–protein adducts identified; the X-ray crystal structure of the cisplatin lysozyme derivative, solved at 1.9 Å resolution, reveals selective platination of imidazole Nε of His15.

The interactions of anticancer platinum complexes with proteins have attracted renewed interest during the last few years as they are considered crucial for the pharmacokinetics, the biodistribution, the resistance processes and the toxicity of these metallodrugs.[1,2]

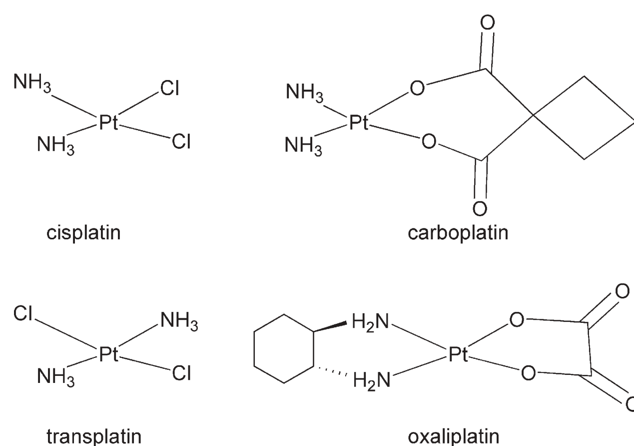
Powerful analytical methods are required to unravel the extreme complexity of platinum–protein interactions; thus, relevant progresses in this field were eventually recorded following the implementation of the latest mass spectrometry techniques and of advanced metalloproteomics protocols.[3,4] Notably, newly reported studies of platinum–protein adducts have highlighted peculiar features of platinum reactions with proteins such as the occurrence of large kinetic differences in the protein platination profiles (strictly dependent on the nature of both the protein and the metal complex)[5] and the eventual release of ammonia ligands from platinum, following protein complexation.[6–8]

Within this frame, we have investigated the interactions of classical platinum drugs (Scheme 1) with hen egg white lysozyme (HEWL), used here as a model protein. Indeed, lysozyme, owing to its small size and to the prevalence of positively charged groups, is a particularly suitable protein for ESI MS investigations as previously shown.[9,10] Moreover, HEWL is well known among crystallographers as a protein very prone to crystallisation, thus turning out very appropriate for X-ray diffraction studies of its metallodrug adducts.[11,12]

In the present study, the platinum–lysozyme interactions were primarily addressed through ESI MS measurements owing to the ease, the rapidity, the sensitivity and the relevant information content of this technique.[3,5,7] Fig. 1 shows the deconvoluted ESI MS spectra of HEWL adducts with cisplatin, transplatin, carboplatin and oxaliplatin, taken after 72 h incubation, at 37 °C.†

A number of observations can be made upon inspection of these ESI-MS spectra. Notably, the ESI-MS peak corresponding to the non-platinated enzyme, at 14 305 Da, is always the one of higher intensity, implying that protein metallation takes place only partially even after challenging the protein with a three fold excess of the metallodrug over long incubation times. This observation was confirmed by ICP-OES (inductively coupled optical emission spectroscopy) measurements† that revealed rather low platination levels for all platinum protein adducts; indeed platination levels of about 50% were measured in the case of cisplatin while values lower than 15% were afforded in all the other cases (oxaliplatin, carboplatin and transplatin). On the other hand, detection of well resolved ESI-MS peaks, with mass values falling in the 14 500/15 000 Da interval, provided unambiguous evidence for adduct formation, also giving detailed information on the nature of protein bound metallic fragments.

The ESI-MS spectrum of the cisplatin derivative (Fig. 1A) shows two peaks of similar intensity at 14 569 and 14 605 Da that formally correspond to either $[Pt(NH_3)_2Cl]^+$ or intact cisplatin bound to the native protein. A similar situation was formerly described by Dyson and coworkers in the case of the cisplatin–transferrin system and interpreted in terms of a two-step cisplatin to protein binding process.[13,14] In our case, additional peaks of similar shape but lower intensitiy are observed at 14 868 and 14 904 Da consistent with the corresponding doubly platinated species. The ESI-MS spectrum of the carboplatin (Fig. 1B) adduct reveals a multiplet centered at 14 676 Da; such a mass corresponds to the addition of a $[Pt(NH_3)_2CBD]$ (CBD = *cis*-(1,1-cyclobutanedicarboxylate)) fragment to the protein, most likely as the result of a classical ring opening process. The ESI-MS spectrum of the oxaliplatin derivative (Fig. 1C) exhibits a main peak at 14 612 Da



Scheme 1 Schematic drawings of selected anticancer platinum complexes.

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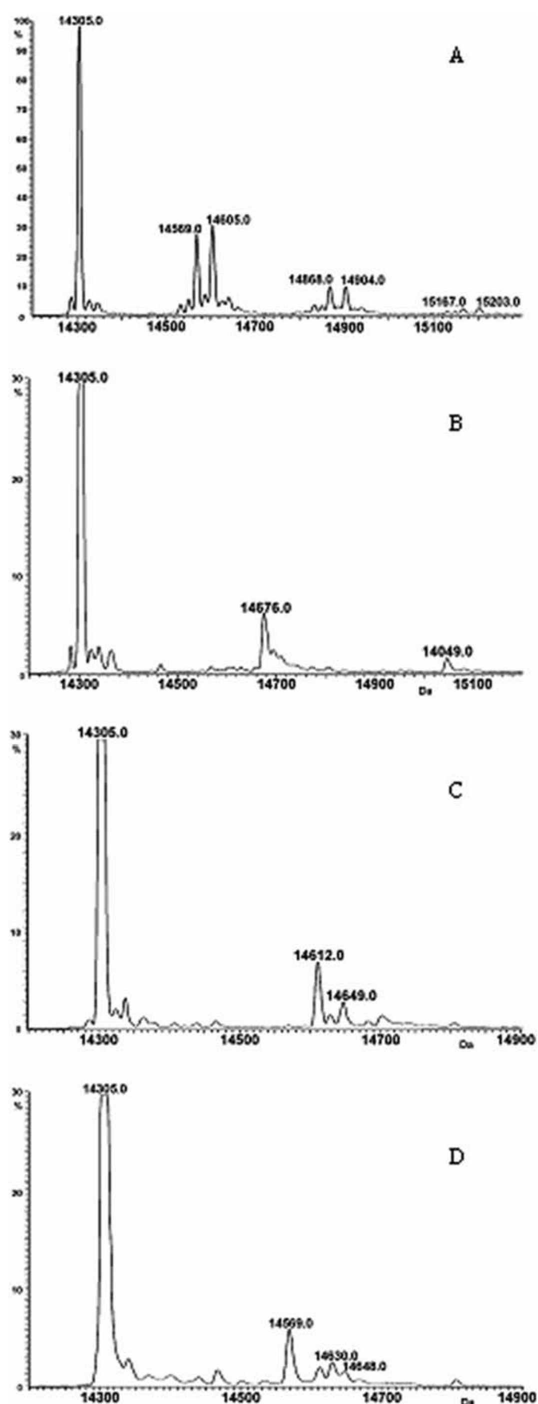

Fig. 1 Deconvoluted ESI-MS spectra of lysozyme adducts with the selected platinum complexes recorded in H_2O after 72 h incubation at 37 °C: (A) cisplatin, (B) carboplatin, (C) oxaliplatin, (D) transplatin. The stoichiometry of each platinum/protein adduct is 3 : 1.

that well corresponds to the binding of a $[Pt(R(NH_2)_2)]^{2+}$ (R = cyclohexane) moiety to HEWL (with concomitant release of the oxalate ligand). This is in accordance with a previous report on the interaction of oxaliplatin with cytochrome *c*.[5] Finally, the mass spectrum of the adduct formed between HEWL and transplatin (Fig. 1D) evidences a peak at 14569 Da that corresponds to protein binding of a $[Pt(NH_3)_2Cl]^+$ fragment (264 Da) after release of a single chloride ligand.

Thus, the above ESI-MS measurements have turned out very valuable to monitor the processes of metallodrug–lysozyme adduct formation and to elucidate the exact nature of the protein bound metallic fragments. We have learned that, under the employed solution conditions, adduct formation is rather slow, that cisplatin is by far the most efficient in producing HEWL platination, that monoplatinated species are the predominant ones, thus suggesting the presence of a highly preferential platinum binding site.

Afterward, in order to gain more precise molecular information on the formed adducts and on the possible sites of metal binding, soaking experiments were carried out in which HEWL crystals were incubated with an excess of each selected platinum drug. Crystals suitable for X-ray diffraction analysis were obtained only in the case of cisplatin. These crystals were subject to X-ray data collection and the structure of the adduct solved at 1.9 Å resolution. Details of data collection and structure refinement are given below.‡

The structure of the cisplatin–HEWL adduct is very similar to that of the native protein (193 L). Platination occurs at the only histidine residue, His 15, which is situated on the surface of the protein and is highly accessible (Fig. 2). Indeed, the analysis of the electron density maps reveals a specific binding of the platinum ion to the Nε of the imidazole ring of His15. In contrast, no other significant modifications of the electron density map were observed ruling out the presence of additional (secondary) binding sites. Particular attention was paid to monitoring the local environment of the two methionine residues (Met 12 and Met 105) that commonly represent preferred anchoring sites for platinum(II) compounds; no significant modifications could be appreciated at these sites in agreement with their scarce accessibility. Platinum binding to HEWL active site (see ref. 12) could be ruled out as well.

We tried to refine the local environment of the protein bound platinum(II) center by assuming a classical square planar geometry and the presence of only nitrogen/oxygen ligands. Within these assumptions the platinum(II) ion appears to bind the protein with an occupancy value of 0.3. The resulting, moderately distorted, square planar coordination environment is represented in Fig. 3. The platinum atom is bound to the the Nε of His 15 and, tentatively, to the nitrogens of two ammonia molecules in cisplatin. The fourth ligand is not detectable: it might well correspond to a loosely bound/disordered platinum coordinated water molecule. A careful analysis of the thermal factors and of the interaction distances suggests that the platinum center, once bound to the protein, retains its ammonia groups. In fact, if the ligands are refined with the assumption that the coordination positions are occupied by nitrogen atoms, the B-factor value goes down to a realistic value (around 22.0). Furthermore, the resulting distances between the platinum atom and the ammonia groups (2.03 Å) is well comparable to usual values for Pt–N distances (around 2.1 Å). Conversely, the presence of chloride ligands coordinated to the platinum center, as it was previously found for the cisplatin/superoxide dismutase derivative,[6] can be ruled out in this case. In a way, this finding contrasts with ESI-MS results of cisplatin–lysozyme showing the persistence of one, or even two, chloride ligands on the protein bound platinum center; however this apparent contradiction might be due to the intrinsic differences of the two experimental approaches.

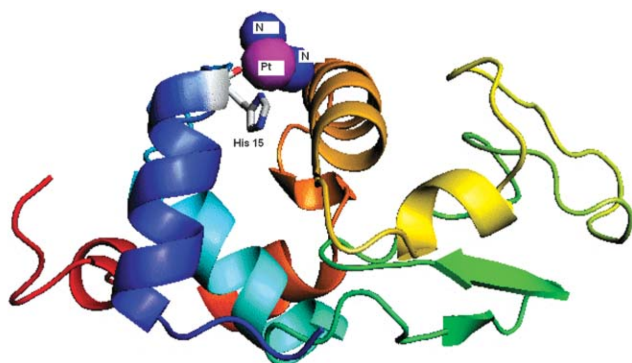

Fig. 2 Schematic representation of the asymmetric unit describing the surface interaction of cisplatin with hen egg white lysozyme; the side chain of His 15 is shown along with platinum, ammonia ligands.

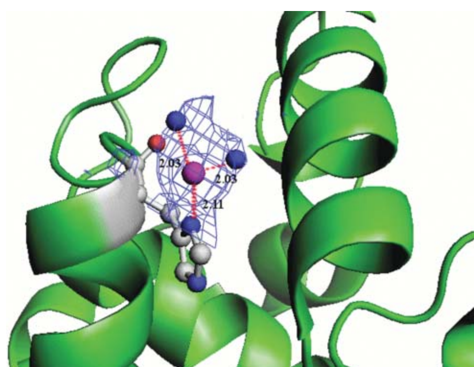

Fig. 3 $2F_o - F_c$ map at 1σ covering cisplatin that interacts with Nε of His15 and with two ammonia ligands and the relative bond lengths (Å).

Overall, the present study has highlighted some relevant features of the interactions of platinum drugs with the model protein HEWL. All tested compounds are able to form specific adducts with lysozyme although these metallation processes appear to be less efficient than in the case of cytochrome *c*, previously reported.[5] The nature of the protein bound metallic fragments has been determined, being in good accord with results obtained on similar systems.[5] Very interestingly, in the case of cisplatin, the crystal structure of its adduct with HEWL has been solved at 1.9 Å resolution. Although details of platinum coordination have not been fully solved, it is evident that platinum is anchored to the imidazole Nε of His 15, in agreement with previous structural results of ruthenated lysozyme.[11] It is very likely that this residue-His15- represents a general binding site for platinum drugs and other late transition metal complexes. Remarkably, the present structure is one of the few reported examples of crystal structures of cisplatin–protein adducts.

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Notes and references

† *Analysis by positive-ion electrospray ionisation mass spectrometry.* Cisplatin, transplatin, carboplatin and oxaliplatin were purchased from Sigma-Aldrich (Codes P4394, P1525, C2538 and O9512 respectively). Hen egg white lysozyme was also from Sigma (Code L7651). HEWL adducts were prepared in ammonium carbonate buffer 25 mM, pH 7.4, with a protein concentration of 10^{-4} M, and a platinum to protein ratio of 3 : 1. The reaction mixtures were incubated for different time intervals, over a period of 72 h, at 37 °C. Samples were extensively ultrafiltered using Centricon YM-3 (Amicon Bioseparations, Millipore Corporation) in order to remove the unbound platinum complex. After a 100 fold dilution with MilliQ water, ESI MS spectra were recorded by direct introduction at a 3 μl min^{-1} flow rate in a LTQ linear ion trap (Thermo, San Jose, California), equipped with a conventional ESI source. The specific conditions used for these experiments were as follows: the spray voltage was 3.5 kV, the capillary voltage was 32 V and the capillary temperature was kept at 353 K. Sheath gas was set at 16 (arbitrary units) whereas sweep gas and auxiliary gas were kept at 0 (arbitrary units). ESI spectra were acquired using Xcalibur software (Thermo) and deconvolution was obtained using Bioworks software (Thermo). The mass step size in deconvolution calculation was 1 Da and the spectrum range considered was 1100–2000 *m*/*z*. The same experiments were repeated varying capillary temperature (180 °C), but the peak patterns and relative abundances were not influenced (data not shown). ICP-OES analysis of the platinum content in each sample were recorded using an Optima 2000 instrument (Perkin Elmer, Europe).

‡ *Crystallization and X-ray data collection.*HEWL crystals were grown at 277 K using the hanging drop method. The reservoir buffer contained 5 mM sodium acetate buffer, pH 6.5 and 0.05 M NaCl, and the drop contained hen egg white lysozyme (10 mg ml^{-1}) in acetate buffer (5 μl) and reservoir solution (5 μl). After 5 days lysozyme crystals were formed, we soaked them with a solution of cisplatin. Final concentration of cisplatin in solution was 10 times higher than the lysozyme. After 3 days of incubation at 277 K, a monochromatic experiment at the Cu-α wavelength was performed on a selected crystal by the rotation method on a PX-Ultra sealed tube diffractometer (Oxford Diffraction) at 100 K. Data were processed using the programs MOSFLM[15] and SCALA. The initial structure was solved using a reported lysozyme structure (pdb code 193 L), and refinement performed using the program REFMAC5.[16] Model building and map inspections were performing using the COOT program.[17] The coordinates have been deposited in the Protein Data Bank (PDB) under the accession code 2I6Z. Data statistics: space group, $P4_32_12$; unit cell, 77.45 Å, 77.45 Å, 37.35 Å, 90°, 90°, 90°; resolution range: 20–1.90 Å; observed reflections (unique) = 18677 (2476); I/σ (I) = 13.2 (4.7); completeness = 97.4% (90.0%); R_{merge} = 5.2% (31.5%). Refinement: R_{fact}, 18.9%, R_{free} 24.8%; rms bonds = 0.008 Å, rms angles (°) = 1.068. Values in parenthesis relate to the highest resolution shell (2.00–1.90).

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