Reactions of a tetranuclear Pt-thiosemicarbazone complex with model proteins

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Abstract

The tetranuclear Pt complex ***(PtL)$_4$*** *(where L^{2-} is the anion derived from para-isopropyl thiosemicarbazone) was first described in 1998.* ***(PtL)$_4$*** *manifests interesting antiproliferative properties in vitro toward various cancer cell lines being a promising anticancer drug candidate. Yet, details of its reactivity with biomolecules have not been elucidated. To this end, we investigated the reactions of* ***(PtL)$_4$*** *with a few model proteins, i.e. bovine pancreatic ribonuclease (RNase A), cytochrome c (Cyt c) and hen egg white lysozyme (Lysozyme), mainly through electrospray ionization mass spectrometry (ESI MS), but also with the support of other biophysical methods. ESI MS revealed a rich reactivity of* ***(PtL)$_4$*** *with the above-mentioned model proteins, leading to the formation of numerous metallodrug-protein adducts. Remarkably, upon reaction with proteins, the tetranuclear complex breaks down and various monometallic fragments are formed that bind proteins up to high metal/protein ratios; this typically results into very complicated ESI MS spectral patterns. However, some of the main ESI MS peaks could be assigned in the case of the Lysozyme adduct working at lower* ***(PtL)$_4$****/protein ratios and shorter incubation times. In addition, crystallographic data were obtained for the* ***(PtL)$_4$****/Lysozyme and* ***(PtL)$_4$****/RNase A adducts pointing at His side chains as the primary binding sites for monometallic Pt fragments. Notably, a few selected features of the interactions observed in the* ***(PtL)$_4$****/protein adducts were reproduced by reacting* ***(PtL)$_4$*** *with a small molecule, i.e. N-methylimidazole, as documented by MS and NMR measurements. In conclusion, the present study confirms the prodrug nature of the tetraplatinum complex, clarifies one possible pathway for its activation through cluster disassembly and allows initial identification of adducts formed with a representative protein.*

INTRODUCTION

The discovery of cisplatin and its great clinical success in cancer treatment during the last 40 years has triggered a lot of attention on the synthesis and the chemical and biological characterization of novel Pt compounds that might be promising anticancer drug candidates. In particular, much interest was paid to the preparation and characterization of novel Pt species that bear innovative and peculiar features both in terms of structure and reactivity. Within this frame the **$(PtL)_4$** complex (**I**), where L stands for the anion derived from *para*-isopropyl thiosemicarbazone (Figure 1), that was developed and characterised a few years ago at Universidad Autónoma de Madrid UAM,[1] represents a nice example for this kind of strategy. The **$(PtL)_4$** complex consists of a Pt atom linked to a terdentate thiosemicarbazone ligand (**L)**, through S, N and C donors. In turn, the S atom of an adjacent **PtL** group occupies the fourth coordination position of the square planar Pt(II) center thus producing a strong Pt-S-Pt bridging interaction. Typically, four distinct **PtL** moieties assemble to generate the compact tetranuclear cluster, where all Pt centers are coordinatively saturated. The resulting platinum core is an eight-membered ring of alternating Pt(II) and S atoms arranged in a boat conformation. The Pt-S bond distances are quite similar one another, all falling into the 2.29 to 2.35 Å range, while the distance between platinum atoms has been determined to be 3.32 Å (Figure 1B).

$(PtL)_4$ exerts very favourable cytotoxic activity against human and murine tumor cell lines sensitive or resistant to cis-DDP (Jurkat, Pam-Ras), normal murine keratinocites (Pam), and two cis-DDP resistant primary cultures of glioma cells, derived from biopsies of cancer patients. Moreover, the analysis of the interactions of **$(PtL)_4$** with DNA underscores its ability to form DNA interhelical cross-links.[1]

This latter observation supports the view that its biological activity may primarily arise from a direct DNA damage. Yet, conclusive information has not been gained so far concerning its mode of action;[2] in particular, the possible interactions taking place between **$(PtL)_4$** and proteins have never been considered before and merit, in our opinion, some attention. Indeed, protein targets, have attracted growing attention in the last years; today their role in the pharmacological and toxicological profiles as well as in the resistance mechanisms of several metal-based anticancer drugs has been well documented .[3] In this view it is interesting to investigate these aspects even for **$(PtL)_4$**.

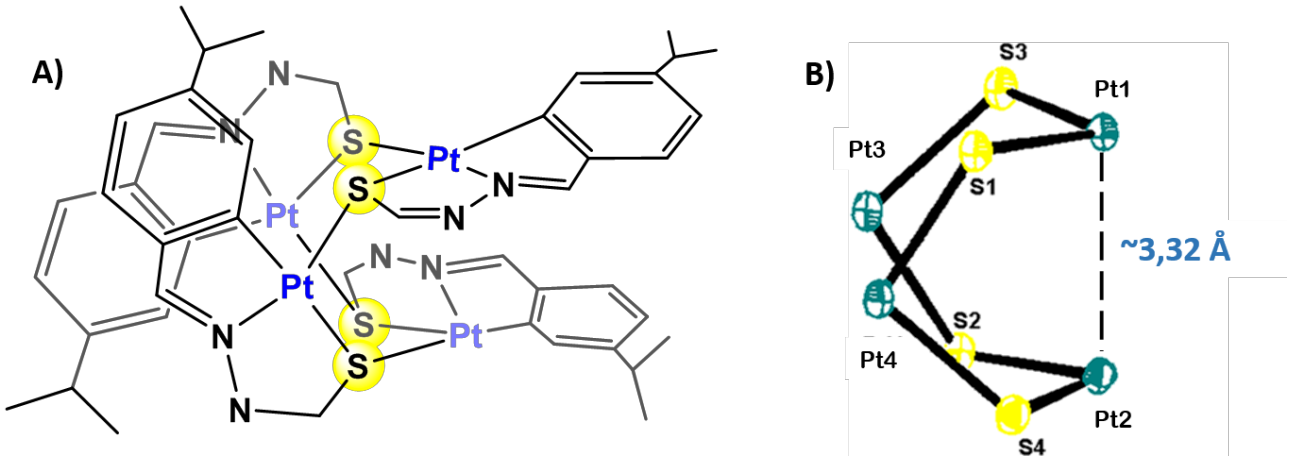


Figure 1. Schematic structure of **(PtL)$_4$**, for clarity the terminal groups -NH_2 of ligands have been omitted.

These arguments led us to explore the behaviour of **(PtL)$_4$** in solution and its reactivity with a few model proteins. In particular, we used small model proteins such as hen egg white lysozyme (HEWL) or RNase as their structure is well known and have been often used to describe the occurring interactions with metal-based anticancer drugs. Also, having afar lower molecular weight compared to some real protein targets e.g. human serum albumin (HSA), these proteins are better suitable for ESI-MS investigations as well as for X-Ray crystallography. Thus, they offer a valid tool to investigate the general mechanistic aspects involved in protein binding.[3]

In this frame ESI MS spectrometry turned out to be a highly suitable tool to characterise the occurring processes and the underlying chemical transformations. Yet, independent valuable information was gained through application to the same systems of other biophysical methods, including X-ray crystallography. Altogether, the obtained results allow a satisfactory description of the occurring interactions and of the activation mechanism of this interesting tetraplatinum species.

RESULTS

1. Solution behaviour of $(PtL)_4$

$(PtL)_4$ was prepared and characterised as previously reported;[1] then we started a detailed study of its solution behaviour over time, first relying on spectrophotometric determinations (Figure 2). A full understanding of the stability of this complex in physiological-like conditions is indeed a prerequisite to optimize the conditions for the biological studies and for the interaction studies with biomolecules. **$(PtL)_4$** (10^{-4} M) was solubilised in the presence of 15% dimethyl sulfoxide (DMSO) in phosphate buffer (50 mM, pH 7.4) or ammonium acetate buffer (20 mM pH 6.8, see supporting information). Under these conditions, **$(PtL)_4$** revealed a stable UV-Vis profile over 24 h, at room temperature, as shown in Figure 2. The spectrum is characterised by two absorption bands, with the first one falling at ~240 nm and the second one, a broader one, centred at ~300 nm.

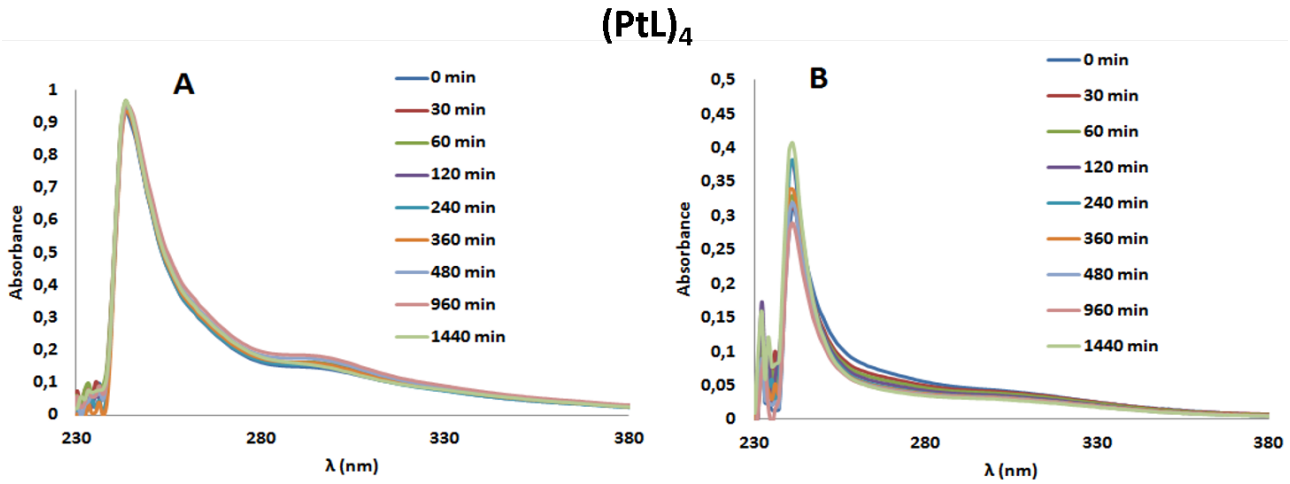


Figure 2. Time dependent UV-Vis analysis of **$(PtL)_4$** (10^{-4} M) in (A)phosphate buffer (50 mM pH 7.4) and (B) 20 mM ammonium acetate buffer (pH 6.8) in presence of 15% of DMSO monitored up to 24 h. TO BE REPLACED

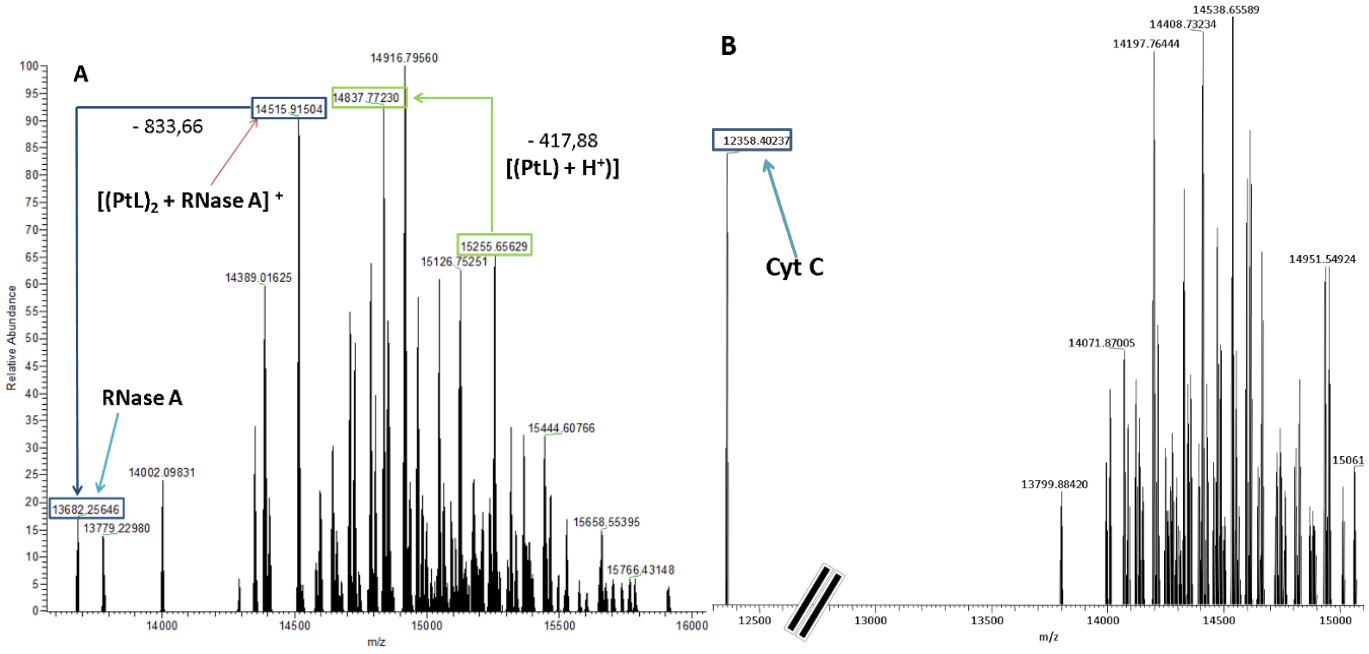
2. Preliminary ESI MS studies of the reactions of $(PtL)_4$ with various model proteins

Subsequently, we have considered the reactions of **$(PtL)_4$** with a few model proteins. Hen egg white lysozyme (Lysozyme), horse heart cytochrome c (Cyt c) and bovine pancreatic ribonuclease (RNase A) were chosen as models for globular proteins, in line with previous studies conducted in our laboratories on these systems and on their reactions with metallodrugs.[3-6]

At first, **$(PtL)_4$** was reacted with the three model proteins above-mentioned at a fixed molar ratio of 3:1 according to standard experimental protocols[7, 8] ; the respective electrospray ionization mass spectra, collected after 48 hours of incubation, are shown in Figure 3. In all cases, several peaks are

observed with mass values greater than those of the corresponding ligand-free proteins. Despite residual peaks assignable to the unreacted proteins are still detectable, these ESI MS results are strongly indicative of a large reactivity of **(PtL)$_4$** with all these model proteins and of consequent adduct formation. It emerges that a great number of metallodrug-protein adducts are formed in large amounts, after relatively short incubation times. The fact that the masses of protein bound metallic fragments are on the whole much smaller than the mass of the intact tetrameric **(PtL)$_4$** complex –i.e. 1630 Da- implies that the starting species breaks down, generating a number of smaller fragments (most likely monoplatinated species), capable of binding these proteins.

The assignment of the peaks in the above ESI MS spectra to precise metallated species is quite problematic owing to the intrinsic spectral complexity; in any case the analysis of fragment isotopic patterns confirms the presence of coordinated Pt atoms.[3, 9] The large number of peaks –with mass shifts ranging from 100/200 to 1000/1200 Da - also points out that metallic fragments of variable mass are formed, supporting the view that the original tetranuclear complex breaks down upon protein interaction, generating a variety of monoplatinated fragments. It is plausible that these metallic fragments may undergo further chemical transformations under harsh conditions, during the ESI ionization process.



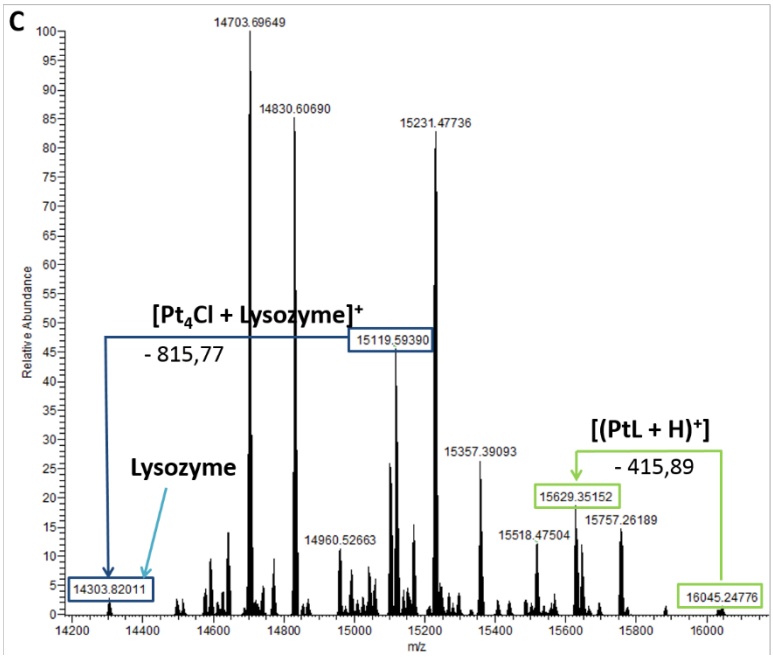


Figure 3. Deconvoluted ESI MS spectra of (A) RNase A,-(B) Cyt c (the peaks in the spectra have been shown in two different areas separated by //) and (C) Lysozyme. Proteins have been treated with 3 x 10^{-4} M of **(PtL)$_4$** and spectra have been recorded after 48 h of incubation at 37 °C, in a metal to protein molar ratio of 3:1, in the presence of 15% DMSO and 20 mM ammonium acetate pH 6.8.

3. The case of Lysozyme

In order to assign the new peaks potentially ascribable to metallodrug-protein adducts that are detected in the above spectra, we performed additional ESI MS experiments aimed at simplifying the complicated spectral patterns shown above. In particular, we decided to work at a lower metallodrug/protein ratio (1:1) and shorter incubation times. This will lead to formation of adducts with a smaller number of bound Pt atoms that are easier to assign. This kind of studies was limited to the reaction of **(PtL)$_4$** with Lysozyme, as the spectrum for this protein (Figure 3C) seemed to us the less complicated and the better resolved. Remarkably, under the newly applied experimental conditions, a simplified spectral pattern was indeed obtained, as shown in Figure 4. Only a few peaks are now observed with *m/z* greater than the native protein (Figure 4). The fact that in the latter conditions we obtained simplified spectra is ascribed to the lower degree of protein metalation as a consequence of the lower **(PtL)$_4$** /Lysozyme ratio and the shorter incubation time. Indeed, under these conditions, adducts bearing a smaller number of metallic fragments are obtained with simpler and clearer spectra.

Also, we decided to check whether the nature of the organic solvent might influence importantly the overall aspect of the ESI MS spectra of Lysozyme derivatives. Interestingly, replacement of DMSO

with acetonitrile did not change significantly the signal pattern that, despite some slight differences in intensities, remained virtually unchanged (Figure 4). Worthy of consideration, in these conditions the protein does not undergo degradation or changes in the aminoacidic chain, according with our previous results gathered applying the same protocol under the same conditions[3]. Indeed in the last years in our laboratories, we obtained a number of evidences confirming that even in presence of pure DMSO, the ESI-MS molecular peak of HEWL is well detectable with no evidences of fragmentation. [10]

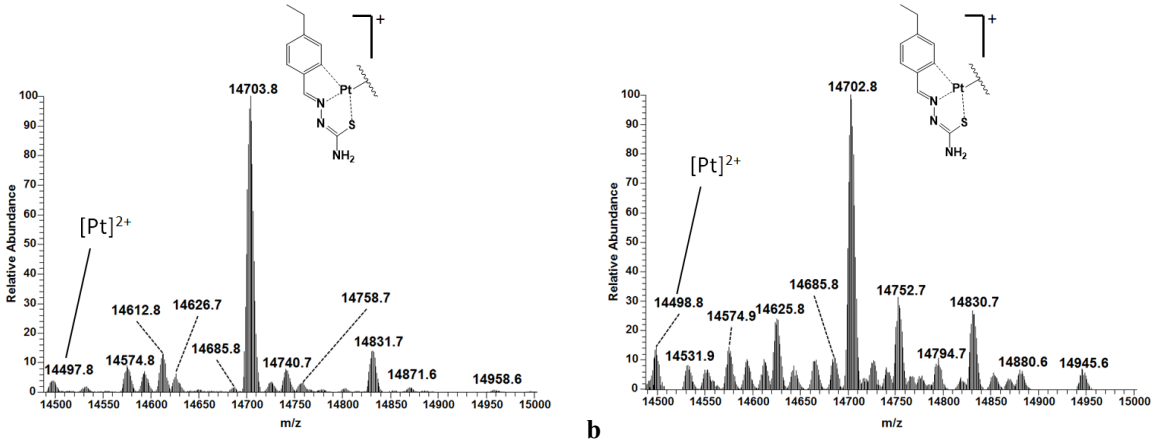


Figure 4. Close-up of deconvoluted ESI MS spectra of Lysozyme (experimental MW= 14303.8 Da) treated with 10^{-4} M of **(PtL)$_4$** after 6 h of incubation at 37° C, in a metal to protein molar ratio of 1:1, in the presence of 15% DMSO (a) or acetonitrile (b) and 20 mM ammonium acetate pH 6.8. See supporting material for full spectra (difference of one Da in the mass shift for the two peaks indicated in figure is due to the software arbitrary assignment).

Upon inspection of Figure 4 a few peaks are detected in the spectral region 14500-14950 Da, that may be tentatively referred to adducts produced by coordination of platinum fragments. Notably, the biggest peak situated at 14703 Da corresponds to a mass shift of 400 Da compared to the Pt-free protein. This mass shift is slightly smaller than the expected mass shift of 415 Da, exactly matching the mass of a single PtL moiety, in other words one fourth of the whole tetraplatinum species. Mass shifts of 415 or 400 Da are indeed recurrent in these ESI MS spectra. The mass shift of 400 Da might be ascribed tentatively to the **PtL** moiety minus 15, which could be due to degradation of the isopropyl group occurring during ESI ionisation process. Moreover, a less intense peak falling at 14498 Da is detected with a mass shift of 195 Da, that could be attributed to protein coordination of a naked platinum ion. Peaks assignments were validated through theoretical simulations (see SI).

Overall, these ESI MS results point out that the tetraplatinum cluster undergoes disassembly upon reaction with the protein in such a way that monoplatinum fragments of molecular mass 415 are released. These fragments are able to bind the protein most likely through formation of a coordinative

bond at the free Pt coordination position. Candidate binding sites on the protein surface are the side chains of histidines[4], methionines[11] or the side chains of Lys[12], and Asp[7] in line with previous results. As the metallic fragment sometimes shows a molecular mass that is slightly lower than 415 Da, it can be hypothesized that under the applied experimental conditions the isopropyl group may undergo chemical transformation during the ESI ionisation process with a loss of 15 Da.

4. Crystallographic results

To obtain a clearer picture of the adducts formed in the reaction between **(PtL)$_4$** and proteins, crystals were grown for **(PtL)$_4$**/RNase A and **(PtL)$_4$**/Lysozyme and the structures of these adducts solved by X-ray crystallography. The structures of the **(PtL)$_4$**/RNase A and **(PtL)$_4$**/Lysozyme adducts have been refined both at 1.78 Å resolution. In both structures, the presence of the Pt fragment(s) does not significantly alter the overall structure of the protein (root mean square deviations between alpha carbon position of the adducts with respect to ligand free proteins are within the range 0.25-0.54 Å) and inspection of difference Fourier electron density maps offers evidence for electron density peaks compatible with the presence of Pt atoms close to His side chains. In particular, in the **(PtL)$_4$**/Lysozyme adduct, electron densities compatible with the presence of Pt atoms are observed at the level of His15 ND1 and NE2 atoms (Figure 5a). Here, the Pt atom is bound to three additional atoms beyond the N atom of His side chain in the well-known planar square geometry or to N atom from Arg14 and other two atoms. His15 has been identified as one of the main Pt binding sites in many other structures of adducts formed in the reaction between Lysozyme[12] and metallodrugs.[14-19] Similarly, in the crystal of the **(PtL)$_4$**/RNase A adduct, which contains two molecules in the asymmetric unit, four Pt binding sites have been observed, close to side chains of His105 and His119 of the two protein chains in the a.u. (Figures 5b and c). These Pt atoms are tetracoordinated and adopt the usual square planar geometry.

The assignment of the Pt ligands in both **(PtL)$_4$**/RNase A and **(PtL)$_4$**/Lysozyme structures is not straightforward, as it often occurs,[20] but in all fragments bound to the two proteins, (at least) one of the atoms coordinated to the Pt centre is an anomalous scatterer, as judged on the basis of the anomalous electron density map (Figure SM5). Considering the presence of sulfur atoms as Pt ligands in the original structure of **(PtL)$_4$** and the finding that the compound was dissolved in DMSO to allow the reaction with the proteins, we believe that at least one of the Pt coordination positions is occupied by a S atom; the other two atoms completing the Pt coordination sphere should be considered as uninterpreted, because no additional evidences were available.

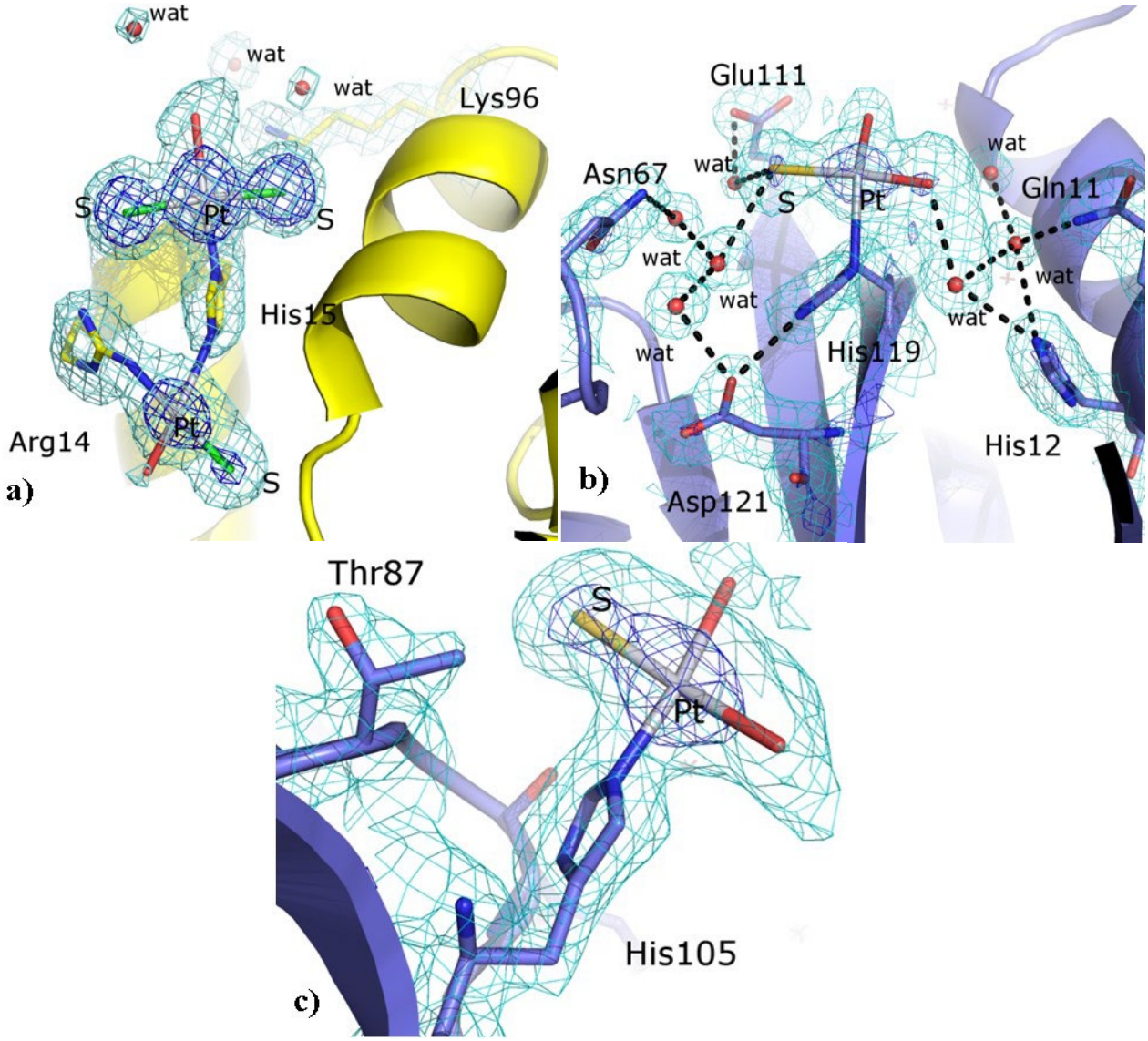


Figure 5. Pt binding sites in the **(PtL)$_4$**/Lysozyme and **(PtL)$_4$**/RNase A adducts. a) Pt binding site close to His15 of **(PtL)$_4$**/Lysozyme structure; b) Pt binding site in the active site of molecule B of **(PtL)$_4$**/RNase A structure. C) Pt binding site close to His105 of molecule B of **(PtL)$_4$**/RNase A structure. 2Fo-Fc electron density maps are contoured at 1.0 (cyan) and 4.0 (blue) σ level. Unassigned Pt ligands are reported in red, as oxygen atoms.

5. The reaction of (PtL)$_4$ with 1-methylimidazole analysed via FAB-MS and ^{1}H NMR

As the above results point out that the tetraplatinum complex undergoes disassembly upon reaction with proteins and releases monoplatinated moieties of molecular weight around 415 Da (Figure 3A and C), we tried to provide further support to this hypothesis working on a simple model system, i.e. 1-methylimidazole. Accordingly, the tetraplatinum complex was reacted with 1-methylimidazole (MeIm) under similar solution conditions and the reaction monitored through ^{1}H NMR spectroscopy.

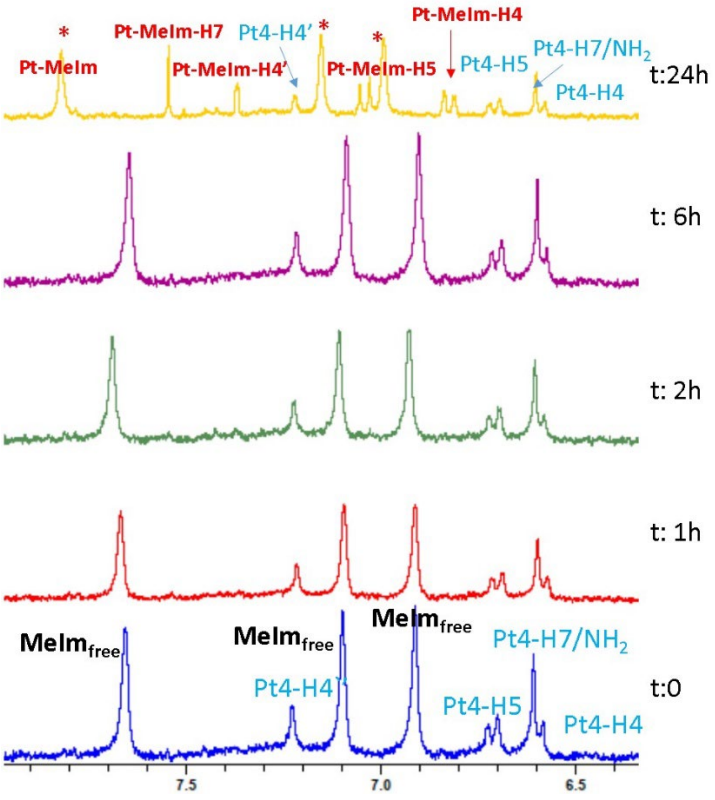


Figure 6. Time dependence of the reaction of **(PtL)$_4$** with MeIm (1:1)

The resulting ^{1}H NMR spectra are shown in Figure 6. These NMR results highlight progressive transition from the starting species (*i.e.* the tetraplatinum cluster) to the presumed final species (PtL-MeIm). In our opinion, these NMR results prove coordination of MeIm to the metal center after cluster disassembly. Figure 6 clearly shows how the tetranuclear compound **(PtL)$_4$** is mostly intact during the first 6 hours (Figure 6, four first spectra); then **(PtL)$_4$** slowly reacts with MeIm affording an adduct (PtLMeIm) where the cyclometallated unit (H4, H5 and H4`) is strongly de-shielded to higher ppm values. The phenyl ring is the most affected area, as expected for cyclometalated complexes, where the ring is directly linked to the Platinum atom by a Pt-C bond. We also observed that the signals of coordinated MeIm (*)de-shielded compared to free MeIm, which proves its coordination. Once we discovered this reactivity, we tried to achieve the mononuclear PtLMeIm complex for full characterization.

We assayed the reaction through varying the temperature and the stoichiometry and found a particularity in the reactivity of this species using ^{1}H NMR and proving it later by mass spectrometry. While complex **(PtL)$_4$** reacts with two equivalents of MeIm affording the mononuclear and tetranuclear complex in a mixture 1:1, even after 24h (Figure 6), using a larger excess of MeIm the mononuclear complex was detected almost pure (Figure 7A). Yet, attempts to purify the mononuclear complex always led to formation of a polynuclear complex (Figure 7 B).

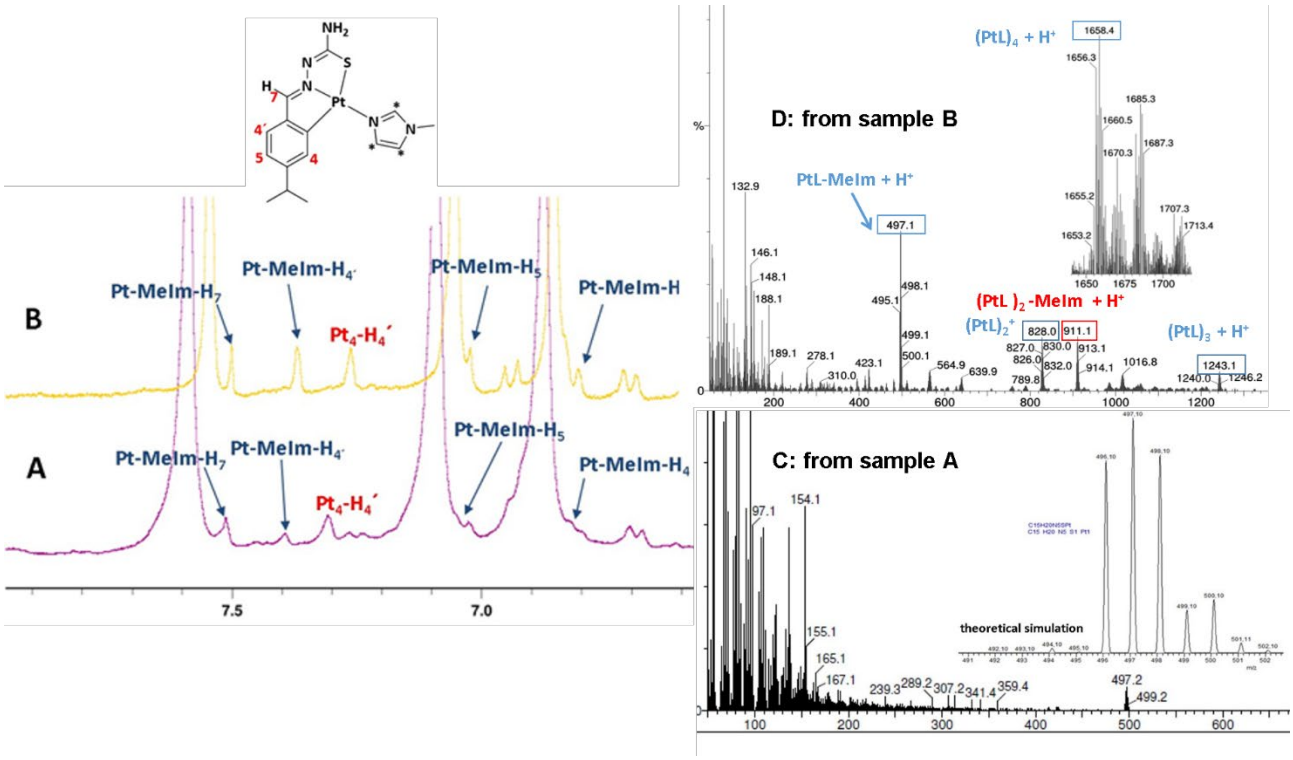


Figure 7. Spectra A) ^{1}H NMR aromatic region from the reaction of **(PtL)$_4$** with an excess of MeIm (1:8) after 24h. Spectra B) ^{1}H NMR aromatic region from the purification attempt of sample. Spectra C) FAB spectra from sample 7A D) FAB spectra from sample 7B

This reactivity was confirmed by FAB that allowed investigating the nature and molecular weight of the adducts formed. The FAB spectra (Figure 7C) proved the presence of the monoadduct (peak at 497) in the NMR experiment (without previous purification). The FAB spectra from the purification attempts (Figure 7C and 7D) proved the association in solution of the mononuclear species with 1-methylimidazole (main molecular ion). The apparent differences between the bound fragment obtained upon reaction with lysozyme or MeIm i.e. the **PtL** moiety minus 15 is not detectable in the FAB experiment when complex is reacted with MeIm, is most probably due to the ionisation process in the ESI-MS experiment. This process is able to determine the degradation of the isopropyl group that is not occurring in FAB experiment.

Concluding, the ^{1}H-NMR results, corroborated by MS/FAB measurements, reveal the formation of a species of MW 497 that is perfectly consistent with the LPt-MeIm complex.

Conclusions

In conclusion, we have shown here that the **(PtL)$_4$** complex reacts eagerly with proteins as it was nicely documented by ESI MS analysis. Similar reactions take place for the three distinct proteins that we have used here as model systems, i.e. Lyszoyme, RNase A and Cyt c . The ESI MS patterns of the resulting adducts are rather complex. Yet, a remarkable spectral simplification could be achieved by working at lower metallodrug/protein ratios and lower incubation times with Lysozyme;

this allowed the assignment of the main ESI MS peaks attributable to the adducts. It is inferred that, upon reaction with proteins, the tetranuclear complex breaks down and releases monometallic fragments capable in turn of binding these proteins. Binding occurs at the level of exposed His side chains, according to crystallographic data collected on both $(PtL)_4$/Lysozyme and $(PtL)_4$/RNase A adducts. This binding scheme is supported by studies on the reaction of **$(PtL)_4$** with 1-methylimidazole analysed through ^{1}H NMR and FAB-MS. Overall, our results demonstrate that the tetraplatinum cluster undergoes disassembly and becomes able to metallate proteins mostly through coordination of monoplatinated fragments to exposed His side chains.

EXPERIMENTAL

Materials and methods

$(PtL)_4$ was synthesized as described in a previous publication[1] and its structural and biophysical characterization was in agreement with previous data. HEWL, Cyt c and RNase A were purchased from Sigma-Aldrich as well as 1-methilimidazole (MeIm).

Mono-dimensional ^{1}H-NMR experiments were performed in DMSO-d^6 and D_2O using a Bruker AMX-300 (300 MHz) and DRX-500 (500 MHz) spectrometers at room temperature (25 ºC). UV-Visible experiments have been performed using a Varian Cary 50 Bio UV-Vis or a Thermo Fisher ScientificEvolution 260 Biospectrophotometers. Mass spectra were registered at SIDI-UAM Department (Servicio Interdepartamental de Investigación-Universidad Autónoma de Madrid) using: a) FAB, b) ESI in a Hybrid Quad-Tof (QTOF) mass spectrometer [QSTAR (ABSciex)] and at Chemistry Department of University of Florence using an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA, USA), equipped with a conventional ESI source.

Crystallization and X-ray diffraction data collection

Lysozyme was crystallized through the hanging drop vapour diffusion method using 20% ethylene glycol and 0.6 M sodium nitrate as precipitants and 0.1 M sodium acetate pH 4.4 as buffer. RNase A crystals were obtained through the same technique, but using a reservoir containing 22% PEG4K and 0.01 M sodium citrate pH 5.1. In order to obtain an adduct between the model proteins and the tetraplatinum compound, crystals of both Lysozyme and RNase A were soaked in a solution consisting of 50% reservoir and 50% **$(PtL)_4$** dissolved in DMSO. The protein to metal molar ratio, evaluated considering the protein concentration used to grow ligand-free protein crystals, is 1:3. After three days of soaking, crystals suitable for X-ray diffraction analysis were fished with nylon loops

and flash-cooled at 100 K without any cryoprotectants, only removing the solvent around the crystal[20].

X-Ray diffraction data were collected using a Saturn944 CCD detector equipped with CuKα X-ray radiation from a Rigaku Micromax 007 HF generator. Processing and scaling were performed using HKL2000.[21] Data were collected at 1.78 Å resolution for both protein-metallodrug adducts at the CNR Institute of Biostructures and Bioimages, Naples, Italy.

The structures of the **(PtL)$_4$**/RNase A and **(PtL)$_4$**/Lysozyme have been solved by molecular replacement method, using protein coordinates from PDB files 1JVT [22] and 193L [23], respectively, as starting models. These models have been also used to compare the structures of the adducts with those of the ligand free proteins, by calculating the root mean square deviation of the carbon alpha atomic coordinates. Refinement of the **(PtL)$_4$**/RNase A and **(PtL)$_4$**/Lysozyme structure has been carried out with CCP4 Refmac5,[24] while model building and map inspections have been manually performed using Wincoot[25].

Pt atoms have been unambiguously identified by comparing 2Fo-Fc, residual and omit Fo-Fc and anomalous difference electron density maps. The interpretation of Pt ligands is very difficult, as it often occurs,[20] since the exact ligands bound to the metal centre are unknown. Considering the presence of at least one peak corresponding to the position of Pt ligands in the anomalous map and the finding that sulphur atoms are Pt ligands in the original compound, at least one of the ligand has been assigned to a S atom. Unknown Pt ligands completing the square planar Pt coordination sphere, well visible in the electron density maps, have been refined as oxygens. Large positive peaks remain in the residual Fo-Fc electron density map on the Pt ligands, suggesting that probably other interpretations are possible.

The structures refine up to R-factor/R-free values of 0.181/0.238 and 0.196/0.250 for **(PtL)$_4$**/Lysozyme and **(PtL)$_4$**/RNase A, respectively. Refinement statistics are reported in Table S1. The refined models and structure factors have been deposited in the Protein Data Bank under accession codes 5OLE (**(PtL)$_4$**/Lysozyme) and 5OLD (**(PtL)$_4$**/RNase A).

UV-Vis analysis

Solution behaviour of complex (PtL)$_4$

Solution behaviour of the complex **(PtL)$_4$** was assessed through spectrophotometric studies performed with a Varian Cary 50 Bio UV-Vis spectrophotometer in buffered solutions. A solution of complex **(PtL)$_4$** (10^{-4} M) was prepared in phosphate buffer (50 mM, pH 7.4) or in ammonium acetate

buffer (20 mM, pH 6.8), using DMSO (15%). The previous biological assays were performed using 1% of DMSO, but in order to perform spectrophotometric studies the concentration of the compound needs to be higher. The absorbance was then monitored in the wavelength between 200 and 800 nm for 24 h at 25 ºC.

NMR studies

Reactivity of complex $(PtL)_4$ and MeIm

A solution of MeIm (0.99 mg, 12.06 µmol) in D_2O was added to a solution of complex **$(PtL)_4$** (10 mg, 6.03 µmol) in DMSO-d_6 to a final volume of 0.5 mL (1:1) in an NMR tube. The tube was kept in a thermoshaker at 37 ºC under slight stirring during the entire experiment (24 h). ^{1}H NMR spectra were recorded fresh, at 1h, 2 h, 6h and 24 h.

Following all the steps performed in experiment 1, the compound was allowed to react 24h. Afterwards a large excess of MeIm (2-98 mg, 36.18 µmol) was added (1:8) and the ^{1}H NMR spectra was recorded (Figure 6A) and sent to mass spectrometry. The remaining sample was precipitated with water, washed with hexane and vacuum dried overnight at 40 ºC. The final solid was characterised by ^{1}H NMR and FABB mass spectrometry.

ESI-MS experiments

Interaction of complex $(PtL)_4$ and Lysozyme

A solution of complex **$(PtL)_4$** (10^{-4} M) with HEWL or RNase A (protein to complex molar ratio 3:1 or 1:1) in ammonium acetate buffer (20 mM, pH 6.8), acetic acid buffer (20 mM, pH 4.5) or acidic water (pH 5.5), using 15% of DMSO, CH_3CN or DMF, was incubated at 37 °C for different time intervals as reported within the text.

After a 20-fold dilution with water, ESI MS spectra have been recorded by direct introduction at 5 µL/min flow rate in an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA, USA), equipped with a conventional ESI source. The working conditions were the following: spray voltage 3.1 kV, capillary voltage 45 V, capillary temperature 220 ºC, tube lens voltage 230 V. The sheath and the auxiliary gases were set, respectively, at 17 (arbitrary units) and 1 (arbitrary units). For acquisition, Xcalibur 2.0. software (Thermo) was used and ±monoisotopic and average deconvoluted masses were obtained by using the integrated-Xtract tool. For spectrum acquisition, a nominal resolution (at m/z 400) of 100,000 was used.

Theoretical peaks have been calculated with ProMass for Xcalibur (version 2.8 rev. 2)

Acknowledgments

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Highlights

- The reactions of a tetraplatinum organometallic complex with various model proteins are investigated
- Adducts formed with model proteins, and in particular with lysozyme are characterised by ESI MS and crystallographic determinations.
- The possible activation pathway of the tetraplatinum complex is delineated.