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Characterization of Pt-protein complexes by nHPLC–ESI-LTQ MS/MS using a gel-based bottom-up approach

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ABSTRACT

The suitability of in-gel digestion for the characterization of Pt-binding proteins by gel-based bottom-up MS approaches has been evaluated regarding the preservation of Pt-protein bonds during the process. Standard proteins (albumin, transferrin, carbonic anhydrase, myoglobin and cytochome c) incubated with cisplatin were separated by nrSDS-PAGE and in-gel trypsin-digested. The whole in-gel digestion protocol included treatment with reagents such as: ammonium bicarbonate, acetonitrile, formic acid, trypsin as enzyme and alternatively, dithiotreitol and iodoacetamide as reducing and alkylating agents. Digests were analyzed by nHPLC–ESI-LTQ-MS/MS and Pt-peptides were recognized in all the proteins studied on the basis of their isotopic pattern. Only when the reducing and alkylating reagents were used, the amount of detectable Pt-peptides decreased due to the high reactivity of thiol containing reagents towards Pt. Furthermore, the repeated use of acetonitrile could lead to the replacement of ligands originally attached to Pt by CN^-, but does not affect the Pt-protein binding. Platinum-binding sites on the proteins were elucidated from the CID–MS/MS fragmentation spectra and assessed by evaluation of protein structures. Several histidines, cysteines and methionines were identified as platinum binding sites in the different standard proteins. Results were in accordance to those obtained with in-solution digestions.

1. Introduction

The use of platinum drugs as anticancer agents has been essential for the treatment of numerous solid tumors for the last decades and nowadays they are still employed in more than half of the treatments, usually in combination with other drugs. Their antitumor activity can be ascribed to their ability to form adducts with DNA bases, in which distortion of the double helix is implied, resulting in apoptosis of tumor cells [1]. However, the whole cytotoxic mechanism may be more complex and is still uncertain. In fact, the capacity of Pt drugs to coordinate to other biomolecules containing nucleophilic groups, such as proteins, has also been long-known and should be born in mind in order to understand their activity, as this can influence the transport, uptake, excretion and availability of the drug [2]. Moreover, the interactions with proteins have often been related to the severe side-effects (nephrotoxicity, ototoxicity, myelosuppression) and the occasional acquired or inherent resistance observed during treatments with Pt drugs. The great importance of this issue makes it essential to develop analytical methodologies for the identification of Pt-binding proteins in biological samples, in order to shed some light on the mechanism of Pt-drugs toxicity.

The suitability of soft ionization-based mass spectrometric techniques for the characterization of Pt-binding proteins has been reported before, mainly in numerous studies of standard proteins incubated with Pt drugs (transferrin [3], metallothioneins [4], cytochrome c [5], ubiquitin [6], myoglobin [7], superoxide dismutase [8], insulin [9]). In particular, electrospray ionization (ESI) has turned out to be more appropriate than matrix-assisted laser desorption-ionization (MALDI) for the characterization of Pt-proteins in terms of preservation of the metal and the original ligands attached to it [9,10]. On the other hand, the proteomics bottom-up approach, using CID MS^n fragmentation on peptide ions from digested proteins seems to be more informative regarding the localization of Pt-binding sites in proteins than the top-down approach on intact Pt-protein complexes [11], although the latter involves a lower degree of sample treatment and a lower risk of alteration of the original adducts in a sample.

A great challenge when facing the study of metal-binding proteins in biological samples is to deal with the separation of very complex mixtures of proteins using multidimensional separation methods, followed by the use of analytical techniques that may allow detecting and identifying metal-proteins (or metal-peptides when enzymatic digestions are applied). The critical issue is that the metal-protein (in this case, Pt-proteins) bond should be preserved along all the steps involved in the methodology.

The use of chromatography for the separation of proteins may be problematic in terms of recoveries of the sample, especially

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when dealing with reverse phase chromatography, which is also required for enhancing the resolution of the separation. Nevertheless, a bottom-up shotgun approach can be applied, as the resolution achieved by chromatographic separations at peptide level is considerably higher than when dealing with proteins. In fact, multidimensional protein identification technology (MudPIT) on-line coupled to ESI-MS/MS was used for the identification of platinated peptides from serum samples and *E. coli* extracts incubated with cisplatin [12,13].

Another very appealing possibility is to carry out a bottom-up approach by first separating proteins by 2-D electrophoresis (2-DE), usually applying isoelectric focusing followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which allows the separation of thousands of proteins [14]. In this case, the highly sensitive elemental technique laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has been proposed as a very interesting tool for the direct detection of metals in the protein spots by analysis of the gels after the protein separation [15]. Then, the protein spots in which the metal has been detected can be excised and in-gel digested, followed by nHPLC–ESI-MS/MS analysis, which represents additional separation dimensions. Their identification is possible by searching the results by algorithmic comparison against a protein database, for example by using SEQUEST or MASCOT, where the metal moiety mass is included as a variable modification in the search. Again, the preservation of the metal-protein bond along all the steps involved in the analysis is crucial. For this reason, especially for non-covalent bound metals like Zn, Cu or Fe, the use of non-denaturing and non-reducing conditions during the electrophoretic separations has been proposed [16]. In the case of Pt-protein complexes, denaturing conditions may still be appropriate for their separation but, in general, the use of sulfur containing reagents such as thiourea has been shown to be problematic, as is the case of reducing agents as dithiotreitol (DTT) or β-mercaptoethanol (BME) [17].

There are only few precedents on the use of gel electrophoresis for the separation of Pt-binding proteins, detection of Pt in the gels by LA-ICP-MS and in-gel digestion, followed by HPLC–ESI-MS/MS for their identification. In particular, the methodology was applied on proteins from either *E. Coli* cell extracts [18] or blood serum [19], both incubated with cisplatin, allowing the identification of OmpA [18] or albumin, transferrin and α-2-macroglobulin [19], respectively, in the observed Pt bands or spots. However, in those cases, no Pt-peptides were detected. On the other hand, few works report the identification of other metalloid-containing peptides after in-gel digestion of proteins: Se-peptides from two proteins, SIP 18 and HSP 12, were detected in a Se-enriched yeast sample, by nHPLC–ESI-MS/MS [20]; additionally, a Zn-containing peptide in albumin, on the basis of the mass observed in the analysis by MALDI-TOF MS of the in-gel digest, was assigned [21]. In contrast, in some other works where Zn, Cu or Fe-proteins were studied, the lack of metal-binding peptides in the mass spectra after in-gel digestion was reported [22].

Therefore, it seems essential to examine if the gel-based bottom-up methodology, and particularly the reagents involved along the in-gel digestion process, are suitable for the characterization of Pt-binding proteins. With this purpose, several model proteins were incubated with cisplatin and separated by SDS-PAGE to evaluate the suitability of the conventional in-gel digestion protocol for the detection of Pt-peptides and the identification of Pt-binding sites by subsequent nHPLC–ESI-MS/MS analysis of the digests. The model proteins selected, all of them reactive towards cisplatin [17], have different complexities to be taken into account during the study: equine cytochrome c (CYT C), equine myoglobin (MYO) and bovine carbonic anhydrase (CA) present a relatively small size (12, 17 and 29 kDa, respectively) and lack disulfide bonds. Conversely, human serum albumin (HSA) and human transferrin (TF) are larger (67 and 79 kDa, respectively) and present numerous disulfide bonds.

2. Experimental

2.1. Materials and reagents

Cisplatin (*cis*-[$PtCl_2(NH_3)_2$]) and standard proteins (apo-transferrin from human serum, TF; human serum albumin, HSA; carbonic anhydrase from bovine erythrocytes, CA; myoglobin from horse heart, MYO; and cytochrome c from horse heart, CYT C) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium chloride (Panreac Química S.A., Barcelona, Spain) and Tris(hydroxymethyl)aminomethane (Tris) (Fluka) were used for the preparation of the incubation solution under physiological conditions. High purity nitric acid (Merck, Darmstadt, Germany) was used for the pH adjustment of the mentioned incubation solution. A pH-meter (Crison) or pH indicator strips (pH 6.5–10, special indicator, Merck, Darmstadt, Germany) were used in order to control the pH of the solutions prepared. Solutions were prepared with deionized water (Milli-Q Ultrapure water systems, Millipore, USA) except the ones involved during tryptic digestions and further analysis, in which mass spectrometry (MS) grade water from Scharlab (Barcelona, Spain) was used. MS grade acetonitrile (ACN) was also obtained from Scharlab (Barcelona, Spain). Ammonium bicarbonate (NH_4HCO_3), trifluoroacetic acid (TFA), formic acid (FA), urea, dithiothreitol (DTT) and iodoacetamide (IAA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Porcine trypsin gold mass spectrometry grade was obtained from Promega (Madison, WI, USA).

2.2. Incubation of standard proteins with cisplatin

To produce Pt-protein adducts, standard proteins (TF, HSA, CA, MYO and CYT C (60 μM)) were incubated separately with cisplatin (600 μM) at a molar ratio 1:10 in a buffer containing NaCl (4.64 mM) and Tris–NO_3 (10 mM, pH 7.4), reproducing the physiological intracellular saline and pH conditions, at 37 °C in a thermostatic bath (Neslab RTE-111), for 96 h. Control samples were also prepared by incubating these same proteins under the conditions described but in the absence of cisplatin. Unbound cisplatin was removed after the incubations with 3 kDa spin-cut-off filters (Amicon Ultra-0.5 mL Ultracel-3, Millipore, USA) by centrifugation at 14,000 × *g* for 30 min, followed by a further washing step of the retained protein fraction with the incubation buffer.

2.3. SDS-PAGE

Pt-bound proteins were diluted appropriately and mixed 1 + 1 with Laemmli sample buffer (LSB), containing: Tris–HCl (62.5 mM, pH 6.8), glycerol (25%), SDS (2%) and bromophenol blue (0.01%) (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The mixtures were heated for 1 min at 95 °C for denaturing the proteins.

The non-reducing SDS-PAGE separations were carried out in a Mini Protean Tetra Cell Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA) using 3% and 12.5% of polyacrylamide for the stacking and the resolving gels, respectively. 2 μg of each protein were loaded on the gels, which were run at constant current (12 mA for 20 min and 20 mA for 3 h). The running buffer contained Tris–HCl (25 mM, pH 8.3), glycine (192 mM) and SDS (0.1%). Precision Plus Protein unstained standards (Bio-Rad Laboratories, Inc., Hercules, CA), were also loaded on the gels as molecular weight markers.

After electrophoresis, proteins were fixed on the gel with a solution containing 40% methanol and 10% acetic acid for 30 min. Gels were visualized by staining with Bio-Safe Colloidal Coomassie Blue

G-250 (Bio-Rad Laboratories, Inc., Hercules, CA) for 1 h, and finally washed with water for 1 h.

2.4. In-gel digestions

Protein bands were excised from the gels with a scalpel, transferred to 600 μL siliconized Lo-bind Eppendorf tubes and washed for 30 min in 150 μL of 25 mM ammonium bicarbonate (NH_4HCO_3) at 22 °C, shaking at 1000 rpm in a Thermomixer (Eppendorf AG, 22331 Hamburg, Germany). Washing solutions were always discarded onwards. The gel slices were unstained in 150 μL of 50% acetonitrile in 25 mM NH_4HCO_3 shaking at 1000 rpm for 30 min, this step being repeated as many times as needed. When completely unstained, the gel slices were washed with 25 mM NH_4HCO_3 for 15 min to keep them completely hydrated during the following step. Then, the gel slices were cut into 1 mm × 1 mm pieces and 50 μL of acetonitrile were added to shrink the gel plugs. After 10 min, the solvent was removed and the gel pieces were dried in a vacuum centrifuge, Concentrator Plus (Eppendorf AG, 22331 Hamburg, Germany).

Whenever reduction and alkylation were performed, gel pieces were reswollen and incubated with 40–50 μL of 10 mM DTT at 37 °C for 40 min. After that, the solution was discarded and replaced by 40 μL of 55 mM IAA, followed by incubation at room temperature in the darkness for 40 min. Then, gel pieces were washed with 100 μL of 25 mM NH_4HCO_3 for 15 min and dehydrated with 50 μL of ACN. Afterwards, gel pieces were dried in a vacuum centrifuge.

The dry gel pieces were rehydrated in an ice bath with 12.5 ng μL^{-1} trypsin in 25 mM NH_4HCO_3 (typically 20 μL). Once the gels had been completely reswollen, 10–20 μL of 25 mM NH_4HCO_3 were added to cover the gel pieces and then incubated at 37 °C for 16 h. After digestion, supernatants were collected and transferred to eppendorf tubes, and kept at 4 °C. Peptides remaining in the gels were extracted with 40 μL of 2% HCOOH, with vortexing, and incubation for 30 min at room temperature. Extracted peptides were pooled with the original supernatants. Additionally, 40 μL of a solution containing 50% ACN, 0.1% HCOOH were added to the gel plugs, vortexed and incubated for another 30 min. The extraction solution was pooled with the previous ones and samples were evaporated in a vacuum centrifuge until near dryness. For nHPLC–MS/MS analysis, digests were diluted with a solution containing 2% ACN, 0.1% HCOOH to a final volume of 15 μL.

2.5. In-solution digestions

The generated Pt-bound standard proteins were also digested in-solution. 100 μg of each protein were digested with porcine trypsin (1:30 w/w trypsin:protein ratio) in a buffer containing 50 mM Tris–NO_3 (pH 7.8) and 1 M urea, at 37 °C for 16 h. The digestions were performed in a thermomixer, shaking at 300 rpm. When dealing with disulfide-containing proteins, reducing and alkylating steps were also alternatively included in the protocol, using 10 mM DTT and 50 mM IAA, as described elsewhere [11]. Prior to the nHPLC–MS analysis, digests were desalted by micro-solid phase extraction, using Omix tips (C_{18}, 10 μL, Agilent Technologies), the samples being eluted in 70% ACN, 0.1% HCOOH and finally diluted at least 1:10 with 2% ACN, 0.1% HCOOH.

2.6. NanoHPLC–ESI-LTQ MS/MS

ESI-MS/MS data were acquired using a linear ion trap LTQ XL with ETD (Thermo Scientific, San Jose, CA, USA) equipped with a nano-electrospray source from Proxeon (Odense, Denmark). Instrumental parameters were tuned with a solution of 2 μM human [Glu^1]-Fibrinopeptide B (Sigma–Aldrich), prepared in 70% ACN, 0.1% HCOOH. During the analysis, the parameters were typically set to: capillary temperature: 200 °C, capillary voltage: 30 V, tube lens: 105 V. A triple play scan method was employed, consisting on acquisition of full enhanced MS scans in the positive ion mode, over the m/z range 400–1600, followed by zoom scans and further full enhanced MS/MS, acquired in profile mode, of the three most intense peaks in the full MS scan. CID was used for the fragmentation of ions in MS/MS experiments. Typically, the conditions applied during the CID experiments were: 35% relative collision energy, activation time of 30 ms, and isolation width of the precursor ions was set to 4. Dynamic exclusion was enabled with a repeat count of 1, using a 180 s exclusion duration window.

A dual-gradient system nanoHPLC pump (nanoLC ultra 1D Plus, Eksigent) with a Thermo Electron Micro AS autosampler was used. Aliquots of samples (5 μL) were injected, using a 20 μL loop and a pick-up method, and loaded on a trap column (Reprosil pur C_{18}, 3 μm particle size, 0.3 mm × 10 mm, 120 Å pore size, SGE) at a 3 $\mu L\,min^{-1}$ flow rate using 2% ACN, 0.1% HCOOH as mobile phase. The preconcentrated peptides were eluted and transferred to a reverse phase microcapillary analytical column (Acclaim PepMap 100, C_{18}, 3 μm particle size, 75 μm × 15 cm, 100 Å pore size, Dionex, LC Packings) by reversing the flow at 200 nL min^{-1}. Peptide elution was performed applying a three-step gradient: 5–15% B linear for 5 min, 15–40% B linear for 40 min and 40–80% B linear for another 5 min, holding the system at 80% B for 10 min. Mobile phases used were: A, 2% ACN, 0.1% HCOOH; and B, 99.9% ACN, 0.1% HCOOH. The end of the column was connected to a stainless steel nano-bore emitter (O.D. 150 μm, I.D. 30 μm, Proxeon, Odense, Denmark) for spraying and coupling with the LTQ. The spray voltage was set at 1.70 kV.

For data analysis, spectra were assessed with Xcalibur Qual Browser software (Thermo Electron). Search on human, equine and bovine Uniprot KB protein databases using SEQUEST allowed the confirmation of the identity of proteins. Platinum peptides were recognized and identified taking into account the isotopic patterns both in the zoom scans of the precursor ions and in the MS/MS spectra.

3. Results and discussion

A number of platinated standard proteins (TF, HSA, CA, MYO, CYT C) were subjected to SDS-PAGE separation in a set of conditions that were previously reported to be the most appropriate for the preservation of platinum-protein bonds [17], in essence, in the absence of thiol-containing reducing agents. The bands containing these Pt-proteins were in-gel digested and analyzed by nHPLC–ESI-MS/MS, in order to determine if the steps and reagents involved in this particular type of digestion procedure may be compatible with the preservation of Pt-protein bonds, and thus appropriate for the study and characterization of Pt-binding proteins. The effect of reduction and alkylation steps during the in-gel digestions on the yield of Pt-peptides was also evaluated. Additionally, for comparison purposes, in-solution digestions were performed on the same protein models. Platinum binding sites were determined in the proteins by using the gel-based bottom-up approach. The selected proteins present a great variety of potential binding sites for Pt (II). It is well known that, according to Pt (II) solution chemistry, it shows a high reactivity with sulfur-containing groups, especially free thiols in Cys and also, with a considerable affinity, the thioether sulfur in Met or the imidazole nitrogens of His, mainly, although other groups such as Thr, Ser, Glu, Asp, Lys, have also been proposed in some cases [13]. Previous studies showed that when both Met and His were present in model peptides, Met were kinetically favoured while His were proposed as the thermodynamically more stable targets on their reaction with Pt(II) compounds, although a

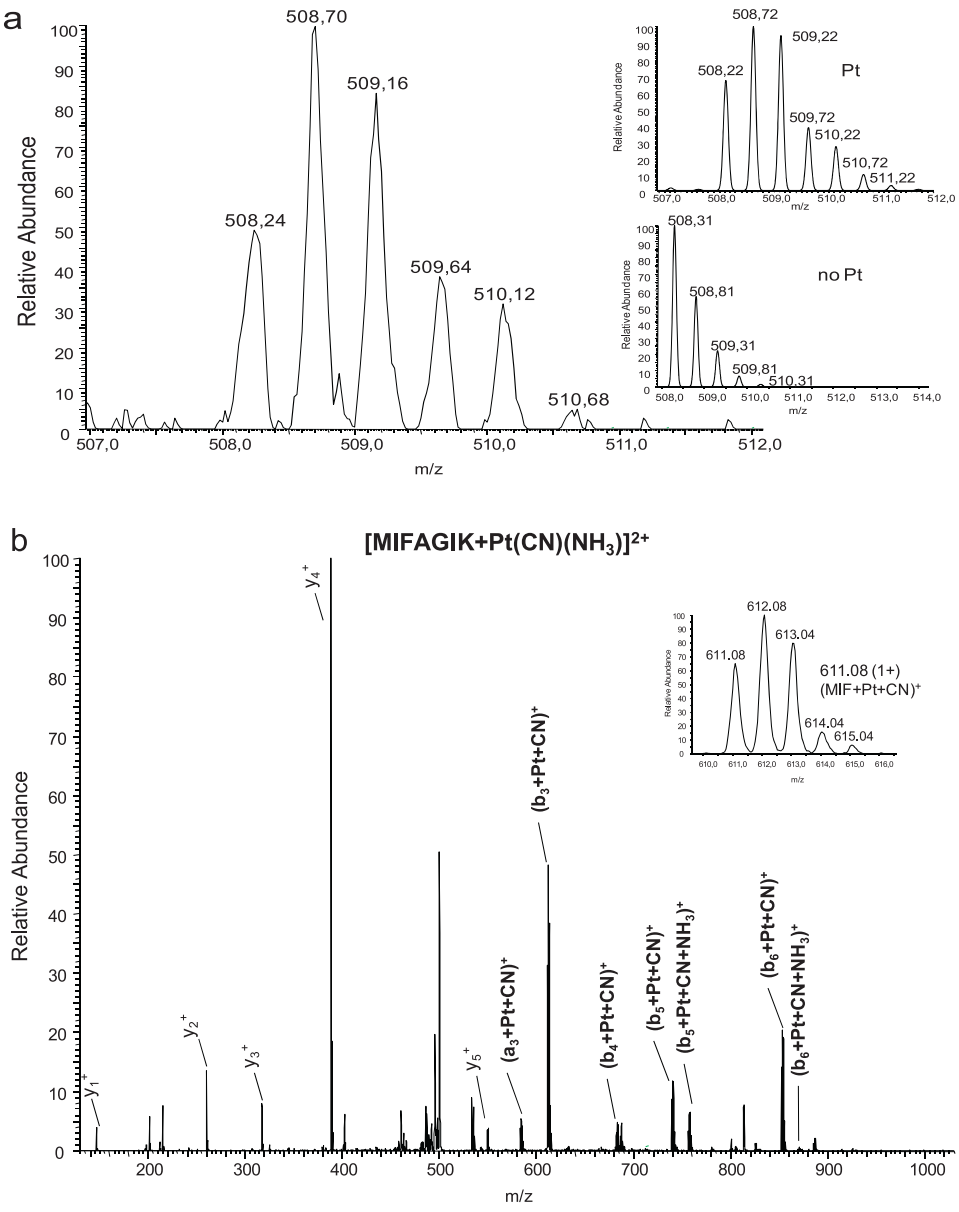

Fig. 1. (a) Zoom scan showing the isotopic pattern of the Pt-modified peptide ion observed at *m*/*z* 508.24 (2+) in the in-gel digest of CYT C–cisplatin, corresponding to $[^{80}\text{MIFAGIK}^{86} + \text{Pt(CN)(NH}_3)]^{2+}$. Insets: theoretical isotopic patterns of a platinated (up) and a regular peptide (down) with the same mass. (b) CID–MS/MS spectrum of the ion at *m*/*z* 508.24. Inset: Pt-fragment ion observed at *m*/*z* 611.08.

competition and even a simultaneous coordination was also observed [23,24].

3.1. Equine cytochrome c–cisplatin digests

Analysis by nHPLC–ESI-MS/MS of the solution resulting from an in-gel digestion of platinum-bound CYT C allowed its identification with SEQUEST with 99% protein coverage. Furthermore, several peptide ions were detected on the basis of the recognition of a particular isotopic pattern typical of platinum-containing peptides. Equine Cyt C presents 2 methionines and several histidines within its sequence that could be considered as potential binding sites in principle, considering that its cysteines reactivity might be impeded, being in this case involved in heme-group binding. Fig. 1a shows the zoom scan for the peptide ion at *m*/*z* 508.24 (2+) with a clear isotopic pattern characteristic of Pt-modified peptides. For comparison, the theoretical isotopic patterns of a peptide with the same mass with and without Pt are shown. The CID–MS/MS spectrum for this ion allowed its identification as $[^{80}\text{MIFAGIK}^{86} + \text{Pt(CN)(NH}_3)]^{2+}$, as can be seen in Fig. 1b. Taking a look at the fragments, the presence of a platinated b_3^+ fragment ion at *m*/*z* 611.08 (1+), corresponding to $[\text{MIF} + \text{Pt} + \text{CN}]^+$, implies the probable binding of platinum to Met 80 in CYT C. As shown in Fig. S-1, another Pt-containing peptide ion was recognized by its fragmentation pattern at *m*/*z* 852.68 (2+), and identified as $[^{61}\text{EETLMEYLENPK}^{72} + \text{Pt(NH}_3)]^{2+}$. In this figure an almost complete b series of peptides carrying platinum can be observed, being detectable the platinated a_3 fragment at *m*/*z* 523.92 (1+), corresponding to $[\text{EET} + \text{Pt}]^+$. This may be ascribed to the binding of Pt to Glu 61/Glu 62 (E) or Thr 63 (T). However, due to the lack of detectable platinated fragment ions on the y series, the possible binding to the Met 65 present in the peptide cannot be clearly elucidated and the likely dual coordination of platinum to both the potentially reactive Met 65 and the Glu or Thr residues cannot be discarded. Indeed, although there is a slight possibility that NH_3 ligands are released to some extent along the ionization process,

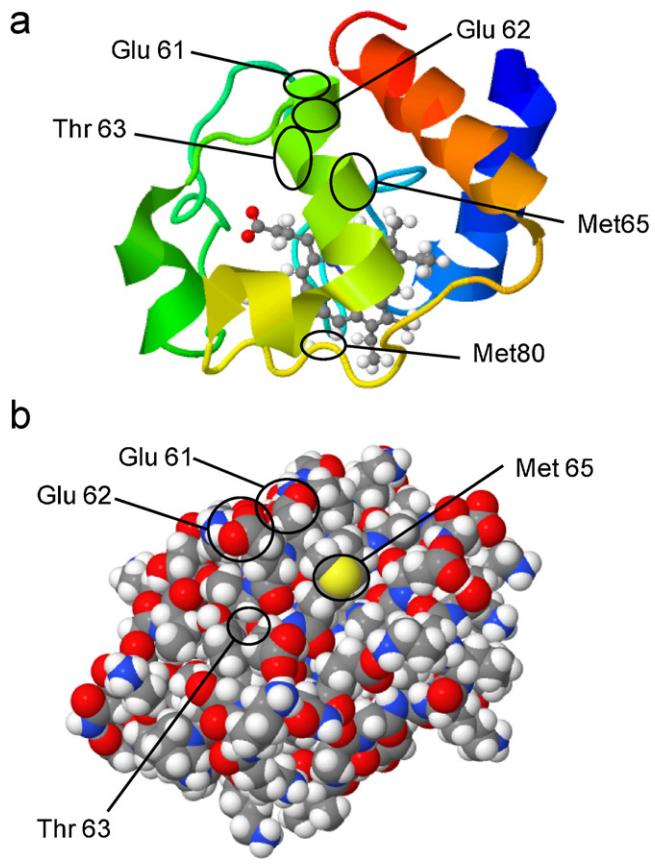

Fig. 2. (a) Schematic and (b) CPK model structures of horse heart cytochrome c, showing the identified cisplatin binding sites. In the CPK model, the atoms color code is the following: H: white, C: grey, O: red, N: blue, S: yellow (Structures retrieved from RSCB Protein Data Bank. ID: 1LC1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in this case, the high trans effect induced by the coordination of Pt to S [25] in Met (after the release of the labile Cl^-, or H_2O in the case of aquo-complexes) would facilitate the release of the trans NH_3 ligand and may explain the coordination of Pt to the peptide as a tridentate ligand, as in the case of cisplatin-ubiquitin adducts, where Pt was found to simultaneously coordinate to the sulfur in Met 1, a deprotonated backbone amide NH and the free carboxylate group of the Glu 16/Glu 18 residues [26]. These same results were confirmed by performing in solution digestion of the CYT C–cisplatin adducts. In this case, peptide ions with a very clear Pt-modified isotopic pattern were found at *m/z* 494.60 (2+) and 852.62 (2+). The CID fragmentation of the platinum-containing ions, allowed their identification as: $[^{80}\text{MIFAGIK}^{86} + \text{Pt}(\text{NH}_3)]^{2+}$ and again $[^{61}\text{EETLMEYLENPK}^{72} + \text{Pt}(\text{NH}_3)]^{2+}$, respectively (Fig. S-2). Similar results regarding the Pt-binding sites were observed compared to the in-gel digests and further MS^3 experiments performed on the in-solution digests allowed the confirmation of Met 65 as Pt-binding site (not shown). The results on the Pt binding sites are in accordance with others previously described [5], in which Met 65 has been proposed as the main binding site for cisplatin in CYT C, while, though less frequently, Met 80 has also been proposed as a secondary site for carboplatin [27]. Taking a look at the 3D structure of the protein (Fig. 2a), Glu 61, Glu 62, Thr 63 and Met 65 are located in an α-helix while Met 80 is located in a loop, being the latter Met coordinated to the Fe atom in the heme group. Considering the surface of the protein based on a CPK model (Fig. 2b), the sulfur atom of Met 65 and the –COOH groups of Glu 61 and Glu 62 present total superficial accessibility, this not being the case of –OH in Thr 63. It could be postulated that the coordination of Pt to S in Met 65 occurs in the first place, possibly followed by coordination to N from an amide backbone and finally to Glu 61/Glu 62 promoted by the strong trans effect induced after S coordination. On the other hand, the S atom of Met 80 lays in the inner part of the folded protein. Therefore, considering the location and the surroundings of Met 65, it is reasonable to think that it may be the primary binding site. The fact that cisplatin may also be able to bind to sulfur in Met 80 and therefore, to displace the Fe atom originally coordinated to it, may have biological implications, as recently cleavage of the Fe-Met 80 binding has been related to the translocation of cytochrome c to the cytoplasm and nucleus in non-apoptotic cells [28]. Moreover, an observed release of cytochrome c from the mitochondria membrane was related to conformational changes induced by Pd drugs [29].

When in-solution digestions are performed, notable differences in the ligands attached to the platinum atom bound to Met 80, compared with those found under in-gel conditions, are evidenced. The insertion of a CN^- ligand is only observed during the in-gel digestion and may be related with the repeated use of ACN during several steps of the digestion protocol. Promotion of cleavage of the C–CN bond in nitriles upon coordination to transition metals has been previously observed [30–32]. So in this case, Pt may be acting in such way with ACN (which is able to coordinate to the metallic center) and, in the experimental conditions used, may result in cleavage of the CH_3–CN bond. Interestingly, no CH_3 ligands were found to remain attached to Pt during the ESI experiments. Also, in light of the fragmentation data, it is worth to mention the notable stability of CN^- coordinated to Pt along the ESI-MS/MS experiments, in contrast to NH_3 ligands, which tend to be easily lost from the fragment ions during the CID activation. Despite the observed exchange of the ligands during the in-gel digestion, Pt is not released from the original binding site and therefore, the location of Pt-binding sites seems not to be affected.

It must be mentioned as well that when in-gel digestion was performed in the presence of DTT and IAA no Pt-peptides were detected by nHPLC–ESI MS/MS analyses. This may be due to the high reactivity of thiol groups towards platinum, which may be sequestered by DTT to a significant extent, impairing the detection of Pt-peptides. The deleterious effect of DTT on metal-binding proteins has been described before. However, in a previous work, Pt-peptides from insulin–cisplatin adducts produced by in-solution digestions were still detectable after DTT and IAA treatments [11]. Consequently, the detection of Pt-peptides probably depends on the amount of Pt-adducts present in the original sample, but also on the strength of the binding, and thus the location in the protein. Therefore, the in-gel digestion protocol, in the absence of steps involving DTT and IAA, seems to be compatible with the preservation of Pt-protein bonds in cytochrome c, and thus appropriate for the characterization of Pt-binding sites. Notably, in this case, Met instead of His were found to be coordinated to Pt.

3.2. *Equine myoglobin–cisplatin digests*

In-gel digests from myoglobin–cisplatin adducts, analyzed by nHPLC–ESI-MS/MS afforded a 100% protein coverage after SEQUEST search. Equine myoglobin also presents several histidines and methionines available for Pt-coordination. The analysis gave rise to several platinum-containing peptides with a clear platinum profile as can be seen in the zoom scans of ions at *m/z* 861.30 (2+) and 1055.98 (2+) shown in Figs. 3 and S3, respectively. Fragmentation of 1055.98 (2+) revealed the peptide sequence $[^{100}\text{YLEFISDAIIHVLHSK}^{115} + \text{Pt}(\text{NH}_3)_2]^{2+}$ as can be seen in Fig. S-3. Aside from a prominent peak corresponding to the neutral loss of both ammine ligands, Pt-containing fragments could be observed along both b and y series. In particular, the first platinated y fragment was found at 563.2 (1+) and the first platinated

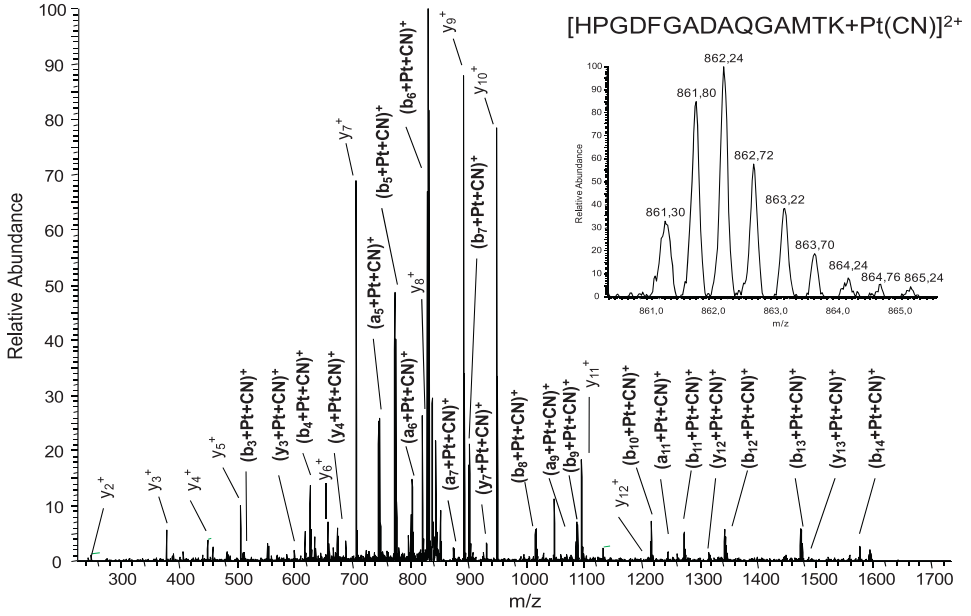

Fig. 3. CID–MS/MS spectrum of the platinated ion at *m/z* 861.30 (2+), corresponding to $[^{119}HPGDFGADAQGAMTK^{133} + Pt(CN)]^{2+}$, observed during the nHPLC–ESI-MS analysis of in-gel digests of MYO–cisplatin. Inset: zoom scan MS of the platinated precursor ion.

b fragment appeared at 1494.5 (1+), corresponding to $[HSK + Pt]^+$ and $[YLEFISDAIIH + Pt]^+$, respectively. In light of the fragments observed, it can be concluded that both His 113 and His 116 can be considered as platinum-binding sites in myoglobin. On the other hand, as is shown in Fig. 3, the CID–MS/MS spectrum for the peptide at 861.30 (2+) allowed its identification as $[^{119}HPGDFGADAQGAMTK^{133} + Pt(CN)]^{2+}$. A closer look at the fragment ions present at the MS/MS spectrum reveals that the platinated b_3^+ ion $[HPG + Pt + CN]^+$ at 511.16 (1+) can be observed, in addition to the platinum-containing y^{3+} ion at 599.08 (1+), corresponding to $[MTK + Pt + CN]^+$. Consequently, His 119 and Met 131 can be proposed as additional Pt-binding sites in myoglobin. In this case, considering that Pt bound to the peptide has only one additional ligand attached to it, a possible dual coordination to the His and Met residues, in addition to nitrogen from either the N-terminus or the amide backbone, facilitated by the ammine release induced by the trans effect induced by S-coordination in Met, could be proposed.

The analysis of in-solution digests from the myoglobin–cisplatin model, also gave rise to platinated peptides at *m/z* 1056.00 and 856.16. The fragmentation of the previous ions allowed their identification as the previously observed $[^{100}YLEFISDAIIHVLHSK^{115} + Pt(NH_3)_2]^{2+}$ and as $[^{119}HPGDFGADAQGAMTK^{133} + Pt(NH_3)]^{2+}$, respectively, involving the same binding sites as the ones described above (Fig. S-4). Again, a remarkable difference on the ligands attached to platinum in the peptide involving His 119 and Met 131 compared to in-gel digestion could be observed, being the original ligands displaced by a CN^- ligand during the in-gel digestion process, as was previously detected for the case of cytochrome c. In any case, similar results were obtained both with in-gel and in-solution digestions. Furthermore, results are in agreement with data previously reported, where His 116 and His 119 were found as binding sites for cisplatin in myoglobin [7] but, in this case, His 113 and Met 131 have also turned out to be other possible binding sites in the protein. Regarding the protein structure, His 113, His 116 and His 119 are located in the same α-helix and Met 131 is in an adjacent α-helix (Fig. S-5a). Considering the CPK model for myoglobin (Fig. S-5b), all the imidazole rings in histidines are present with higher accessibility at the surface of the protein, while Met 131 seems to be located in an inner part of the protein structure, thus less reachable for cisplatin. Considering these facts, the primary binding to His 113, 116 and 119 seems to be likely. Remarkably, although the protein has both Met and His within several parts of its sequence, in this case Pt seems to have preference for the accessible His-rich area.

On the other hand, the use of DTT and IAA during the in-gel digestion process, yielded Pt-peptides that were visible in the nHPLC–ESI-MS/MS analysis only at *m/z* 1056.00 (2+) (data not shown), involving His 113 and His 116, as previously mentioned. However, no evidence on the binding of Pt to His 119 or Met 131 was found using this approach, evidencing once more the deleterious effect of thiol-containing reagents on the Pt-peptide bonds.

3.3. *Bovine carbonic anhydrase–cisplatin digests*

nHPLC–ESI-MS/MS analysis of the in-gel digests prepared from CA–cisplatin bands was carried out, yielding a 78% protein coverage. Several ions were detected with an isotopic pattern expected from Pt-containing peptides. Figs. 4 and S-6 show the zoom scans corresponding to such ions, found at *m/z* 413.88 (3+) and 678.78 (2+). The CID–MS/MS of the precursor ion at 413.88 (3+), shown in Fig. 4, revealed its sequence as $[^1Ac\text{-}SHHWGYGK^8 + Pt(NH_3)_2]^{3+}$. In this case, the first platinated ion in the b series, b_3^{2+}, was found at 315.60 (1+), corresponding to $[Ac\text{-}SHH + Pt + 2NH_3]^{2+}$. On the other hand, the first Pt-containing ion in the y series was y_6^{2+} at 487.60 (2+), corresponding to $[HWGYGK + Pt + 2NH_3]^{2+}$. Taking a look at the mentioned fragments present in the spectrum, His 3 is disclosed as a clear binding site for platinum in carbonic anhydrase II. However, as no b_2 fragment ion was observed, a bifunctional coordination of Pt to both His 2 and His 3 is likely and could be proposed. Regarding the peptide ion at *m/z* 678.78 (2+), its fragmentation pattern suggested its sequence as $[^9HNGPEHWHK^{17} + Pt(NH_3)]^{2+}$, as can be seen in Fig. S-6. In this case, the main fragmentation pathway observed consisted on the neutral loss of NH_3, while scarce additional fragments were observed. Notably, no b series regular ions could be observed and only platinated b ions were present from b_6^+, $[HNGPEH + Pt]^+$, onwards. On the other hand, unplatinated y ions were observed until y_3 was reached but no platinated y ions were visible along the y series. Therefore His 9 and His 14

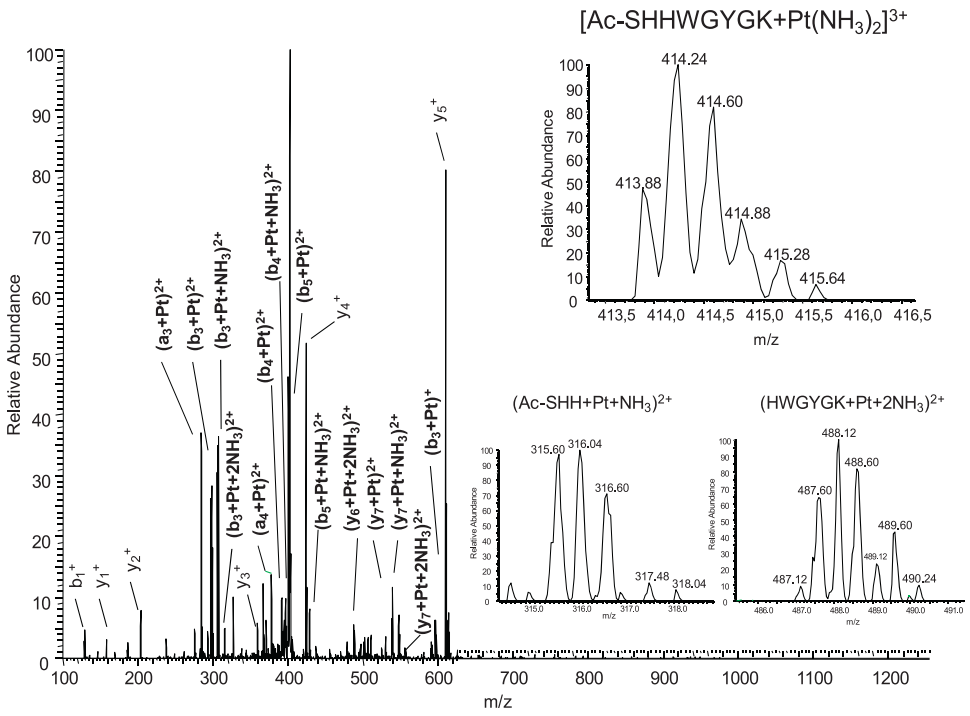

Fig. 4. CID–MS/MS spectrum of the platinated peptide ion at *m/z* 413.88 (3+), corresponding to [^{1}Ac-SHHWGYGK^{8} + Pt(NH$_3$)$_2$]$^{3+}$, observed during the nHPLC–ESI-MS analysis of in-gel digests of CA–cisplatin. Insets: up: zoom scan MS of the platinated precursor ion. Down: platinum-containing fragment ions observed in the CID–MS/MS at *m/z* 315.60 and 487.60.

can be proposed as probable Pt binding sites while the binding to His16 cannot be confirmed in light of the results but should not be discarded. It seems that due to the simultaneous binding of Pt to several His in the peptide, the fragmentation pathways in the peptide are influenced too, in light of the lack of a considerable amount of b and y ions. The previous observations were confirmed after in-solution digestion of CA–cisplatin adducts, which provided similar results. The binding of cisplatin to histidines in a His-rich environment located in the N-terminus side of the protein is not surprising and can be expected. Taking a look at the protein structure displayed in Fig. 5a, His 2 and His 3 are located in the N terminus, His 9 and 14 are located in a turn, while His 16 is placed in an adjacent α-helix. It is interesting to see in the CPK model shown in Fig. 5b that all the mentioned His residues can be found at the surface of the protein, which makes them likely candidates for interacting with cisplatin. Moreover, for His 3, His 9 and His 14, imidazole rings are fully accessible at the surface of the protein, while the rings for His 2 and His 16 are only partially exposed. This fact may be related to the lack of clear evidence on the binding of cisplatin to the latter residues in the MS/MS spectra, pointing out to the most probable binding to the more accessible His 3, His 9 and His 14. Therefore, although CA-II has different His and Met residues that may potentially coordinate to Pt, cisplatin seems to show more affinity for the highly accessible His-rich N-terminus area. To our knowledge, no previous results on Pt-binding sites on carbonic anhydrase have been reported so far. These results point to the coordination of Pt to a region different to the active Zn (coordinated to His 63, His 66, His 126) domain in CA-II, to which the biological activity of CA is due.

On the other hand, the use of DTT and IAA during the in-gel digestion only allowed the identification of the N-terminal peptide at 413.88 (3+) described above, corresponding to [^{1}Ac-SHHWGYGH^{8} + Pt(NH$_3$)$_2$]$^{3+}$, as Pt-binding region, while the peptide ion at 678.78 (2+) could not be detected. As in the case of CYT C and MYO, the identification of platinum binding sites was impaired by the application of the reducing and alkylating steps using the

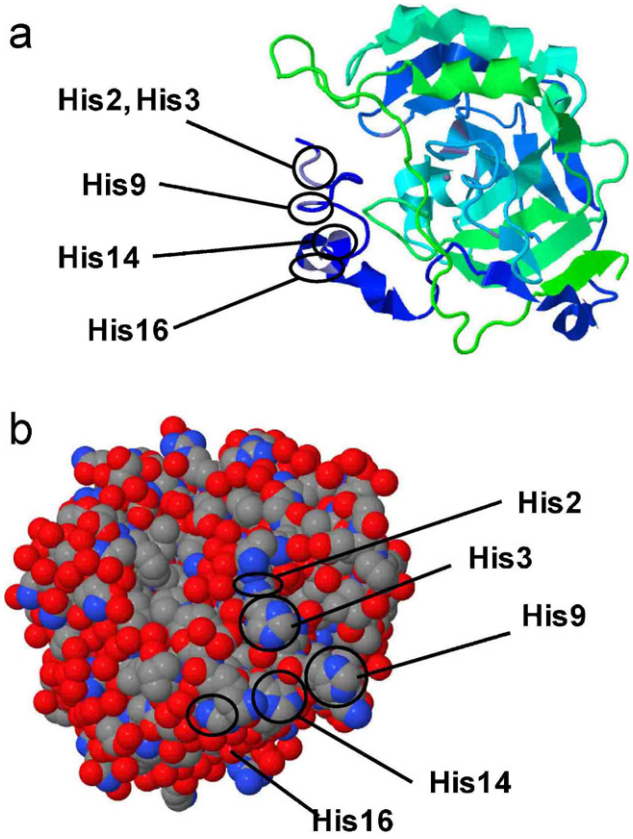

Fig. 5. (a) Schematic and (b) CPK model structures of bovine carbonic anhydrase II, showing the identified cisplatin binding sites. In the CPK model, the atoms color code is the following: C: grey, O: red, N: blue, S: yellow (Structures retrieved from RSCB Protein Data Bank. ID: 1V9E). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

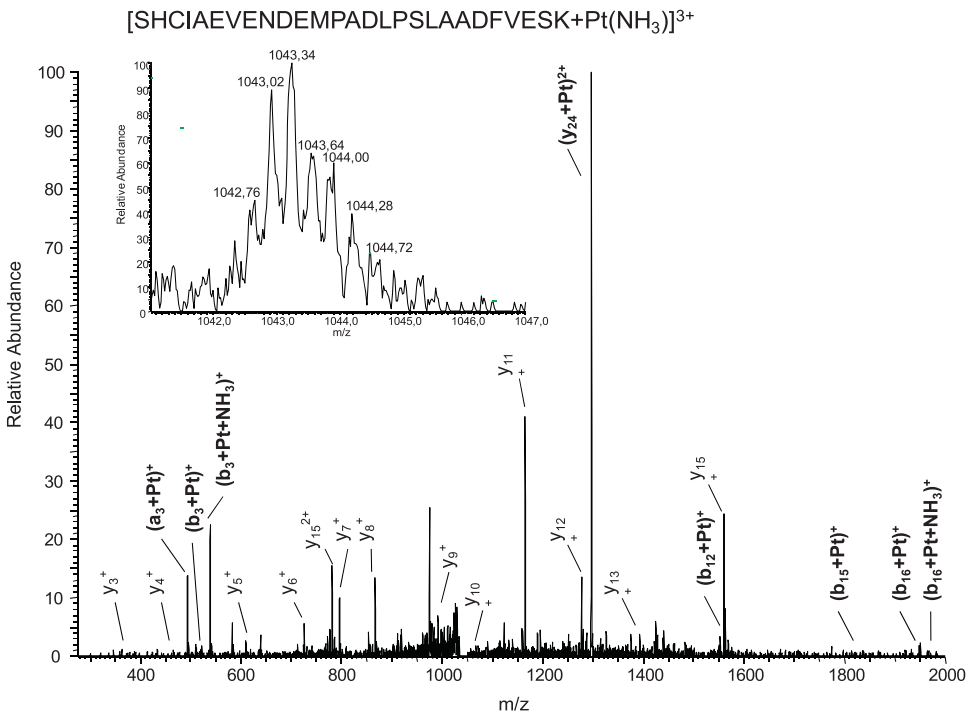

Fig. 6. CID–MS/MS spectrum of the platinated ion at *m*/*z* 1042.73 (3+), corresponding to [^{287}SHCIAEVENDEMPADLPSLAADFVESK^{313} + Pt(NH$_3$)]$^{3+}$, observed during the nHPLC–ESI-MS analysis of in-gel digests of HSA–cisplatin. Inset: zoom scan MS of the platinated precursor ion.

mentioned reagents. Interestingly, the rest of the processes involved in the conventional in-gel digestion have been proved again to be able to allow determining Pt-binding sites in carbonic anhydrase.

3.4. Human serum albumin–cisplatin digests

In-gel digestions in non-reducing conditions were prepared on HSA-Pt bands from SDS-PAGE. Digests were analyzed by nHPLC–ESI-MS/MS, allowing the identification of the protein with 38.9% coverage of the aminoacid sequence. This low coverage obtained is due to the fact that the protein presents numerous disulfide bonds and no reduction was performed. Nevertheless, Pt-containing peptide ions were recognized according to their isotopic pattern at *m*/*z* 925.44, 843.94 and 1042.76, as can be seen in Figs. S-7, S-8 and 6, respectively. The fragmentation by CID of the ion at 925.44 (2+) allowed its identification, turning out to be [^{324}DVFLGMFLYEYAR^{336} + Pt(NH$_3$)$_2$]$^{2+}$, as shown in Fig. S-7. In the CID–MS/MS, non-platinated ions from the y series could be followed up to y_7^+, [FLYEYAR]$^+$, but the sequence could not be continued further, as can be seen in Fig. S-7. Moreover, no unplatinated ions in the b series could be found while b Pt-ions were present indeed, the first one, b_6^+, appearing at 855.20 (1+) and 872.24 (1+), corresponding to [DVFLGM + Pt]$^+$ and [DVFLGM + Pt + NH$_3$]$^+$, respectively. Therefore, considering the fragmentation pattern observed, platinum can be proposed to be coordinated to the Met 329 in HSA. On the other hand, the Pt-ion observed at 843.94 (2+) was fragmented, as shown in Fig. S-8, revealing its sequence as [^{337}RHPDYSVVLLLR^{348} + Pt(CN)]$^{2+}$. This peptide presents again a CN^- ligand attached to platinum probably due to the exchange of the original NH_3 by acetonitrile, followed by C-CN cleavage, as described before for cytochrome c and myoglobin. Among the fragments produced, a platinated b_2^+ ion was observed at *m*/*z* 513.08, corresponding [RH + Pt + CN]$^+$, while exclusively unplatinated fragment ions were observed within the y series, being y_{10}^+, [PDYSVVLLLR]$^+$, at *m*/*z* 1174.56, the last one visible, suggesting that platinum may be bound to His 338 as well. Regarding the peptide ion at 1042.73 (3+), its CID–MS/MS spectrum allowed its identification as [^{287}SHCIAEVENDEMPADLPSLAADFVESK^{313} + Pt(NH$_3$)]$^{3+}$, as can be seen in Fig. 6. Considering the MS/MS spectrum, a platinated b_3^+ ion can be assigned at *m*/*z* 537.08 (1+), [SHC + Pt + NH$_3$]$^+$, being the first Pt-ion in the b series. Moreover, no unplatinated b ions were found. On the other hand, fragment ions belonging to the y series were found with no platinum until y_{15}^+, [DLPSLAADFVESK]$^+$. Onwards, no more y ions in the series were found, except for y_{24}^{2+}, at 1245.48 (2+), corresponding to [IAEVENDEMPADLPSLAADFVESK + Pt]$^{2+}$. As a result, it could be concluded that platinum may be bound to either His 288 or Cys 289, and in light of the detection of the platinated y_{24}^{2+} fragment, the binding to Met 298 would also be most likely. It is interesting to remark that Cys 289 is participating in a disulfide bond in native HSA, so the fact that it is seen free and bound to platinum when digested under non-reducing conditions, may indicate that platinum could be able to disrupt the original S-S bond after coordinating to Cys 289. This fact was previously reported during a top-down insulin–cisplatin adducts characterization [9]. On the other hand, the fact that only one ammine ligand remains attached to Pt can also be explained by the presence of several simultaneous binding sites in the peptide and by the trans effect induced after coordination to S in Met or Cys, enhancing the release of an original NH_3 ligand.

When the in-gel digestion was performed under reducing conditions (using DTT and IAA during the process), the aminoacid coverage reached was considerably higher (77.5%). However, only the platinated ion at 1061.70 (3+), corresponding to [^{287}SHC*IAEVENDEMPADLPSLAADFVESK^{313} + Pt(NH$_3$)]$^{3+}$, could be detected in the chromatogram. In this case, in the MS/MS spectrum (Fig. S-9), the first platinated b ion found was b_{12}^{2+} at 812.76 (2+), corresponding to [SHC*IAEVENDEM + Pt + NH$_3$]$^{2+}$, and no other platinated b ions where detected below b_{12}. Furthermore, a very intense unplatinated y_{15}^+ ion at 1559.68 (1+), [PADLPSLAADFVESK]$^+$, was also observed, in addition to a first

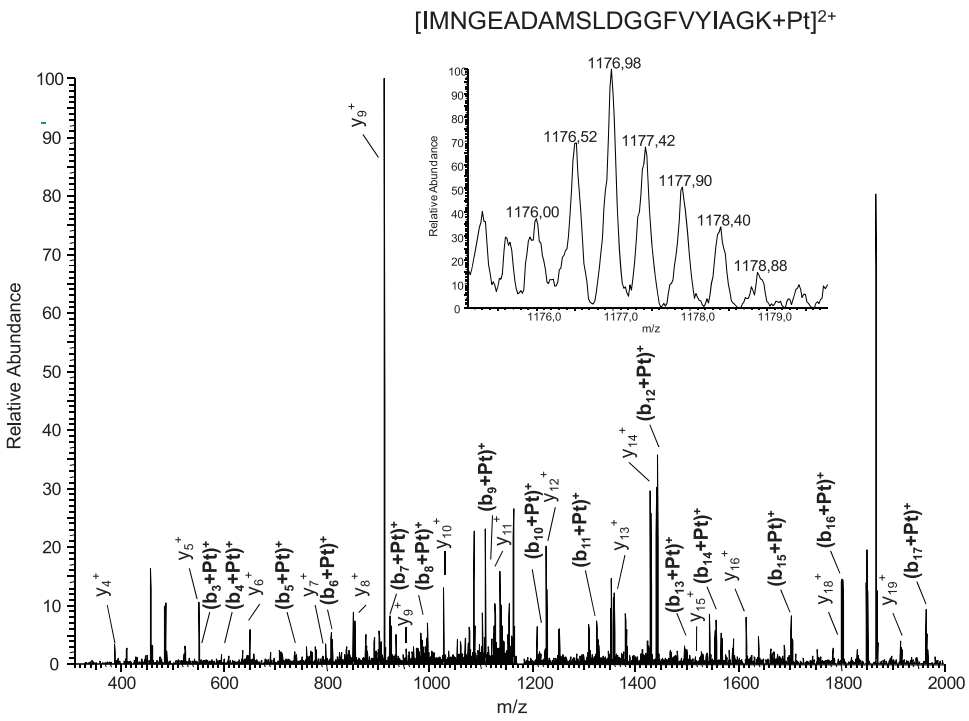

Fig. 7. CID–MS/MS spectrum of the platinated ion at *m*/*z* 1176.00 (2+), corresponding to $[^{381}$IMNGEADAMSLDGGFVYIAGK^{401} + Pt$]^{2+}$, observed during the nHPLC–ESI-MS analysis of in-gel digests of TF–cisplatin. Inset: zoom scan MS of the platinated precursor ion.

platinated ion at the y series at *m*/*z* 1014.88 (2+), [DEMPADLPSLAADFVESK + Pt]$^{2+}$. Therefore, according to these facts, Met 298 would also be clearly involved in the binding to platinum, aside from the previously observed Cys 289 and His 288. In spite of the higher protein coverage, no other Pt-peptides were detected, most probably due to the effect of DTT in the Pt-peptides already observed. Regarding in-solution digestions using reduction and alkylation, the same results were obtained, confirming the previous observations.

As can be seen in the protein structure shown in Fig. S-10a, Cys 289/His 288 and Met 329 are located in different α-helixes while Met 298 and His 338 belong to different loops. On the other hand, taking a look at the protein surface in a CPK model (Fig. S-10b), the sulfur atoms of Met 298, Met 329 and Cys 289 and the imidazole rings of His 338 and partially of His 288, are located at the surface, and therefore, are highly accessible for cisplatin, confirming the observed results on the binding sites. These observations are in accordance with previous NMR results [33] proposing Met 298 as the primary binding site for cisplatin in HSA and that superficial histidines can be potential binding sites too. By using a MudPIT approach, Met 329 was also found as a Pt-binding site [12]. Considering the location of the residues in the protein structure, the neighboring His 288 and Cys 289 are located relatively far from Met 298, being their simultaneous binding to Pt unlikely, although they can be proposed as binding sites separately. Therefore, in this case both His and Met residues were found to be competing binding sites for cisplatin.

3.5. Human serotransferrin–cisplatin digests

Gel bands containing transferrin–cisplatin adducts were also digested in-gel and analyzed by nHPLC–ESI-MS/MS. It should be noted that due to the high amount of disulfide bonds in transferrin, only 39% aminoacid coverage was obtained. In the chromatogram, a doubly charged peptide ion bearing a platinum-like isotopic pattern was observed at *m*/*z* 1176.00. Fig. 7 shows the CID–MS/MS spectrum of the mentioned ion, which turned out to be $[^{381}$IMNGEADAMSLDGGFVYIAGK^{401} + Pt$]^{2+}$. Taking a closer look at the fragment ions produced, it can be seen that platinated ions from the b series were detected from b_3^+ onwards, at 551.32 (1+), corresponding to [IMN + Pt]$^+$, while no unplatinated b ions were observed. On the other hand, clear fragments without platinum belonging to the almost complete y series were observed, until y_{19}^+, [NGEADAMSLDGGFVYIAGK]$^+$, was reached. However, no platinated ions could be assigned in the y series. According to these results, platinum would be most probably coordinated to Met 382. This is in agreement with a previous work, where Met 382 was found as a cisplatin binding site in the protein too, being also Glu 385 proposed [12]. Considering the protein structure (Fig. S-11a), Met 382 is located in an α-helix while Glu 385 is in a loop, but the Glu 385 residue is located at the surface and thus, in principle, would be more accessible for cisplatin, as can be seen in Fig. S-11b. In this case, the lack of ligands remaining attached to Pt in the adduct detected may reflect the enhanced NH_3 release due to the trans effect promoted after Met S-coordination and would point to the possible binding to several simultaneous sites in the peptide. When the in-gel digestion was performed including reduction with DTT and alkylation with IAA, the protein coverage was increased to 69%. However, no Pt-peptides could be recognized after nHPLC–ESI-MS/MS analysis despite the higher protein coverage, probably due to the deleterious effects of DTT on the Pt-protein binding. In-solution digestions produced similar results but in this case, either under the use of reduction and alkylation steps or omitting the previous steps, the mentioned peptide ion could be detected. Probably, during the in-solution digestions performed, which involved less amount of steps for sample treatment, platinum could be preserved better. Again, as observed for albumin, surprisingly, the increase of aminoacid coverage obtained after the use of DTT and IAA did not result in the identification of a higher amount of Pt-peptides probably due to the effect of DTT. However, the in-gel digestion process itself allowed the identification of Pt-peptides in the protein.

4. Conclusions

In light of the results by nHPLC–ESI-MS/MS for the standard proteins studied, the conventional tryptic in-gel digestion protocol seems to be suitable for the characterization of Pt-proteins. The use of ammonium bicarbonate, acetonitrile and formic acid along the steps involved in the tryptic digestion seems to be appropriate for the preservation of Pt-protein bonds. However, the repeated use of acetonitrile may result in the substitution by CN^- of some of the original ligands attached to platinum, which does not affect the original binding sites in the protein. Anyway, this modification has to be born in mind if a search engine is used for assisting on the recognition and identification of Pt-peptides, considering the mass of the platinum modification.

By using this in-gel approach, relatively simple Pt-binding proteins, such as cytochrome c, myoglobin and carbonic anhydrase have been fully characterized regarding the binding sites for cisplatin: Met 65, Glu 61/Glu 62/Thr 63 and Met 80 for CYT C; His 113, His 116, His 119 and possibly Met 131 for MYO, His 2/His 3, His 9/His 14 for CA-II. On the other hand, during the study of more complex proteins, such as albumin or transferrin, the in-gel digestion also proved to be appropriate for the identification of Pt binding sites: Met 329, His 338, His 288/Cys 289, Met 298 in HSA; Met 382 in TF. In general, results point out the competition of His and Met residues towards platinum binding, and the fact that their selectivity may also depend on the accessibility of their side chains. However, for a comprehensive study of the binding sites in such proteins, with a considerably high amount of cysteines, reduction and alkylation of cysteines involved in disulfide bonds may be required. In this work, the use of DTT as a reducing agent has proved not to be recommendable, as it seems to hamper the localization of Pt-binding sites. Thiol groups in DTT, reactive towards platinum may, probably, cleave the original Pt-protein binding to some extent, being the increase in protein coverage at the cost of the preservation of certain Pt-protein bonds. Therefore, other non-thiol-containing reducing agents will be investigated for this purpose in future work.

Overall, the in-gel digestion procedure seems to be appropriate for the characterization of Pt-binding proteins, implying that the whole bottom-up approach based on the separation of proteins by 2-DE, followed by tryptic digestion of Pt-spots and analysis by nHPLC–ESI-MS/MS may be suitable for the detection of Pt-peptides and therefore the identification of Pt-binding proteins in biological samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.11.044.

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