Insights into the Molecular Mechanisms of Protein Platination from a Case Study: The Reaction of Anticancer Platinum(II) Iminoethers with Horse Heart Cytochrome *c*[†]

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ABSTRACT: The interactions of anticancer metallodrugs with proteins are attracting a growing interest in the current literature because of their relevant pharmacological and toxicological consequences. To understand in more depth the nature of those interactions, we have investigated the reactions of four anticancer platinum(II) iminoether complexes, namely, *trans-* and *cis-EE* (*trans-* and *cis-*[PtCl$_2${(*E*)-HN═C(OCH$_3$)CH$_3$}$_2$], respectively) and *trans-* and *cis-Z* (*trans-* and *cis-*[PtCl$_2$(NH$_3$){(*Z*)-HN═C(OCH$_3$)CH$_3$}], respectively), with horse heart cytochrome *c* (cyt *c*). Our investigation was performed using mainly electrospray ionization mass spectrometry (ESI MS) but was also supported by NMR, inductively coupled plasma optical emission spectroscopy (ICP OES), and absorption electronic spectroscopy. ESI MS spectra clearly revealed the formation of a variety of platinum−protein adducts predominantly corresponding to monoplatinated cyt *c* species. From a careful analysis of the major ESI MS peaks, specific information on the nature of the protein-bound metallic fragments and on the underlying metallodrug−cyt *c* reactions was gained for the various cases. We found that *trans-EE* produces a major cyt *c* adduct (12 667 Da) that is different from that produced by either *cis-EE* or by *trans-Z* and *cis-Z* (12 626 Da). In particular, occurrence of extensive hydrolysis/aminolysis (the latter fostered by ammonium carbonate buffer) of the iminoether ligands and formation of the corresponding amides/amidines has been unambiguously documented. The reactivity of the iminoether ligands is greatly enhanced by the presence of cyt *c* as inferred from comparative NMR solution studies. Additional ESI MS measurements recorded on enzymatically cleaved samples of platinated cyt *c* adducts, together with NMR investigation of the cyt *c/trans-EE* adduct, strongly suggest that protein platination primarily occurs at Met 65. The biological and pharmacological implications of the described protein platination processes are discussed.

Metallodrugs are known to behave, in most cases, as "prodrugs". In other words, an activation step, usually consisting of a ligand-exchange and/or a redox process is required before they can exert their pharmacological effects (*1−7*). The resulting "activated" metal-containing species are, thus, the "chemical entities" truly responsible for the observed biological actions. Remarkably, these latter species manifest a high propensity to react with biomolecules and to transfer them "metal-containing molecular fragments", commonly through simple ligand substitution reactions.

For instance, the mechanism of action of cisplatin is thought to rely on coordination to adjacent DNA guanine nucleobases of a bidentate [Pt(NH$_3$)$_2$]$^{2+}$ fragment that is formally obtained through release of two water molecules from the "activated" [Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$ cation (*8−10*). Notably, introduction of kinetic restrictions to the production of these metallic fragments and to their transfer to target biomolecules results into a substantial loss of biological activity for the metallodrug, as was clearly demonstrated in the case of some representative gold(III) and ruthenium(III) anticancer compounds (*11, 12*).

During the last 20 years, the interest of the scientific community working on anticancer platinum compounds has mostly focused on platinum interactions with DNA, the putative "primary" target, that were described and analyzed in hundreds of papers (*8−10*). In contrast, rather surprisingly, the reactions of platinum drugs with proteins have received very scarce attention.

Just a few studies were devoted to the analysis of the *in vitro* interactions of platinum drugs with the main serum proteins albumin and transferrin. In particular, in 1998, Ivanov et al. reported a pioneering NMR investigation on

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thereaction of cisplatin with serum albumin and on the characterization of the resulting adducts (*13*). Subsequently, Khalaila et al. described and modeled the binding of cisplatin to transferrins (*14*). More recently, Mandal et al. have analyzed the interactions of platinum drugs with hemoglobin (*15*). Some other studies have analyzed the reactivity of platinum drugs with small model proteins. For instance, Gibson's group produced a few fundamental studies on the platinum/ubiquitin system (*16−20*), and very recently, some of us have reported high-resolution crystal structures of the adducts of cisplatin with superoxide dismutase (*21*) and lysozyme (*22*). The most relevant achievements obtained in this field during the past 20 years have been summarized by Timerbaev et al. in a comprehensive review that appeared in 2006 (*23*).

Yet, we believe that this topic deserves more and more attention as it is increasingly evident that the interactions of platinum drugs with proteins may play crucial roles in their uptake and biodistribution processes as well as in determining their toxicity profile. In addition, reactions of platinum drugs with proteins might be also involved in some aspects of their overall mechanism of action through direct interactions with "secondary" protein targets.

Nowadays, the study of the interactions occurring between metallodrugs and proteins may take new and considerable advantage from the availability of very sophisticated analytical tools. For instance, a number of papers have highlighted the great potential of modern mass spectrometry methods to characterize metal−protein adducts at a molecular level (*24−33*). Conversely, the rapid development of proteomic technologies and the use of advanced protein separation techniques, coupled to very sensitive metal detection methods, hold promise for the successful analysis of complex mixtures of platinated proteins and for the identification of those proteins that act as "platinum receptors" and/or "platinum targets".

We have recently shown that ESI MS is able to provide valuable and detailed information on the reactivity of classical anticancer platinum(II) complexes with various model proteins (*24, 25*). In particular, a well-known and intensely studied small protein, namely, the horse heart cytochrome *c* (cyt *c* hereafter),[1] has been selected as the "test protein" for this kind of investigation upon consideration of a number of favorable properties (*24, 34*). Accordingly, we report here on the reactions of cyt *c* with a few representative platinum(II) iminoether complexes, a family of promising anticancer agents developed in the laboratory of Bari.

Pt(II) iminoethers are indeed very interesting metallodrugs that exhibit innovative and well-documented antitumor properties (*35, 36*). These compounds may be straightforwardly prepared by alcoholysis of the parent platinum(II) nitrile complexes (*37*). The overall geometry around the platinum center is preserved during the alcoholysis reaction; however, the formed iminoether ligands can have either *Z* or *E* configuration, depending upon the relative positions of the alkoxide and platinum ions with respect to the C═N double bond (Chart 1). Within this family of platinum

Chart 1: Schematic Drawing of the Selected Platinum Iminoether Complexes

trans-EE   *cis-EE*

trans-Z   *cis-Z*
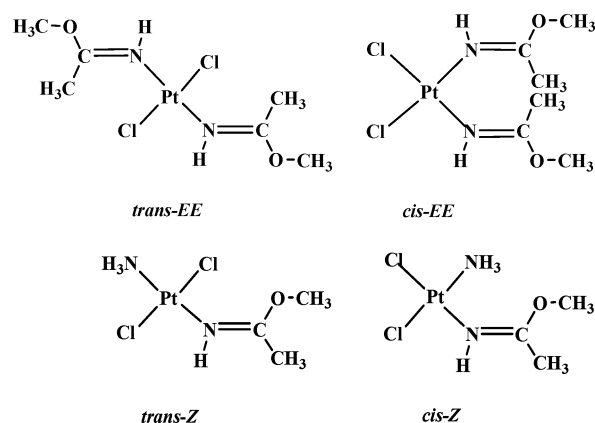
iminoethers, *trans*-[PtCl₂{(E)-HN═C(OCH₃)CH₃}₂] (*trans-EE*) was found to be as active as cisplatin toward P388 leukemia and Lewis lung carcinoma in mice (*38, 39*) through formation of stable DNA monofunctional adducts (*40*). Remarkably, the presence of only one iminoether ligand resulted to be sufficient for promoting the antitumor activation of the trans geometry. Accordingly, the complex *trans*-[PtCl₂(NH₃){(Z)-HN═C(OCH₃)CH₃}] (*trans-Z*), turned out to be highly active against murine P388 leukemia and SKOV-3 human cancer cell xenograft in nude mice (*41*).

Four representative platinum iminoether complexes, abbreviated as *trans*- and *cis-EE* (*trans*- and *cis*-[PtCl₂{(E)-HN═C(OCH₃)CH₃}₂], respectively), and *trans*- and *cis-Z* (*trans*- and *cis*-[PtCl₂(NH₃){(Z)-HN═C(OCH₃)CH₃}], respectively) were selected for the present study. All these compounds were previously characterized, both chemically and pharmacologically (*37, 38, 41*), and their chemical structures are represented in Chart 1.

The main reason for the present investigation and also its primary goal is to describe the reactions that occur between selected platinum−iminoether compounds and cyt *c* and to elucidate the nature of the resulting adducts. Through the study of a specific case, we hoped to unravel representative and mechanistically relevant interactions that take place, within biological fluids, between reactive platinum species and the pool of soluble proteins.

MATERIALS AND METHODS

Sample Preparation for ESI Mass Spectrometry. The synthesis of the iminoether complexes has been carried out as already reported (*37*). Horse heart cytochrome *c* was purchased from Sigma (code C7752). Metal complexes/cyt *c* adducts were prepared in ammonium carbonate buffer (25 mM, pH 7.4), with a protein concentration of 5×10^{-4} M and platinum to protein ratio of 3:1. The reaction mixtures were incubated for different time intervals (3, 6, 24, 72, and 168 h) at 310 K. 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 3 μL/min flow rate, in a LTQ linear ion trap (Thermo, San Jose, CA) equipped with a conventional ESI source. The specific conditions used for these experiments were as follows: spray voltage 3.5 kV, capillary voltage 40 V, and capillary

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[1] Abbreviations: cyt *c*, cytochrome *c*; ESI MS, electrospray ionization mass spectrometry; *trans-EE*, *trans*-[PtCl₂{(E)-HN═C(OCH₃)-CH₃}₂]; *cis-EE*, *cis*-[PtCl₂{(E)-HN═C(OCH₃)CH₃}₂]; ICP OES, inductively coupled plasma optical emission spectroscopy.
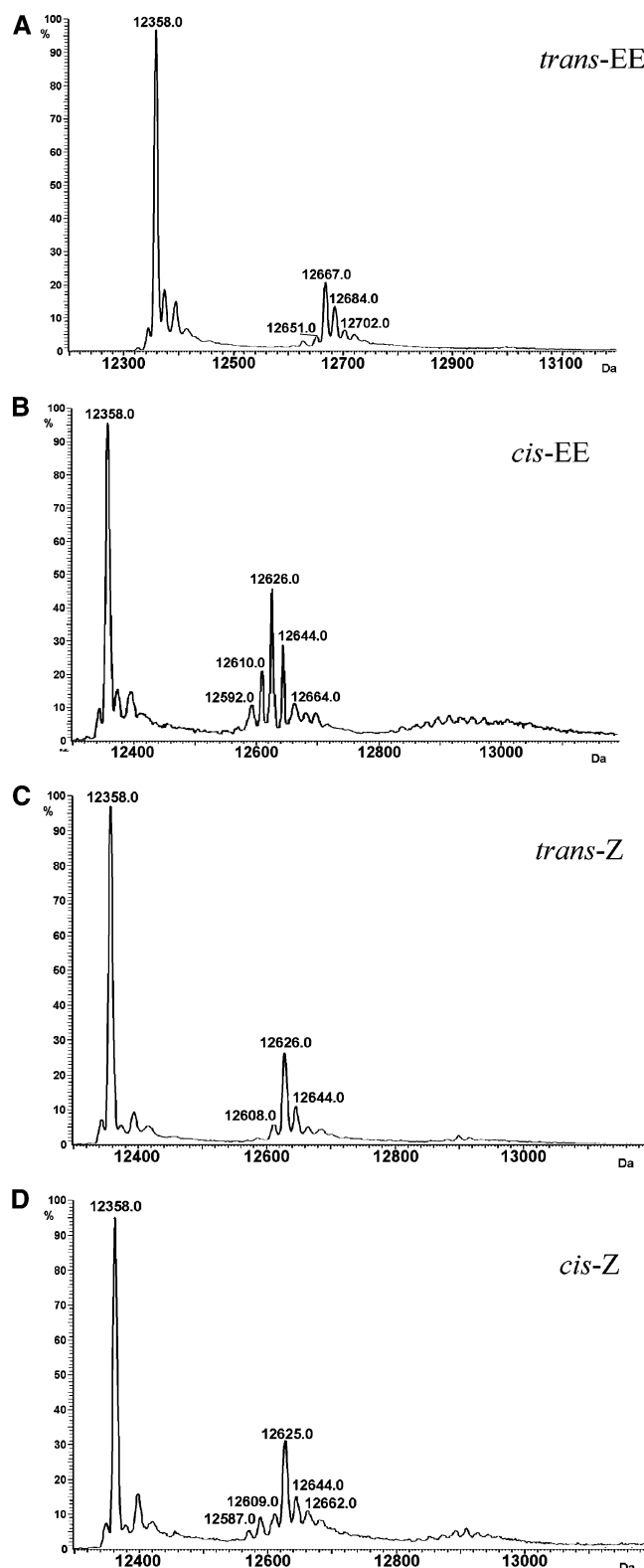
FIGURE 1: Deconvoluted ESI MS spectra of cyt *c* adducts with *trans-EE* (A), *cis-EE* (B), *trans-Z* (C), and *cis-Z* (D). The platinum/protein ratio is 3:1, and the incubation time at 310 K is 24 h.

temperature 353 K. Sheath gas was set at 18 a.u. (arbitrary units), whereas sweep gas was set at 5 a.u. and auxiliary gas was kept at 0 a.u. 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 (363 to 453 K), but the peak patterns and relative abundances were not influenced (data not shown).

Spectra of the same samples were also recorded on an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA), and the obtained results were fully consistent (data not shown). The instrument was equipped with a conventional ESI source. The working conditions were the following: spray voltage 2.3 kV, capillary voltage 20 V, and capillary temperature 403 K. Sheath gas was set at 12 a.u., the sweep gas and the auxiliary gas were kept at 0 a.u. For acquisition, Xcalibur 2.0 software (Thermo) was used, and monoisotopic and average deconvoluted masses were obtained by using integrated Xtract tool. For spectra acquisition a nominal resolution (at *m/z* 400) of 60 000 was used.

Determination of the Binding Site. For the enzyme-digestion analysis, 40 μL of cyt *c* (10^{-4} M), either free or reacted with *trans-EE* or *cis-EE*, was diluted at a concentration of 5 μM in 10 mM ammonium bicarbonate (pH 7.4), and endoproteinase Asp-N (Sigma, P3303) was added to a ratio of 1:50 (w/w). The mixture was incubated for 16 h at 310 K and after digestion was acidified with 0.05% formic acid before ESI MS analysis, using the LTQ Orbitrap mass spectrometer and the instrumental parameters mentioned before.

ICP OES Measurements. ICP OES analyses were recorded using an Optima 2000 instrument (Perkin-Elmer, Europe). The samples containing adducts of cyt *c* with the various platinum complexes were prepared as described above for the ESI MS studies.

NMR Studies. The complex *trans*-[PtCl$_2$\{(*E*)-H^{15}N=C(OCH$_3$)CH$_3$)\}$_2$] was prepared as already reported (*37*) and then used in two types of experiments. In one case the complex (1 mg, 0.0024 mmol) was dissolved in 1 mL of ammonium carbonate buffer (25 mM) prepared by dissolving (NH$_4$)$_2$CO$_3$ (2.4 mg, 0.025 mmol) in H$_2$O/D$_2$O (9:1, 1 mL). In a second experiment the same complex was dissolved in ^{15}N-enriched ammonium carbonate buffer prepared by dissolving Ag$_2$CO$_3$ (6.7 mg, 0.024 mmol) and (^{15}NH$_4$)Cl (2.7 mg, 0.048 mmol) in 1 mL of H$_2$O/D$_2$O (9:1) and filtering the solution in order to remove the precipitated AgCl. The pH of both ammonium carbonate buffers was adjusted to 7.4 by addition of HClO$_4$ (1 M solution). The time-dependent transformations of the two samples were monitored by 1D and 2D NMR spectroscopy using Bruker Instruments Avance 300 UltraShield, equipped with a broad-band probe, and Avance 600 UltraShield Plus, equipped with a triple-resonance (TXI) probe with pulsed field gradients along the *z*-axis.

The complex *trans*-[PtCl$_2$\{(*E*)-HN=C(O^{13}CH$_3$)CH$_3$)\}$_2$] was prepared as reported in the Supporting Information. For monitoring the hydrolysis of this ^{13}C methoxide-enriched complex in the presence of cyt *c*, a solution of the complex in H$_2$O/D$_2$O (9:1 v/v, pH 6.9; 1.5 mM concentration) was treated with an equimolar amount of cyt *c*. For comparison purposes, a second complex solution similar to the previous one, but deprived of cyt *c*, was also investigated. The two solutions were incubated for 1 week at 310 K and monitored through 1D ^{13}C and 2D ^{1}H,^{13}C-edited HSQC (heteronuclear single quantum correlation) NMR spectra at 600 MHz.
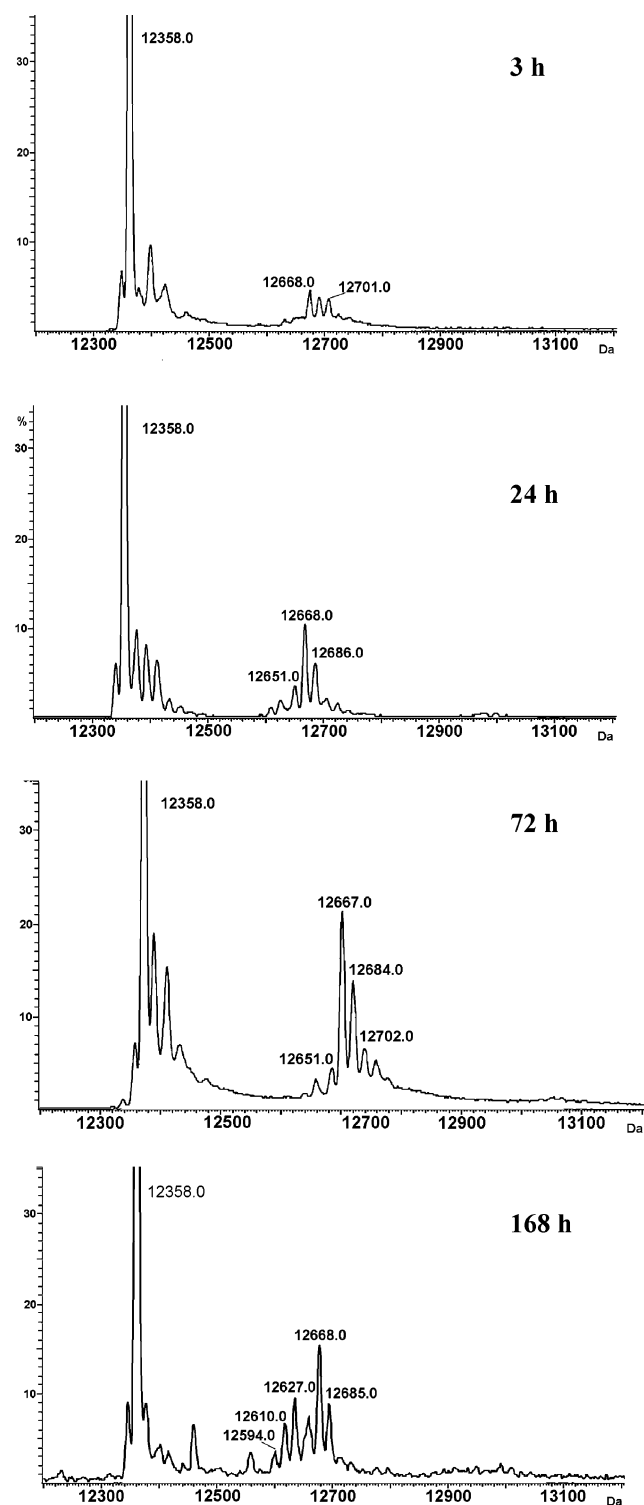
FIGURE 2: Time-dependent spectral profiles for the *trans-EE*/cyt *c* derivative. Spectra were recorded after 3, 24, 72, and 168 h of incubation of the sample at 310 K.

For the identification of the platinum coordination site of cyt *c*, natural abundance ^{1}H,^{13}C-edited HSQC (*42−44*) spectra were recorded at 293 K for a sample of pure cyt *c* and for a sample containing a 1:1 mixture of cyt *c* and *trans-EE* incubated for 1 week at 310 K.

For HSQC experiments, 16 transients were acquired over an F2 (^{1}H) spectral width of 14 ppm into 1024 complex data points for each of 256 t_1 increments in TPPI mode (*45*) with an F1(^{13}C) spectral width of 70 ppm centered at 40 ppm. All 2D NMR data were acquired using a gradient-enhanced

sequence in which coherence selection and water suppression are achieved via gradient pulses. The sequence was optimized with a delay $1/(4J_{CH})$ of 1.72 ms. Decoupling during the acquisition time was achieved using a GARP decoupling scheme (*46*). Data zero-filled in F1 were subjected to apodization using a squared cosine bell function in both dimensions prior to Fourier transformation and phase correction. The data were analyzed with the program CARA (The Computer Aided Resonance Assignment Tutorial, R. Keller, 2004, CANTINA Verlag). Resonance assignment was carried out by using available ^{1}H and ^{13}C chemical shifts data at 293 K (*47, 48*), with the aid of 2D TOCSY (total correlation spectroscopy) and NOESY (nuclear Overhauser enhancement spectroscopy).

RESULTS

Preparation and Characterization of Metal−Protein Adducts. In order to investigate comparatively their interactions with the chosen test protein, all four platinum iminoether compounds were individually reacted with horse heart cyt *c* at a molar ratio of 3:1 (metal/protein) and at physiological pH. Samples of the individual reaction mixtures, taken at increasing time intervals after mixing, were subjected to extensive ultrafiltration and then analyzed for Pt content by ICP OES. Under the applied experimental conditions, the protein platination was found to be relatively fast and nearly comparable for the four samples. The Pt/cyt *c* ratio was ≥ 1.0 already after 1 h of incubation and increased only marginally for longer incubation times. Even after very long incubation times (168 h), the platination levels never exceeded a Pt/cyt *c* ratio of 1.5; in all cases a significant amount of Pt was recovered in the low molecular weight fraction. Remarkably, *cis-EE*, at variance with the other three tested compounds, was shown to cause some direct cyt *c* damage, possibly as a consequence of partial proteolysis, that could be directly monitored through spectrophotometric analysis (however, the total protein loss never exceeded 15%).

ESI MS Studies of the Adducts. Characteristic features were detected in the ESI MS profiles of platinated cyt *c* samples that are diagnostic of the formation of stable platinum−protein adducts. Representative deconvoluted ESI MS spectra of the protein adducts formed after 24 h of incubation with the four platinum(II) iminoether compounds, and subsequent extensive ultrafiltration, are shown in Figure 1. Beyond the peak characteristic of native cyt *c*, located at 12 358 Da, a number of intense additional peaks of higher molecular mass are also observed, which likely correspond to platinum−cyt *c* adducts.

Inspection of the ESI MS spectra provides straightforward insight into the stoichiometry of the resulting metallodrug−cyt *c* adducts. In full agreement with the ICP OES results, indicating that the monoplatinated adducts are the dominant species, also the majority of the ESI MS peaks correspond to *monoplatinated* cyt *c* with peaks falling in the 12 550−12 700 range. In a few cases, some weak features with masses higher than 12 800 Da (notably in the 12 800−13 100 Da range) were also observed, which are indicative of *doubly platinated* species.

To identify the precise nature of the protein-bound Pt fragments, a detailed analysis of the major ESI MS peaks belonging to the various platinated species was carried out.

Scheme 1: Proposed Reaction Scheme for *trans-EE* in Ammonium Carbonate Buffer, pH 7.4, and 310 K
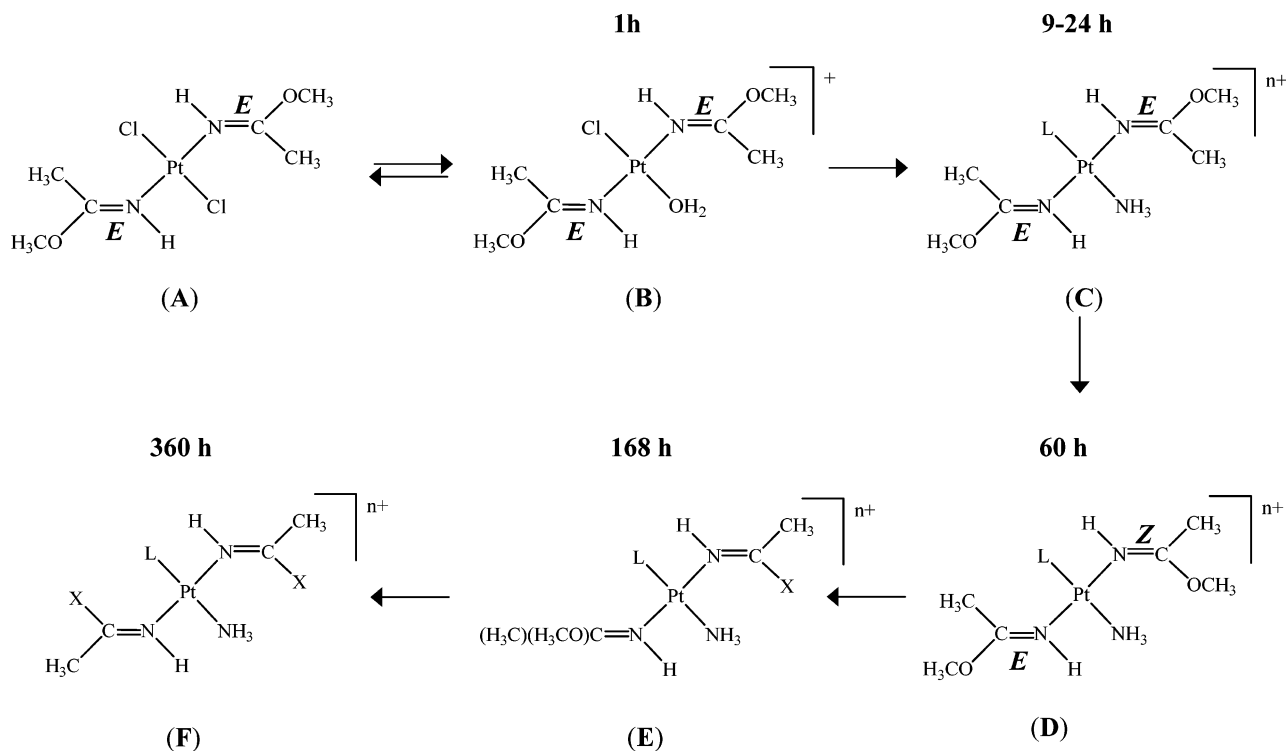
X = OH (**E**) or NH$_2$ (**F**); L= OH$_2$ or NH$_3$

Notably, the ESI MS spectrum of the *trans-EE*/cyt *c* derivative is dominated by a peak at 12 667 Da, which corresponds to addition to the protein of a molecular fragment of mass 309 Da. Rather surprisingly, this mass does not correspond to retention of the two intact iminoether ligands on the platinum center (expected mass of ∼340 Da) but to protein binding of a molecular fragment containing the platinum ion and two acetamide/acetamidine ligands (expected mass of ∼311 Da). These latter ligands are thought to originate from hydrolysis/aminolysis of the iminoether ligands (the aminolysis being fostered by the presence of a high concentration of ammonium carbonate buffer; it is to be noted that the mass of acetamide is only 1 Da greater than that of acetamidine). So far the hydrolysis/aminolysis of the iminoether ligand was only observed at high pH.

Conversely, in the case of *cis-EE*, *cis-Z*, and *trans-Z*, the ESI MS spectra of the Pt/cyt *c* adducts are dominated by a peak corresponding to a mass increase of 268 Da (peak at ∼12 626 Da in the deconvoluted spectra). This latter mass value corresponds well to a platinum(II) ion coordinated to both an acetamide/acetamidine and an aqua/amine ligand.

In all cases, the ESI MS results point out that, under the solution conditions used in our experiments, the platinum-bound iminoether ligands are not lost but undergo important chemical transformations (hydrolysis/aminolysis). Only in the case of *cis-EE* there was release of one iminoether ligand probably due to trans labilization induced by the coordinated cyt *c*.

Our interpretation of the ESI MS results was further supported by additional experiments in which cyt *c* was reacted with either *trans-EE* or *cis-EE* bearing deuterated C-methyl groups. A mass increase of 6 units was observed in the case of *trans-EE*, whereas in the case of *cis-EE*, the measured mass increase was of only 3 units. A complete

assignment of the peaks detected in the ESI MS spectra is given in the reaction Schemes 2 and 3 reported later on in the discussion.

Time-Dependent ESI MS Studies. A number of additional ESI MS measurements were carried out, at different intervals over 1 week time, to monitor the time-dependent behavior of the various adducts. A representative example is reported in Figure 2, where the evolution of the ESI MS spectra of *trans-EE*/cyt *c* over 168 h is shown. After 3 h of incubation, there is already a weak multiplet centered at ∼12 668 Da corresponding to addition to the protein of the mentioned 311 Da platinum fragment. For longer incubation times (24, 72, and 168 h) at 310 K, similar spectral features are observed (the peak at 12 668 Da is always the most intense) implying that the nature of the main protein-bound platinum fragment does not change. However, some significant variations in peak intensities are observed between 72 and 168 h, implying that the system is still subject to slow changes. A similar behavior was found for the ESI MS peaks of cyt *c* adducts with *cis-EE*, *cis-Z*, and *trans-Z*, when expanding the observation period to 168 h.

NMR Studies on the Solution Behavior of trans-EE. The hydrolysis/aminolysis reactions documented by ESI MS for platinum-coordinated iminoether ligands were rather unexpected. Thus, additional experiments were carried out to better elucidate this critical point and to gain independent information on this controversial issue. Specifically, high-resolution NMR studies were performed on *trans-EE* in the same medium (ammonium carbonate buffer, pH 7.4) at 310 K.

After dissolution of *trans*-[PtCl$_2${(*E*)-HN=C(OCH$_3$)-CH$_3$}$_2$] in ammonium carbonate buffer, the first observed transformation (1 h reaction time) is a solvolytic process in which one of the two trans chlorido ligands is replaced
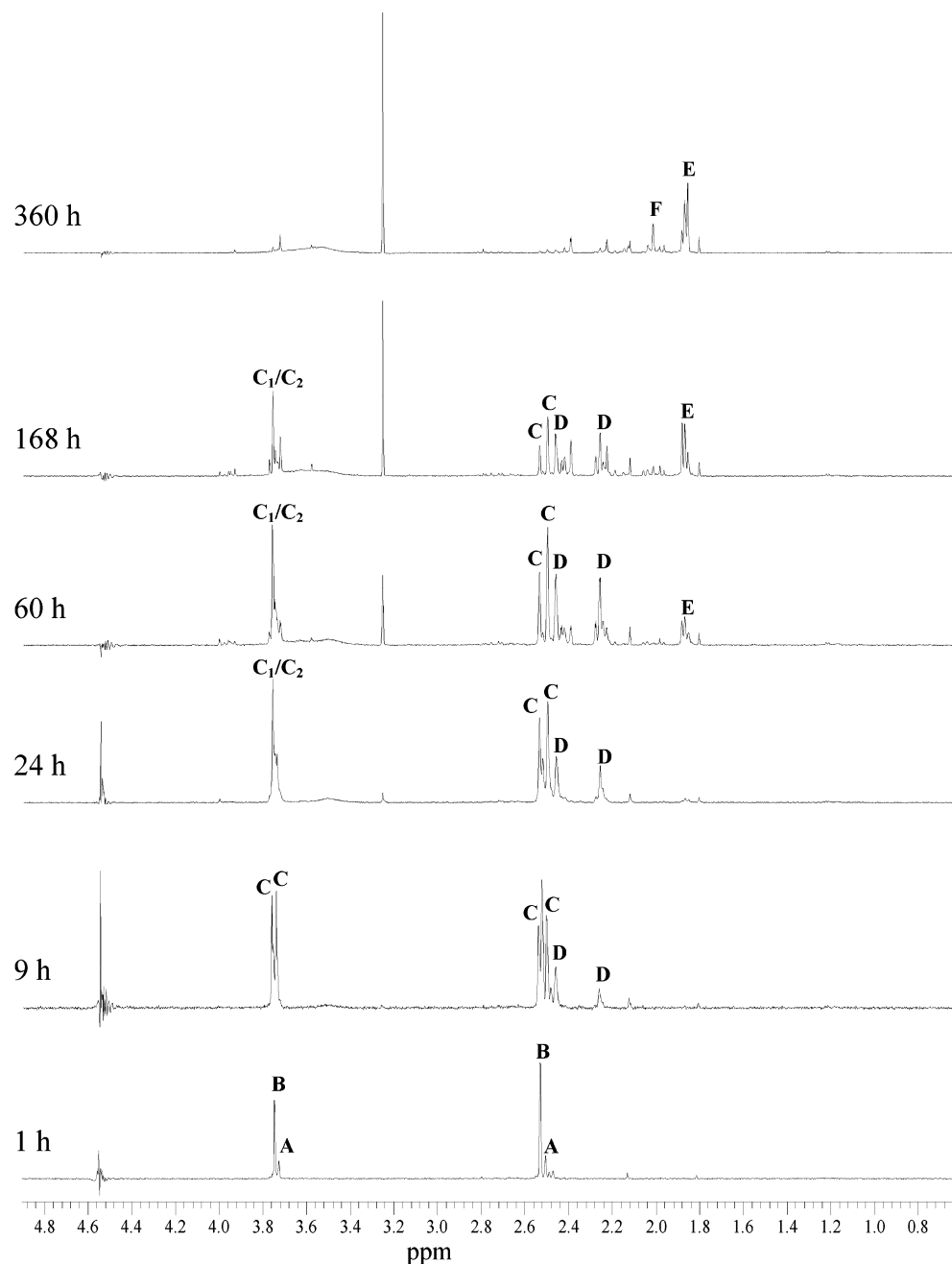
FIGURE 3: ^{1}H NMR spectra (600 MHz) in H_2O/D_2O (9:1) of *trans*-[PtCl$_2${(*E*)-H^{15}N=C(OCH$_3$)CH$_3$}$_2$] (2.4 mM) in ammonium carbonate buffer (25 mM, pH 7.4) at 310 K.

by a molecule of solvent (H_2O). The starting complex (**A** in Scheme 1) has ^{1}H signals at 2.50 (CH$_3$,^{13}C ~21.0 ppm), 3.72 (OCH$_3$,^{13}C ~55.0 ppm), and 7.35 ppm (NH, ^{15}N ~90.5 ppm), whereas the new species (**B**) has signals at 2.54 (CH$_3$, ^{13}C 21.5 ppm), 3.75 (OCH$_3$, ^{13}C 55.0 ppm), and 7.50 ppm (NH, ^{15}N 91.5 ppm) and is assigned to the monosolvated species. One hour after dissolution the ratio between **A** and **B** is ca. 1:9 (Figure 3).

For longer reaction times (9−24 h) a further transformation leads to the formation of complex species bearing coordinated amine (broad signals around 3.5 ppm). Two new species (**C$_1$** and **C$_2$** in Figure 3) have proton signals at 2.55 and 2.51 ppm (CH$_3$, ^{13}C ~21.0 ppm) and at 3.77 and 3.74 ppm (OCH$_3$, ^{13}C ~55.5 ppm), which are in accordance with the new compounds **C$_1$** and **C$_2$** having the iminoether ligands in the original *E* configuration. Another newly formed species

(labeled **D** in Figure 3) has signals at 2.46 (CH$_3$, ^{13}C ~21.0 ppm), 2.30 (CH$_3$, ^{13}C ~19.0 ppm), and 4.00 ppm (OCH$_3$, the intensity of this signal is comparable to those of the other two signals if the spectrum is acquired without suppression of the solvent signal; ^{13}C ~58.0 ppm), which can be ascribed to a complex having one iminoether ligand in *E* and the other in *Z* configurations (an upfield shift of the methyl protons and a downfield shift of the methoxy protons are common features in the switch from *E* to *Z* configuration of an iminoether ligand coordinated to platinum; an analogous trend is also observed for the chemical shifts of the ^{13}C nuclei).

Iminoether ligands are also characterized by imino protons in the 7.0−8.0 ppm region. Signals for all compounds (**A**−**D**) were observed in this region (Figure 1S in the Supporting Information).
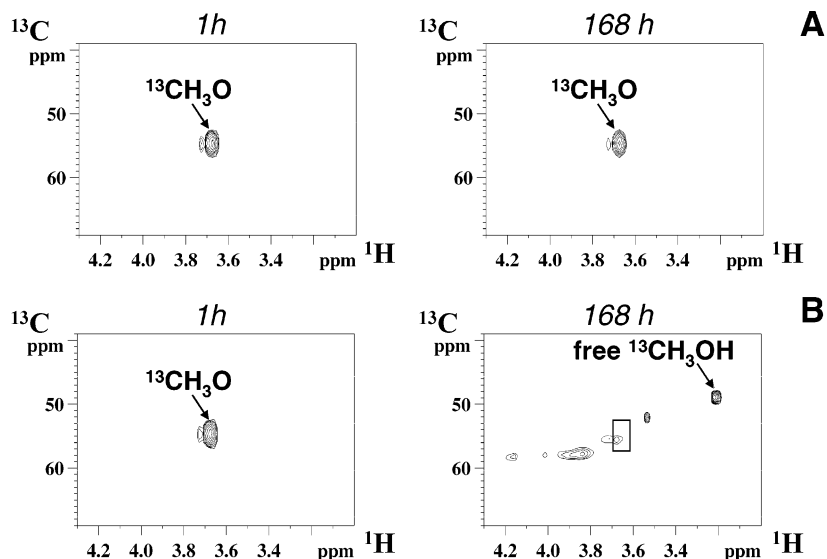
FIGURE 4: 2D ^{1}H,^{13}C-edited HSQC spectra of ^{13}C methoxide-enriched *trans-EE* alone (A) and with 1 equiv of cyt c (B), recorded after 1 h (left panels) and after 1 week (right panels) at 310 K. The peak positions of ^{13}C methoxide in the complex and of free ^{13}C methanol are indicated.

The coordination to platinum of ammonia was investigated in more detail by performing the reaction in ^{15}N-enriched ammonium carbonate buffer. The presence in the [^{1}H,^{15}N] HMQC (Figure 2S in the Supporting Information) of at least four signals (^{15}N chemical shift between −80.0 and −60.0 ppm, ^{1}H chemical shift between 3.5 and 4.1 ppm) coupled with platinum ($^{1}J_{Pt,N}$ ranging between 260 and 280 Hz and $^{2}J_{Pt,H} \sim 57$ Hz), strongly supports the formation of platinum complexes bearing coordinated amine ligand(s).

For still longer reaction times (60 h) a significant amount of free methanol (^{1}H signal at 3.26 ppm and ^{13}C signal at 49.2 ppm) is formed. Simultaneously, new methyl signals (**E** in Figure 3) around 1.85 ppm (^{13}C $\sim$24.5 ppm) and new iminic signals around 5.50 ppm (^{15}N 68.0 ppm) appear. The chemical shifts of the latter set of signals are characteristic of a platinum-coordinated amide. Therefore a hydrolysis of the iminoether ligand with formation of an amide and of free methanol takes place. At still longer times (168 h) the amounts of free methanol and of species containing amide ligands increase at the expenses of species containing iminoether ligands.

After 15 days the iminoether signals disappear almost completely while signals **E** further increase and new signals (**F**) at 2.09 (CH$_3$, ^{13}C 20.5 ppm) and 6.00 ppm (NH) appear. We can assign the **F** signals to amidine complexes by comparison with the chemical shifts of authenticated platinum−amidine compounds. The formation of the different species as inferred from NMR results is shown in Scheme 1.

Thus, the NMR experiments have confirmed the occurrence (*but only over a time intervals of 1−2 weeks*) of extensive hydrolysis/aminolysis with release of methanol and conversion of the iminoether into amide/amidine (see Figure 3).

In the light of the NMR results obtained on the *trans-EE* complex, it is evident that the presence of cyt c leads to a great and generalized enhancement of the reactions taking place at the level of the platinum-coordinated iminoether ligands. Such transformations could take place before platinum coordination to cyt c or soon after coordination of the platinum iminoether complex to cyt c. In any case the

presence of cyt c would have a great effect on the rate of hydrolysis. In order to further prove the catalytic role of cyt c on the hydrolysis of iminoether ligands, a ^{13}C methoxide-enriched *trans-EE* complex was prepared and its hydrolysis in buffered water solution (1.5 mM concentration, pH 6.9) was monitored in the presence or absence of a stoichiometric amount of cyt c. The extent of hydrolysis after 1 week at 310 K (easily monitored by the appearance of ^{13}C-enriched methanol) was negligible in the absence of cyt c, whereas it was quantitative in the presence of cyt c (Figure 4 and Figure 3S in the Supporting Information). It is to be noted that under these experimental conditions the amount of platinum coordinated to cyt c is in the range of 20−25% while the iminoether hydrolysis is almost complete; therefore, it is also possible to conclude from this experiment that cyt c catalyzes hydrolysis of the iminoether ligands also on the free complex. It can be hypothesized that electrostatic interaction between cyt c and *trans-EE* taking place at basic protein surface can foster such a hydrolysis.

Assignment of the Primary Platinum Binding Site. The above-reported results, but in particular the strong tendency to form monoplatinated derivatives, strongly favor the idea that cyt c possesses a primary binding site for platinum drugs. As platinum(II) compounds are known to manifest relatively high affinity only for a few amino acid side chains, namely, Cys, Met, and His, we analyzed the primary sequence of the protein and its crystal structure in order to identify which potential platinum(II) binding sites were characterized by a good solvent accessibility. The protein exhibits seven candidate binding sites: two Cys (14 and 17), two Met (65 and 80), and three His (18, 26, and 33). Among them the two cysteine residues are involved in the covalent binding of the heme group to the polypeptide chain, while Met 80 and His 18 are recruited in the axial coordination of the iron in the heme pocket. Therefore, only Met 65 and His 26 and 33 can be predicted to form adducts with platinum(II) compounds. Moreover, previous studies have supported the view that Met 65 might indeed represent the primary binding site for platinum drugs on horse heart cyt c (*49*). The latter hypothesis can be probed with a simple experiment of cyt c
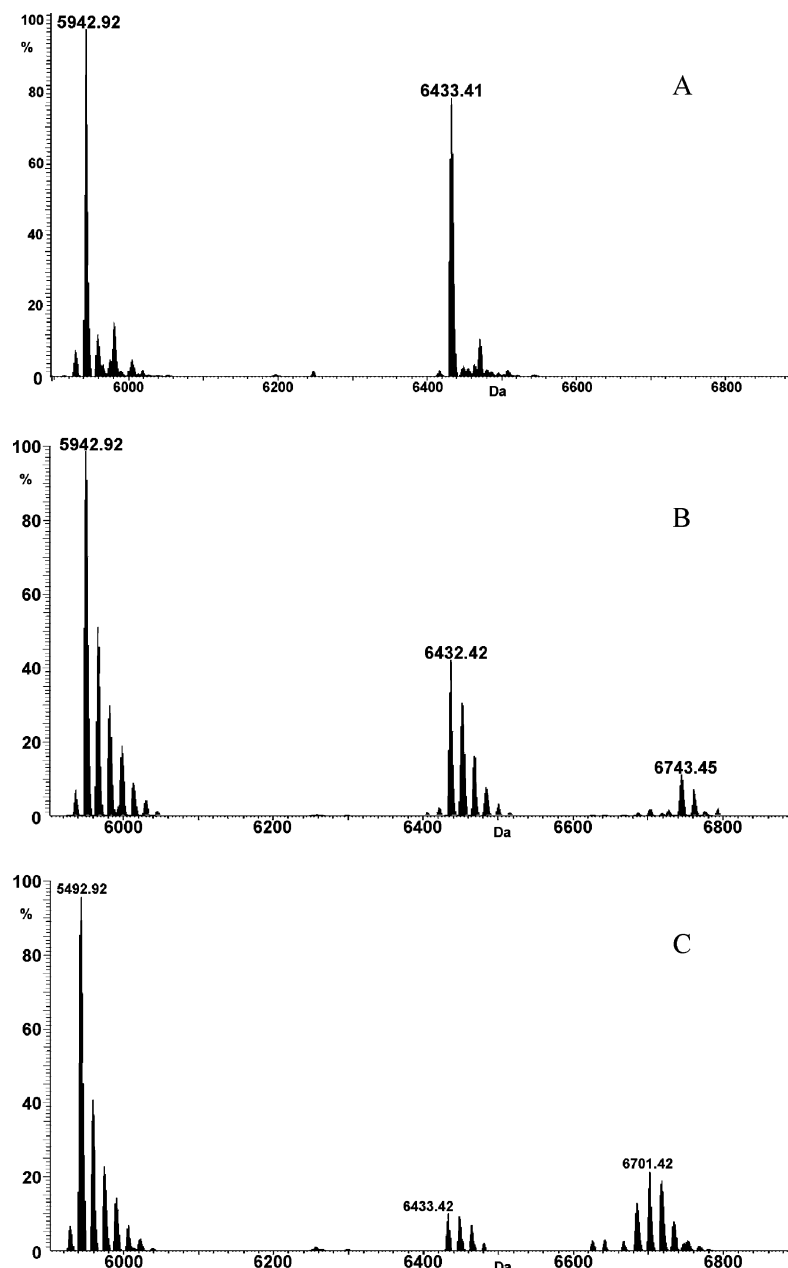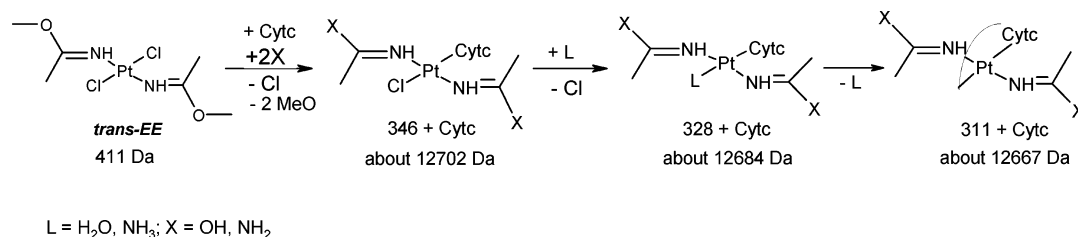
FIGURE 5: Deconvoluted ESI MS spectra of Asp-N treated samples of cyt *c* alone (A) or after incubation with an excess of *trans-EE* (B) or *cis-EE* (C). Data were recorded with an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA).

Scheme 2:   Proposed Reaction Scheme for *trans-EE* with Cytochrome *c*

L = H_2O, NH_3; X = OH, NH_2

proteolysis using the endoproteinase Asp-N. This enzyme is known to hydrolyze peptide bonds on the N-terminal side of aspartic and cysteic acid residues. In the case of horse heart cyt *c*, Asp-N causes selective cleavage only at the N-terminal site of an aspartic residue, and this single cleavage results into the separation of Met 65 from all the other potential binding sites (His 26 and 33). Therefore, a protein sample was treated with either *trans-EE* or *cis-EE*, incubated

with Asp-N, and then analyzed by ESI MS in comparison to a control. The obtained results are shown in Figure 5. The deconvoluted ESI MS spectrum of Asp-N treated nonplatinated cyt *c* reveals two main fragments that correspond to the expected products: the peptide 1−49 (including the heme) and the peptide 50−104 (molecular weights of ~5944 and 6433 Da, respectively; Figure 5A). In contrast, the ESI MS analysis of the platinated species produced the
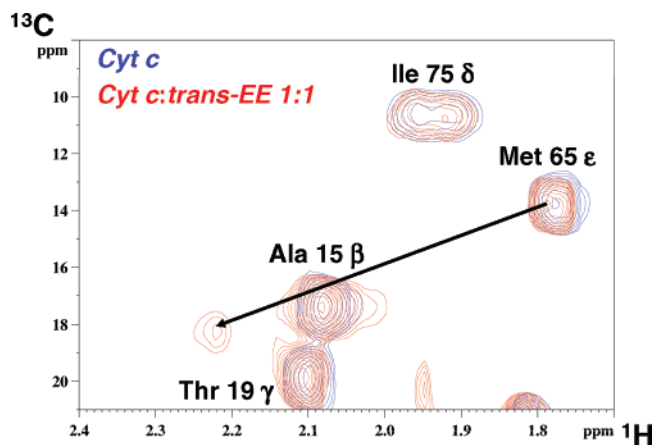
FIGURE 6: Overlay of 2D $^1H,^{13}C$-edited HSQC spectra, recorded at 20 °C, of cyt *c* alone (blue contours) and after 1 week of incubation at 310 K with 1 equiv of *trans-EE* (red contours). Resonance assignments of this spectral region are reported. The spectral change induced by Pt^{II} complexation to Met 65 is indicated with an arrow.

spectra shown in Figure 5B and 5C. It is evident that, in addition to peaks of the two main fragments, a third group of peaks having molecular masses greater than those of the larger fragment is observed. In the case of *trans-EE*, the third multiplet exhibits a mass increase of ~311 Da that nicely corresponds to the mass of the larger protein fragment plus a platinum fragment carrying two acetamide/acetamidine ligands. Conversely, in the case of *cis-EE*, the third multiplet exhibits a mass increase of ~268 Da corresponding to a platinum fragment carrying an acetamide/acetamidine and an aqua/amine ligand. No signs of platination of the smaller fragment were detected.

The latter result provides clear evidence that metalation occurs selectively on the larger fragment (peptide 50−104), i.e., in the peptide containing Met 65. In the light of these data it is rather straightforward to propose that Met 65 represents the primary binding site for platinum(II) imino-ethers in horse heart cyt *c*.

NMR experiments were also set to further confirm the involvement of Met 65 in coordination to platinum residues. Therefore, equimolar amounts of cyt *c* and *trans-EE* were allowed to react in water solution (1.5 mM concentration, pH 6.9) and the $^1H,^{13}C$ HSQC spectrum monitored as a function of time. Although under these experimental conditions only a fraction of cyt *c* reacts with platinum (ca. 20−25%), the newly formed species could be characterized. In particular a new cross-peak (belonging to Met 65 ϵ-CH$_3$) appears downfield of the corresponding peak in native cyt *c* (shift of ^1H and ^{13}C resonances from 1.78 to 2.22 ppm and from 13.8 to 18.2 ppm, respectively, Figure 6) (*50, 51*). Very similar shifts were already observed for platinum coordination to methionine in some model polypeptides. Therefore, it is possible to conclude that, behind any reasonable doubt, platinum coordination occurs at Met 65 of cyt *c*.

DISCUSSION

The present study has highlighted the effectiveness of an ESI MS-based approach for the characterization of platinum−protein adducts at a molecular level. Indeed, relying primarily on ESI MS results, it has been possible to achieve a rather detailed description of the metallodrug/protein adducts,

comprising the identification of the protein-bound metallic fragments and the assignment of the primary metal binding site for cyt *c*.

Preferential formation of monoplatinated adducts was observed for all investigated platinum compounds supporting the view that cyt *c* bears a single, high-affinity, binding site for these platinum(II) species. Upon careful analysis of the various ESI MS peaks, a comprehensive identification of the various platinated species and of the underlying reactions was achieved (see Schemes 2 and 3).

trans-EE is proposed to release a chloride ligand and bind cyt *c*. Concomitantly, replacement of the OCH$_3$ groups by water/ammonia takes place leading to the formation of acetamide/acetamidine ligands (amination is fostered by the presence of ammonium carbonate buffer). In turn, protein binding promotes replacement of the trans chlorido ligand by a water/ammonia molecule, whereas the imine ligands remain coordinated to the platinum center. The postulated sequence of events and peak assignments (specifically at ca. 12 667 and 12 684 Da) are given in Scheme 2.

In the case of *cis-EE*, after the release of the first chlorido ligand and binding of cyt *c*, the iminoether ligand that is trans to the protein is lost, while the iminoether ligand which is cis to the protein is retained (hydrolysis/aminolysis converts the iminoether into the corresponding amide/amidine). The reaction pattern and peak assignment are described in Scheme 3 (specifically at ca. 12 626 and 12 644 Da).

Finally, for *cis-Z* and *trans-Z* it is found that both of them maintain the platinum-coordinated iminoether ligand which, as always, undergoes hydrolysis/aminolysis with transformation into the corresponding amide/amidine.

Remarkably, the above-reported interpretation of the ESI MS data reveals a peculiar and largely unexpected behavior for these iminoether ligands, i.e., occurrence of extensive hydrolysis/aminolysis and formation of the corresponding amide/amidine. It is evident that such a reactivity is greatly enhanced by a direct interaction of the metal complexes with the protein as it emerges from independent NMR measurements performed on *trans-EE* either alone or in the presence of a stoichiometric amount of cyt *c*. Under identical experimental conditions, pure *trans-EE* remains stable, whereas 1:1 mixtures of *trans-EE* and cyt *c* undergo extensive ligand hydrolysis. Overall, these results imply that platinum drugs dissolved in biological media—and thus in the presence of many chemical components, including macromolecules—may manifest a chemical reactivity that is profoundly distinct from that observed when they are just dissolved in simple buffered solutions. These observations pose important "caveats" to extrapolating the behavior observed in solution for metallodrug to that believed to occur inside cells. It is proposed that the hydrolysis of iminoethers is fostered by electrostatic interactions between the metal complex and basic surface areas of the protein.

In addition, important differences have emerged in the reactivities of the various tested compounds with cyt *c*. At variance with *trans-EE*, *cis-EE* was shown to release one of its iminoether ligands, most likely the one which is trans to the bound protein. Thus, the same type of molecular fragments was found in the adducts of cyt *c* with *cis-EE*, *cis-Z*, and *trans-Z*. Remarkably, the time-dependent evolution of the various platinated adducts was found to manifest a

Scheme 3: Proposed Reaction Scheme for *cis-EE* with Cytochrome *c*
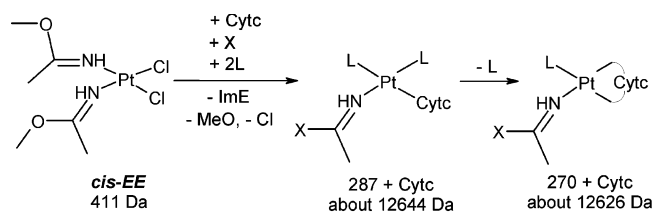
L = H₂O, NH₃; X = OH, NH₂; ImE = iminoether

significant stability even over relatively long time periods (168 h); only in the case of *cis-EE* some relevant protein degradation was observed already within 24 h.

A final comment concerns the specific localization of the platinum binding sites on cyt *c*. On the grounds of the above results (mainly formation of monoadducts), it is evident that cyt *c* has a primary binding site significantly stronger than any other. Previous studies have reported that Met 65 represents a high-affinity binding site for platinum drugs (*49*). This hypothesis is now strongly supported by additional ESI MS studies performed on cyt *c* proteolytic fragments showing that platination occurs selectively on the larger fragment (i.e., the fragment containing Met 65). The latter conclusion is also fully supported by NMR investigations showing a characteristic downfield shift of the Met 65 methyl signal upon reaction with the metallic substrate.

Concluding Remarks and Perspectives. Overall, the strategy here illustrated has turned out to be particularly successful in monitoring the reactivity of platinum metallodrugs with the model protein cyt *c* and in elucidating, at the molecular level, the formation, nature, and evolution of the resulting platinum−protein adducts. It has emerged very clearly that *interactions with this specific protein do profoundly alter the intrinsic reactivity of platinum compounds*, leading to the observation of rather unexpected chemical transformations at the level of the platinum ligands. The comparative analysis of the present results with those previously obtained on classical platinum(II) anticancer drugs, the results of a specific partial proteolysis experiment, and the NMR monitoring of methionine chemical shifts make us confident that Met 65 is the major binding site for platinum(II) iminoethers on cyt *c*.

The mechanistic implications of the present work also deserve a final comment. The results obtained here for cyt *c* are attractive since they might be of general significance and could help in understanding what is occurring inside cells during treatment with platinum drugs. Of course, some specificities in the metal/protein interactions can be expected depending upon nature and surface exposure of different amino acids and their affinity for platinum (consider for instance the case of cysteine-rich metallothioneins, a group of proteins displaying high affinity for platinum and other soft metals). Some literature is indeed available on this issue. However, since it is now firmly established that a large number of proteins (and not only a few ones) are platinated *in vitro*, we can reasonably assume that reactions similar to those described here for platinum iminoethers toward cyt *c* may actually occur inside cells. These arguments reinforce the importance of the present strategy based on the use of specific model proteins.

REFERENCES

1. Special Issue on Medicinal Inorganic Chemistry. (1999) *Chem. Rev. 99*, 2201−2842.
2. Keppler, B. K. (1993) *Metal Complexes in Cancer Chemotherapy*, VCH, Weinheim, Germany.
3. Sigel, A., and Sigel, H. (2004) *Metal Ions in Biological Systems*, Vol. 42, Marcel Dekker, Inc., New York, Basel, The Netherlands.
4. Gielen, M., and Tiekink, E. R. T. (2005) *Metallotherapeutic Drugs and Metal-Based Diagnostic Agents: The Use of Metals in Medicine*, Wiley, Weinheim, Germany.
5. Zhang, C. X., and Lippard, S. J. (2003) New metal complexes as potential therapeutics, *Curr. Opin. Chem. Biol. 7*, 481−489.
6. Guo, Z., and Sadler, P. J. (1999) Medicinal inorganic chemistry, *Adv. Inorg. Chem. 49*, 183−306.
7. Farrell, N. (2004) Polynuclear platinum drugs, *Met. Ions Biol. Syst. 42*, 251−296.
8. Lippert, B. (1999) *Cisplatin: Chemistry and Biochemistry of a Leading Anticancer Drug*, John Wiley & Sons, Inc., New York.
9. Wang, D., and Lippard, S. J. (2005) Cellular processing of platinum anticancer drugs, *Nat. Rev. Drug Discovery 4*, 307−320.
10. Reedijk, J. (2003) New clues for platinum antitumor chemistry: kinetically controlled metal binding to DNA, *Proc. Natl. Acad. Sci. U.S.A. 100*, 3611−3616.
11. Messori, L., Marcon, G., Orioli, P., Fontani, M., Zanello, P., Bergamo, A., Sava, G., and Mura, P. (2003) Molecular structure, solution chemistry and biological properties of the novel [ImH]-[*trans*-IrCl(4)(Im)(DMSO)], (I) and of the orange form of [(DMSO)₂H][*trans*-IrCl₄(DMSO)₂], (II), complexes, *J. Inorg. Biochem. 95* (1), 37−46.
12. Messori, L., Abbate, F., Marcon, G., Orioli, P., Fontani, M., Mini, E., Mazzei, T., Carotti, S., O'Connell, T., and Zanello, P. (2000) Gold(III) complexes as potential antitumor agents: solution chemistry and cytotoxic properties of some selected gold(III) compounds, *J. Med. Chem. 43* (19), 3541−3548.
13. Ivanov, A. I., Christodoulou, J., Parkinson, J. A., Barnham, K. J., Tucker, A., Woodrow, J., and Sadler, P. J. (1998) Cisplatin binding sites on human albumin, *J. Biol. Chem. 273* (24), 14721−14730.
14. Khalaila, I., Allardyce, C. S., Verma. C. S., and Dyson, P. J. (2005) A mass spectrometric and molecular modelling study of cisplatin binding to transferrin, *ChemBioChem 6* (10), 1788−1795.
15. Mandal, R., Kalke, R., and Li, X. F. (2004) Interaction of oxaliplatin, cisplatin, and carboplatin with hemoglobin and the resulting release of a heme group, *Chem. Res. Toxicol. 17* (10), 1391−1397.
16. Gibson, D., and Costello, C. E. (1999) A mass spectral study of the binding of the anticancer drug cisplatin to ubiquitin, *Eur. Mass Spectrom. 5*, 501−510.
17. Balter, L., and Gibson, D. (2005) Mass spectrometric studies of the formation and reactivity of *trans*-[PtCl₂(Am)-(piperidinopiperidine)] × HCl complexes with ubiquitin, *Rapid Commun. Mass Spectrom. 19* (24), 3666−3672.
18. Najajreh, Y., Ardeli-Tzaraf, Y., Kasparkova, J., Heringova, P., Prilutski, D., Balter, L., Jawbry, S., Khazanov, E., Perez, J. M., Barenholz, Y., Brabec, V., and Gibson, D. (2006) Interactions of platinum complexes containing cationic, bicyclic, nonplanar piperidinopiperidine ligands with biological nucleophiles, *J. Med. Chem. 49* (15), 4674−4683.
19. Peleg-Shulman, T., Najajreh, Y., and Gibson, D. (2002) Interactions of cisplatin and transplatin with proteins: Comparison of binding kinetics, binding sites and reactivity of the Pt−protein adducts of cisplatin and transplatin towards biological nucleophiles, *J. Inorg. Biochem. 91*, 306−311.

20. Najajreh, Y., Peleg-Shulman, T., Moshel, O., Farrell, N., and Gibson, D. (2003) Ligand effects on the binding of *cis*- and *trans*-[PtCl$_2$)Am(1)Am(2)] to proteins, *J. Biol. Inorg. Chem. 8* (1−2), 167−175.

21. Calderone, V., Casini, A., Mangani, S., Messori, L., and Orioli, P. L. (2006) Structural investigation of cisplatin−protein interactions: selective platination of His19 in a cuprozinc superoxide dismutase, *Angew. Chem., Int. Ed. 45* (8), 1267−1269.

22. Casini, A., Mastrobuoni, G., Temperini, C., Gabbiani, C., Francese, S., Moneti, G., Supuran, C. T., Scozzafava, A., and Messori, L. (2007) *Chem. Commun. 14* (2), 156−158.

23. Timerbaev, A. R., Hartinger, C. G., Aleksenko, S. S., and Keppler, B. K. (2006) *Chem. Rev. 106* (6), 2224−2248.

24. Casini. A., Gabbiani, C., Mastrobuoni, G., Messori, L., Moneti, G., and Pieraccini, G. (2006) Exploring metallodrug−protein interactions by ESI mass spectrometry: the reaction of anticancer platinum drugs with horse heart cytochrome *c*, *ChemMedChem 1* (4), 413−417.

25. Hartinger, C. G., Ang, W. H., Casini, A., Messori, L., Keppler, B. K., and Dyson P. J. (2007) Mass spectrometric analysis of ubiquitin platinum interactions of leading anticancer drugs: MALDI versus ESI, *J. Anal. At. Spectrom.*, in press.

26. Cristoni, S., and Bernardi, L. R. (2003) Development of new methodologies for the mass spectrometry study of bioorganic macromolecules, *Mass Spectrom. 22*, 369−406.

27. Loo, J. A. (1997) Studying noncovalent protein complexes by electrospray ionization mass spectrometry, *Mass Spectrom. Rev. 16*, 1−23.

28. Hu, P., Ye, Q. Z., and Loo, J. A. (1994) Calcium stoichiometry determination for calcium binding proteins by electrospray ionization mass spectrometry, *Anal. Chem. 66*, 4190−4194.

29. Mann, M., Hendrickson, R. C., and Pandey, A. (2001) Analysis of proteins and proteomes by mass spectrometry, *Annu. Rev. Biochem. 70*, 437−473.

30. Jonsson, A. P. (2001) Mass spectrometry for protein and peptide characterisation, *Cell. Mol. Life Sci. 58*, 868−884.

31. Hartinger, C. G., Alexenko, S., Timerbaev, A. R., and Keppler, B. K. (2005) The binding of platinum complexes to human serum albumin studied by electrospray ionization-ion trap-mass spectrometry (ESI-IT-MS) *Novel Approaches for the Discovery and the Development of Anticancer Agents*, CESAR, Vienna, P16.

32. Yang, G., Miao, R., Jin, C., Mei, Y., Tang, H., Hong, J., Guo, Z., and Zhu, L. (2005) Determination of binding sites in carboplatin-bound cytochrome *c* using electrospray ionization mass spectrometry and tandem mass spectrometry, *J. Mass Spectrom. 40* (8), 1005−16.

33. Mandal, R., and Li, X. F. (2006) Top-down characterization of proteins and drug−protein complexes using nanoelectrospray tandem mass spectrometry, *Rapid Commun. Mass Spectrom. 20* (1), 48−52.

34. Casini, A., Mastrobuoni, G., Ang, W. H., Gabbiani, C., Pieraccini, G., Moneti, G., Dyson, P. J., and Messori, L. (2007) Exploring metallodrug−protein interactions by ESI mass spectrometry: ESI MS characterization of protein adducts of anticancer ruthenium-(II)−arene PTA (RAPTA) complexes, *ChemMedChem 2* (5), 631−635.

35. Natile, G., and Coluccia, M. (2001) Current status of trans-platinum compounds in cancer therapy, *Coord. Chem. Rev., 216/217*, 383−410.

36. Coluccia, M., and Natile, G. (2007) Trans-platinum complexes in cancer therapy, *Anticancer Agents Med. Chem. 7* (1), 111−123.

37. Cini, R., Caputo, P. A., Intini, F. P., and Natile, G. (1995) Mechanistic and stereochemical investigation of imino ethers formed by alcoholysis of coordinated nitriles: X-ray crystal structures of *cis*- and *trans*-bis(1-imino-1-methoxyethane)dichloroplatinum(II), *Inorg. Chem. 34*, 1130−1137.

38. Coluccia, M., Nassi, A., Roseto, F., Boccarelli, A., Mariggiò, M. A., Giordano, D., Intini, F. P., Caputo, P., and Natile, G. (1993) A trans-platinum complex showing higher antitumor activity than the cis congeners, *J. Med. Chem. 36*, 510−512.

39. Coluccia, M., Boccarelli, A., Mariggiò, M. A., Cardellicchio, N., Caputo, P., Intini, F. P., and Natile, G. (1995) Platinum(II) complexes containing iminoethers: a trans platinum antitumour agent, *Chem.−Biol. Interact. 98*, 251−266.

40. Brabec, V., Vrana, O., Novakova, O., Kleinwachter, V., Intini, F. P., Coluccia, M., and Natile, G. (1996) DNA adducts of antitumor *trans*-[PtCl$_2$ (E-imino ether)$_2$], *Nucleic Acids Res. 24*, 336−341.

41. Leng, M., Locker, D., Giraud-Panis, M. J., Schwartz, A., Intini, F. P., Natile, G., Pisano, C., Boccarelli, A., Giordano, D., and Coluccia, M. (2000) Replacement of an NH$_3$ by an iminoether in transplatin makes an antitumor drug from an inactive compound, *Mol. Pharmacol. 58*, 1525−1535.

42. Palmer, A. G., III, Cavanagh, J., Wright, P. E., and Rance, M. (1991) Sensitivity improvement in proton-detected 2-dimensional heteronuclear correlation NMR spectroscopy, *J. Magn. Reson. 93*, 151−170.

43. Kay, L. E., Keifer, P., and Saarinen, T. (1992) Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity, *J. Am. Chem. Soc. 114*, 10663−10665.

44. Schleucher, J., Schwendinger, M., Sattler, M., Schmidt, P., Schedletzky, O., Glaser, S. J., Sørensen, O. W., and Griesinger, C. (1994) A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients, *J. Biomol. NMR 4*, 301−306.

45. Marion, D., and Wüthrich, K. (1983) Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurements of ^{1}H−^{1}H spin-spin coupling constants in proteins, *Biochem. Biophys. Res. Commun. 113*, 967−974.

46. Shaka, A. J., Barker, P. B., and Freeman, R. (1985) Computer optimized decoupling scheme for wideband applications and low-level operation, *J. Magn. Reson. 64*, 547−552.

47. Banci, L., Bertini, I., Gray, H. B., Luchinat, C., Reddig, T., Rosato, A., and Turano, P. (1997) Solution structure of oxidized horse heart cytochrome *c*, *Biochemistry 36*, 9867−9877.

48. Liu, W., Rumbley, J., Englander, S. W., and Wand, A. J. (2003) Backbone and side-chain heteronuclear resonance assignments and hyperfine NMR shifts in horse cytochrome *c*, *Protein Sci. 12*, 2104−2108.

49. Cox, M. C., Barnham, K. J., Frenkiel, T. A., Hoeschele, J. D., Mason, A. B., He, Q. Y., Woodworth, R. C., and Sadler, P. J. (1999) Identification of platination sites on human serum transferrin using (13)C and (15)N NMR spectroscopy, *J. Biol. Inorg. Chem. 4* (5), 621−631.

50. Arnesano, F., Scintilla, S., and Natile, G. (2007) Interaction between platinum complexes and a methionine motif found in copper transport proteins, *Angew. Chem., Int. Ed.*, in press.

51. Lijuan, J., Yu, C., Guozi, T., and Wenxia, T. (1997) Studies on the interaction between cytochrome *c* and *cis*-PtCl$_2$(NH$_3$)$_2$, *J. Inorg. Biochem. 65*, 73−77.