

Mapping the Reactivity of Two Pharmacologically Active Organopalladium Compounds toward the Model Protein RNase A Using a Dual ESI MS and X-ray Diffraction Investigative Approach

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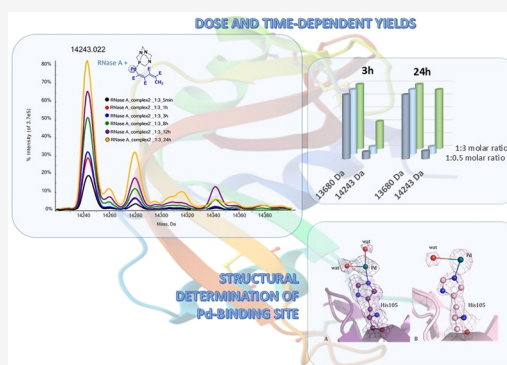


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Supporting Information

ABSTRACT: Organopalladium compounds are a new class of metal-based drugs with significant yet largely unexplored therapeutic potential. Previous studies have suggested that these compounds act as prodrugs that can strongly interact with protein targets. However, the underlying protein metalation processes have scarcely been investigated to date. To gain a deeper insight into this issue, we analyzed the interactions of two representative organopalladium compounds with the model protein RNase A using a strategy that combined electrospray ionization mass spectrometry (ESI MS) and X-ray crystallography. Time-course and titration ESI-MS studies revealed that the two palladium compounds readily react with RNase A to form stable adducts with distinct structural characteristics. The crystal structures of two organopalladium/RNase A adducts were then determined. These structures revealed that the palladium-containing fragments bind to histidine and methionine side chains. Interestingly, one of the two complexes interacts with the catalytically important residues His12 and His119, causing significant inhibition of the protein's enzymatic activity. Overall, these results provide detailed insights into the main features of protein metalation reactions promoted by organopalladium compounds as well as the nature of protein-bound palladium fragments.



INTRODUCTION

Over the past two decades, organopalladium compounds have attracted increasing interest in medicinal chemistry,¹ although research into these compounds remains less developed than that into platinum-, gold-, and ruthenium-based drugs.² Defined by the presence of at least one palladium–carbon bond, organopalladium compounds occupy a privileged position because this linkage enables oxidative addition, migratory insertion, π – σ interconversion, and reductive elimination with extraordinary efficiency.³ While these processes were historically exploited in homogeneous catalysis, they are now increasingly recognized as powerful tools in medicinal chemistry, enabling organopalladium fragments to selectively engage with biological targets.

Within this emerging paradigm, some of us have systematically explored the anticancer activity of a broad range of organopalladium fragments, including Pd(II)–allyl, Pd(II)–butadienyl, Pd(II)–vinyl, Pd(II)–aryl, Pd(II)–indenyl, Pd(II)–imidoyl, and palladacyclopentadienyl complexes, as well as Pd(0)–olefin species and Pd(I) dimers.^{4–11} These studies have covered two-dimensional (2D) cancer cell models, three-dimensional (3D) patient-derived tumor organoids, and selected *in vivo* systems. Of the families investigated, Pd(II)–allyl and Pd(II)–butadienyl complexes have consistently proven to be the most promising. They exhibit high cytotoxicity across

multiple cancer models and pronounced selectivity toward ovarian cancer, with only marginal effects on non-cancerous cells.^{4,8,9} This favorable therapeutic window is preserved in 3D patient-derived organoids and is consistent with the reasonable aqueous solubility and chemical stability of the complexes that is ensured by appropriate ligand frameworks.

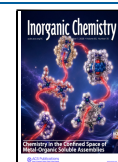
A paradigmatic example is a Pd(II)–butadienyl complex bearing two PTA ligands (PTA = 1,3,5-triaza-7-phosphaadamantane), which is soluble in water at millimolar concentrations and displays submicromolar cytotoxicity in both 2D cultures and 3D tumor organoids.⁹ This compound, hereafter referred to as complex **1** (Scheme 1), also reduces tumor mass *in vivo* while being exceptionally well tolerated, with no detectable systemic toxicity even at doses exceeding 200 mg kg^{−1} in mice. Its mechanism of action is highly unconventional for Pd-based drugs, as it primarily induces ferroptosis via inhibition of glutathione peroxidase 4 (GPX4), a selenoenzyme

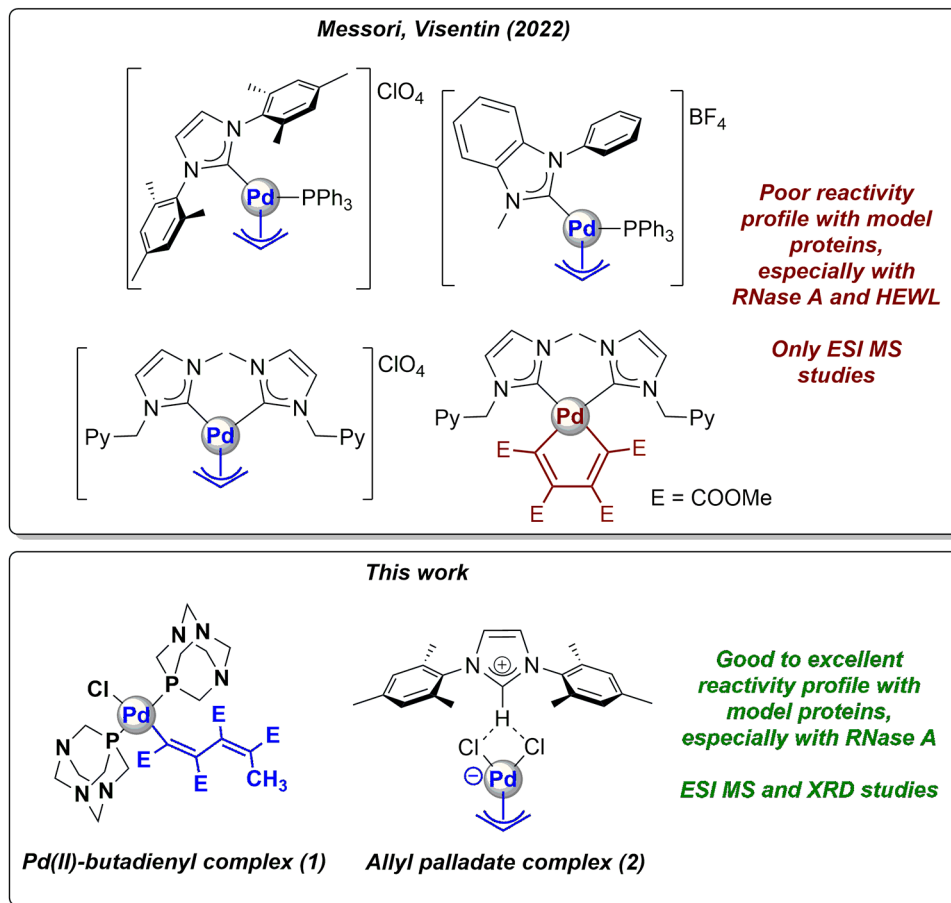
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Scheme 1. Previously Studied¹⁸ Organopalladium Compounds and Those Reported in This Work

that suppresses lipid peroxidation.¹² By triggering iron-dependent oxidative membrane damage rather than DNA lesions, this compound circumvents the classical resistance pathways associated with apoptosis-based chemotherapies.

Pd(II)-allyl complexes operate through a distinct yet equally compelling biological mechanism. Immunofluorescence studies have shown that these compounds accumulate preferentially in mitochondria, where they disrupt redox homeostasis and cellular bioenergetics.⁴ The presence of at least one *N*-heterocyclic carbene (NHC) ligand significantly enhances their biological performance by stabilizing the metal center and modulating its reactivity in the cellular environment. Three main families have been explored: neutral [Pd(NHC)Cl(allyl)] species;⁸ cationic [Pd(NHC)(L)(allyl)]⁺ complexes,^{4,13} where L is a phosphine, NHC, or isocyanide; and allyl palladates of the type [NHC·H][PdCl₂(allyl)].^{14–17} Of these, allyl palladates have consistently proven to be the most potent anticancer agents and thioredoxin reductase (TrxR) inhibitors, demonstrating significantly higher activity than the neutral or cationic Pd-allyl-NHC complexes.^{14–16} Their relevance is further enhanced by their exceptionally simple, modular synthesis. These species can be obtained in essentially quantitative yields through the direct combination of the [PdCl(allyl)]₂ dimer and the appropriate azolium salt. This process can be carried out under mild solid-state grinding conditions, enabling the rapid generation of large compound libraries. Importantly, previous studies from our group have shown that the biological activity of allyl palladates arises from the intimate association between the allyl-palladate anion and

the azolium cation. Even in polar media, a significant portion of these species remain associated as ion pairs.^{14–17} While the corresponding azolium salts are generally poorly cytotoxic or completely inactive toward cancer cells, combining them with the allyl-palladate fragment produces highly active compounds. Moreover, while the palladium-containing anion is essential for the biological activity, the azolium cation is not merely a spectator, as different cation–anion combinations can markedly influence cellular uptake, cytotoxicity, and, in some cases, selectivity toward cancer over non-cancerous cells. Scheme 1 shows the allyl palladate selected, in the present study, as a representative model compound, which is referred to as complex 2 hereafter. Further studies have demonstrated that this framework can be chemically programmed to enhance tumor selectivity using carbohydrate-derived azolium salts. Notably, allyl palladates are the first palladium-based inducers of immunogenic cell death reported to date.¹⁴

Together, the above findings represent a significant shift in our understanding of the role of Pd compounds in biology. Previous studies have largely described Pd(II) and organopalladium complexes as DNA-binding agents with limited potency and selectivity.^{19–21} In contrast, the systems described above primarily act through protein targeting, aligning palladium with other successful metallodrugs, such as gold compounds that inhibit key enzymes.^{18,22,23} Organopalladium complexes therefore emerge as protein-reactive, mechanistically programmable metallodrugs.

In this context, the concept of protein metalation—the formation of metal–protein adducts—has become a central

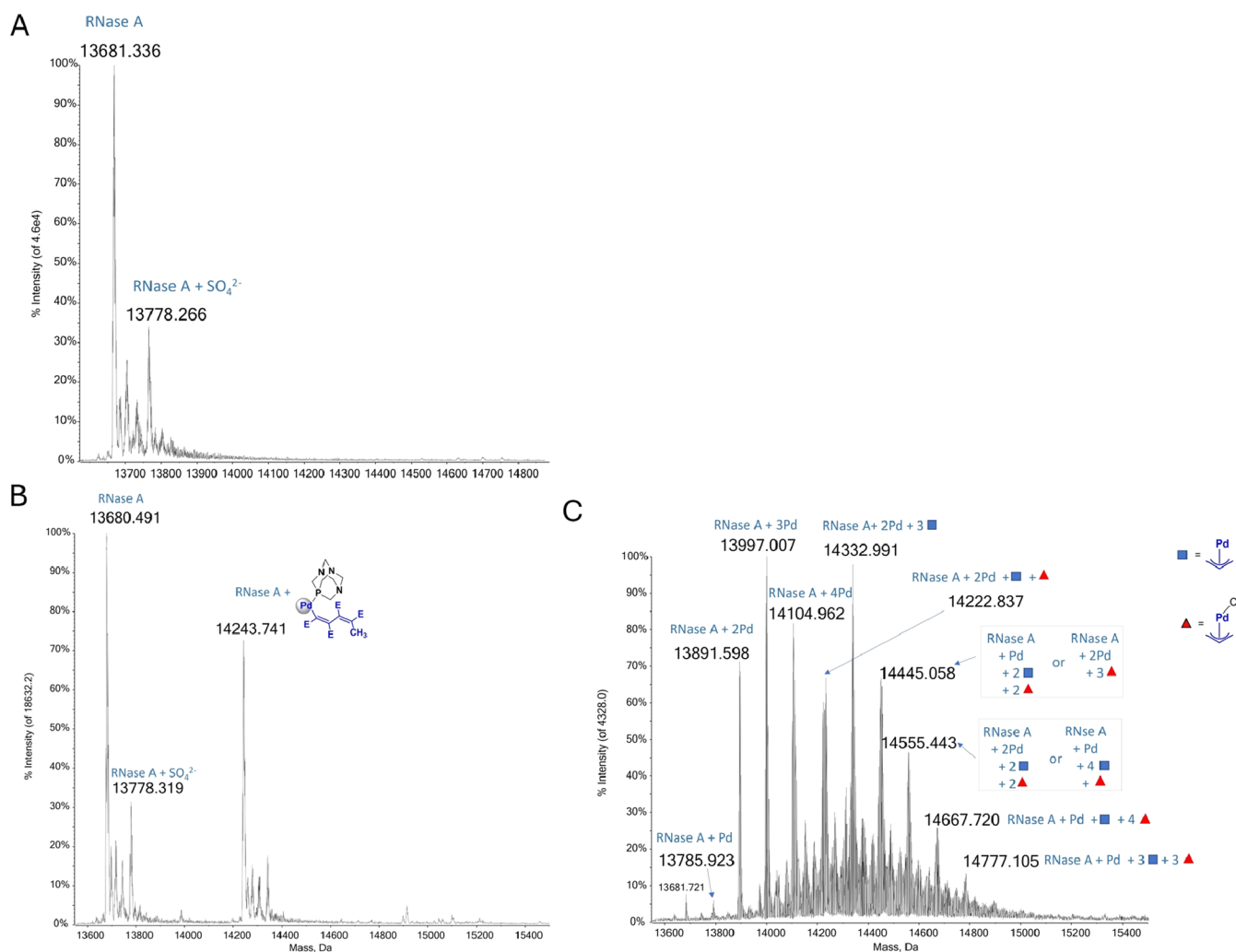


Figure 1. Deconvoluted ESI-Q-TOF spectra of (A) metal-free RNase A and RNase A adducts with (B) complex 1 and (C) complex 2 in a 1:3 protein-to-Pd complex molar ratio. Incubation time: 24 h.

theme in the field of medicinal inorganic chemistry.^{24–27} This process governs drug transport, accumulation, activation, and enzyme modulation. It also underpins the design of artificial metalloenzymes and protein-based delivery systems, where the protein scaffold is integral to the chemical and biological functions of the metal. However, defining this process at the molecular level can be very difficult as it involves metal speciation, ligand exchange, redox chemistry, and selective coordination with amino acid residues.

Recently, a joint investigative protocol involving ESI MS and X-ray crystallography was proposed for characterizing the protein metalation process at the atomic level.^{24–27} Although X-ray crystallography can reveal the location of metal-binding sites within protein structures, it may fail to determine the chemical identity of the metal ligands due to low occupancy of metal-containing fragments in the metal/protein adducts or to high conformational flexibility of these groups. In contrast, ESI MS can directly reveal the stoichiometry, speciation, and ligand composition of metal–protein adducts in solution and allows the definition of some of the dynamic aspects of the process. Therefore, combining the two techniques provides a particularly powerful view of protein metalation.

Nevertheless, few comprehensive studies have employed this integrated approach with metallodrugs that are of genuine

pharmacological relevance and even fewer with organopalladium compounds. Previous work using ESI MS revealed that organopalladium complexes can form well-defined protein adducts;¹⁸ however, their reactivity was insufficient to produce crystallographically tractable species.

Thus, in the present study, we first investigated the interaction of organopalladium complexes 1 and 2 with bovine pancreatic ribonuclease (RNase A) using ESI-MS and X-ray crystallography.

RESULTS AND DISCUSSION

ESI-MS Interaction Experiments with RNase A

ESI MS is a powerful tool for characterizing metallodrug–protein interactions at the molecular level with a high precision. To analyze the possible interactions of Pd complexes 1 and 2 with a protein, ESI MS spectra of RNase A were recorded before and after incubation with the palladium complexes. RNase A was selected as a model protein for these studies since it is relatively small (124 residues, ~13.7 kDa) and has been widely used to investigate the protein metalation process by several metal compounds, including anticancer agents such as cisplatin²⁸ and its derivatives,²⁹ gold,³⁰ ruthenium,³¹ iridium,³² vanadium³³ and rhodium³⁴ compounds and some palladium coordination complexes.³⁵ Its

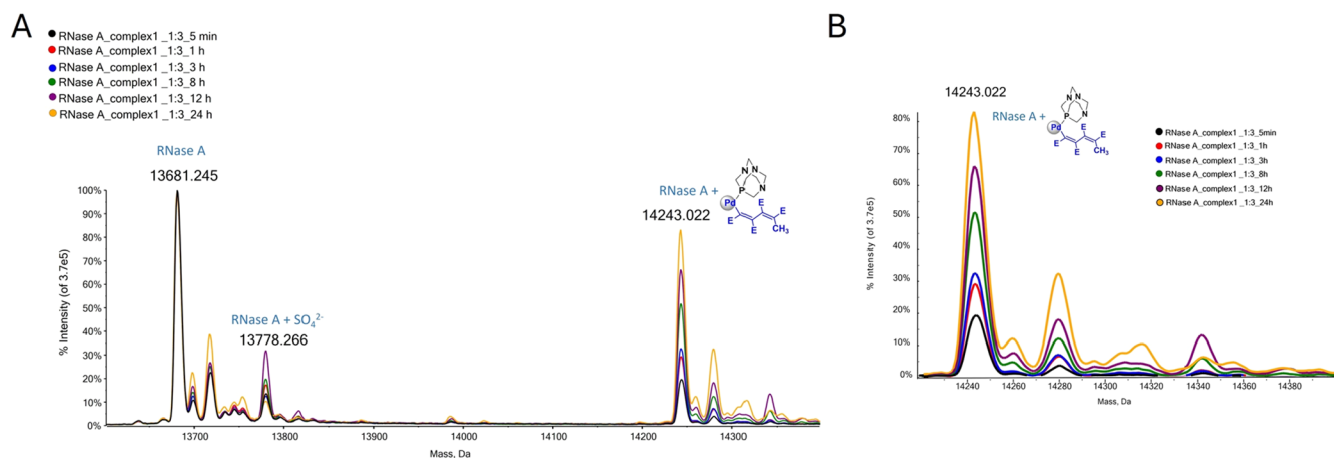


Figure 2. (A) ESI-MS time-course analysis of RNase A incubated with complex **1** for 24 h. (B) Magnified mass spectrum of the 14243 Da adduct, corresponding to the RNase-Pd(II)–butadienyl bearing one PTA.

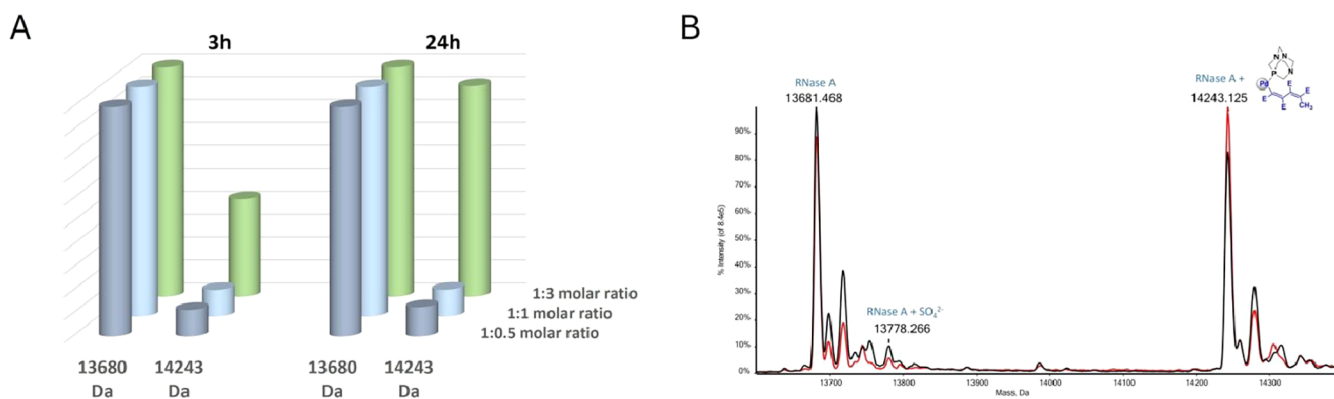


Figure 3. ESI-MS titration of the RNase-complex **1** at 3 and 24 h. (A) Protein-to-metal molar ratios of 1:0.5, 1:1, and 1:3. (B) Comparison of the 1:10 (red line) and 1:3 (black line) stoichiometries.

inherent stability and extensive characterization³⁶ provide a robust framework for the validation and comparison of new experimental data with literature.

First, RNase A was incubated with complexes **1** and **2** at a 1:3 protein-to-metal molar ratio for 24 h, according to established procedures,³⁷ after which the respective ESI MS spectra were recorded. These spectra are shown in Figure 1, together with that of the metal-free protein. The control spectrum of metal-free RNase A displays a primary peak, consistent with the theoretical mass of the RNase A sequence, at 13,681 Da. A secondary peak, at 13,779 Da, corresponds to the sulfate-bound protein form, inherent to the commercial sample.

Inspection of the spectra of the protein incubated with the Pd complexes clearly shows the formation of an adduct. In the case of complex **1**, the MS spectrum reveals the formation of a major adduct characterized by a mass increase of 563 Da (Figure 1B), when compared to the native protein. This mass shift is consistent with the binding to the protein of a Pd(II)–butadienyl fragment bearing one PTA ligand. The relative intensity of this peak is approximately 70% of that of the residual signal of the unmodified protein. In contrast, the mass spectrum obtained upon incubation of RNase A with complex **2** is characterized by the near-complete disappearance of the native protein peak and the appearance of several peaks corresponding to a variety of protein adducts bearing a

multiplicity of Pd-containing fragments of the type PdCl(allyl) or [Pd(allyl)]⁺, as well as palladium ions (Figure 1C).

ESI MS time-course experiments were then carried out to monitor adduct formation and evolution, with the concentrations of the protein and the Pd complexes kept constant throughout. Monitoring of the reaction was possible by observing the relative intensity of the signals at different time intervals. The obtained results demonstrate that the interaction between the Pd(II)–butadienyl fragment bearing one PTA ligand (derived from complex **1**) and the protein reaches equilibrium after 24 h of incubation, as evidenced by the progressive increase of the adduct signal over time (Figure 2).

To investigate how the concentration of palladium complex **1** influences binding, we tested protein-to-metal molar ratios of 1:0.5, 1:1, 1:3, and 1:10 at two time points (3 and 24 h; Figure 3A). Interestingly, the relative abundance of the adduct at a 1:10 ratio remained consistent with that observed at 1:3, suggesting a saturation point at higher ratios (Figure 3B).

The reaction of RNase A with complex **2** yielded more intricate results; specifically, the number of bound Pd ions was modulated by time and concentration, as highlighted by the evolution of adduct signal intensities (see Supporting Information, Figures S1–S3).

X-ray Diffraction Studies

To characterize the above adducts from the structural point of view, crystallization experiments were carried out. Indeed, we

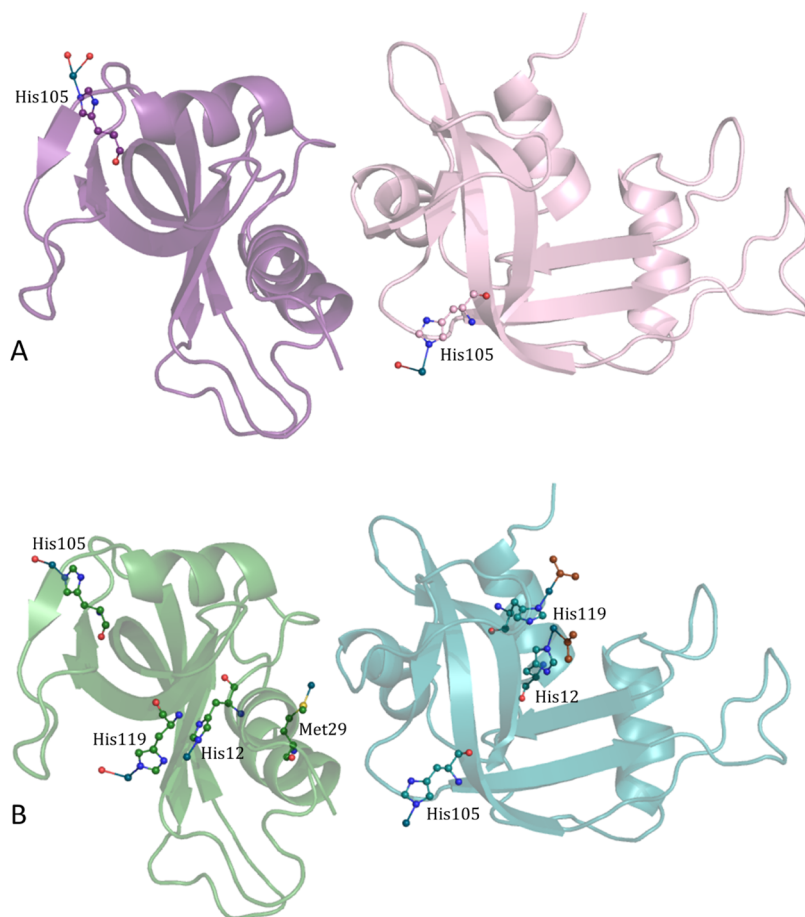


Figure 4. Cartoon representation of the overall structure of the adducts formed by RNase A with Pd complexes **1** (A) and **2** (B). Molecules A of the a.u. are colored in purple in (A) and in forest green in (B). Molecules B of the a.u. are colored in light pink in (A) and in deep teal in (B). In both structures, Pd binding sites are highlighted and shown as bond and stick.

were successful in obtaining crystals for protein adducts with both organopalladium compounds by soaking metal-free RNase A crystals in a 1.0 μ L drop containing the crystallization solution (22% PEG 4K and 10 mM sodium citrate buffer pH 5.1) saturated with Pd complex powder. After 7 days of incubation, crystals were fished, cryo-protected in a solution containing 70% reservoir and 30% glycerol for a few seconds, and analyzed for X-ray diffraction. The crystals belong to the space group C2, contain two protein molecules in the asymmetric unit (a.u.) (molecules A and B hereafter, **Figure 4**), and diffract X-rays at 1.99 and 2.72 Å resolution for the adducts with complexes **1** and **2**, respectively.

The structures were refined to R-factor/ R_{free} of 0.211/0.234 and 0.216/0.258, respectively. The overall structures of RNase A in both adducts with Pd complexes are very similar to that of the metal-free protein; the root-mean-square deviation of carbon α atoms (r.m.s.d.) between the structure of each Pd/RNase A adduct and that of RNase A used as the starting model (PDB code 1JVT³⁸) is within the range 0.26–0.40 Å.

Analysis of Fourier difference electron density (e.d.) maps of the adduct formed by RNase A with complex **1** showed that this Pd complex has limited reactivity with the protein. Indeed, just one naked Pd ion with 0.50 occupancy binds the protein, close to the highly exposed side chains of His105 of both molecules of the a.u. (**Figures 4A** and **5**). The e.d. maps close to these binding sites are not well-defined; thus, we cannot model Pd ligands to complete the metal coordination sphere. This is

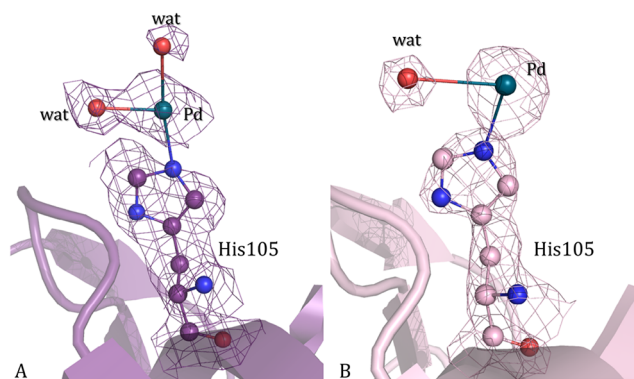


Figure 5. Pd binding sites in molecules A (purple) and B (light pink) of the adduct of RNase A with complex **1**. Pd binds close to the side chain of His105 of both molecules of the a.u. 2Fo-Fc e.d. maps are contoured at 1.0 σ level (purple and light pink).

not surprising, considering the partial occupancy and local flexibility of these Pd-containing fragments. The e.d. map close to the metal suggested to us that Pd is bound to some ligands, which, for simplicity, have been interpreted as water molecules. This e.d. could correspond to a part of the original ligands that are disordered in the crystal.

Inspection of Fourier difference e.d. maps of the adduct formed by RNase A with complex **2** showed a higher reactivity of the Pd compound toward the protein (**Figure 6**). Naked Pd

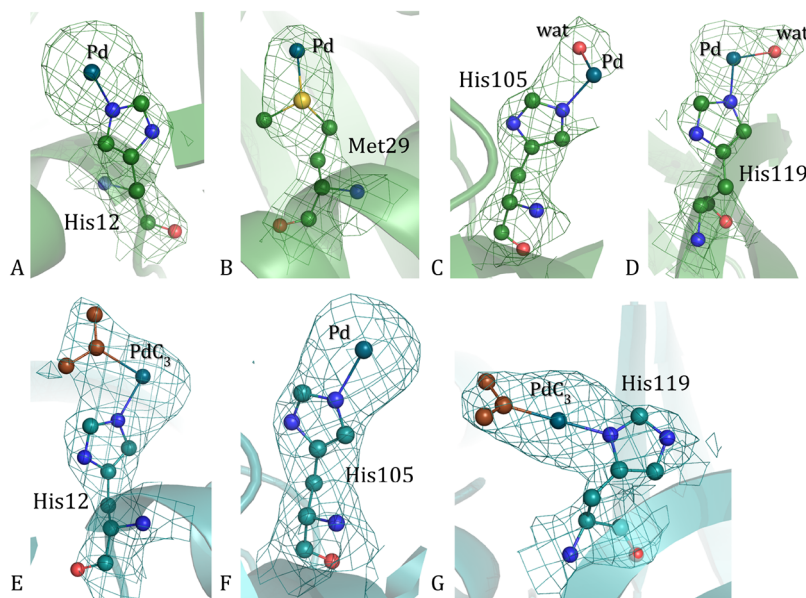


Figure 6. Pd binding sites in molecules A (forest green) and B (deep teal) of the adduct of RNase A with complex 2. Pd binds close to the side chains of His12 (A), Met29 (B), His105 (C), and His119 (D) of molecule A, and close to the side chains of His12 (E), His105 (F), and His119 (G) of molecule B. 2Fo-Fc e.d. maps are contoured at 1.0 σ level (forest green and deep teal in (A–D) and (E–G), respectively).

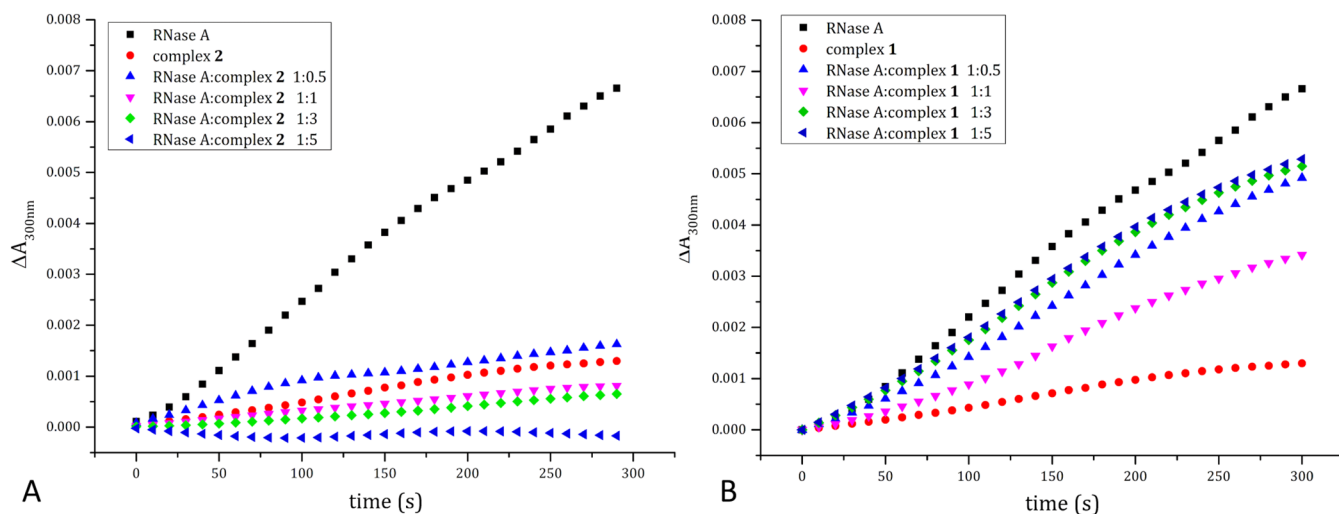


Figure 7. Hydrolysis of yeast RNA in 50 mM sodium acetate buffer at pH 5.0, according to the Kunitz method.³⁹ The variation of absorbance at 300 nm was followed as a function of time upon addition of the protein (alone or after incubation with the Pd complex) to the RNA. Four different protein-to-Pd complex molar ratios were tested: 1:0.5, 1:1, 1:3, and 1:5. RNase A was incubated in the presence of complex 2 (A) and complex 1 (B) for 24 h. Protein concentration was 10 μ M. Experiments were performed in triplicate, and curves were obtained by averaging the three measurements.

ions, with partial occupancies (ranging from 0.25 to 0.70), are found close to the side chains of His12, His105, and His119 of both molecules of the a.u. and close to the side chain of Met29 of molecule A (Figure 6A–G). Even in the case of this structure, the e.d. maps are not well-defined close to the metal center and do not allow us to complete modeling of the Pd coordination sphere. Water molecules have been added to the metal center bound to the side chains of His105 and His119 of molecule A (Figure 6C,D). A slightly larger e.d. was observed close to the Pd center bound to the side chains of His12 and His119 of molecule B. Here, the map has been interpreted with the presence of a Pd-allyl fragment (Figure 6E,G). This interpretation agrees with ESI MS data, which showed the

presence of Pd-allyl fragments in the adduct that RNase A forms with complex 2.

Ribonuclease Activity Assays

Since crystallographic results have indicated that complex 2-derived fragments can bind RNase A close to the side chains of His12 and His119, which are involved in the RNA recognition by the protein,³⁶ it is reasonable to expect that RNase A could lose its ability to degrade nucleic acids in the presence of this Pd complex. To verify this hypothesis, the catalytic activity of the adduct formed upon reaction of complex 2 with RNase A has been investigated using the Kunitz assay.³⁹ Briefly, the assay measures the variation in yeast RNA absorbance at 300 nm over time, which occurs upon RNA hydrolysis following the addition of a defined amount of RNase A.

The experimental data reported in Figure 7A reveal that the presence of complex 2 alters the ribonuclease activity of the protein and that the loss of activity is dependent on Pd concentration. A comparable behavior was already observed in the adducts formed upon reaction of RNase A with V,^{40,41} Ru,⁴² Pd,³⁵ Au,³⁰ and Pt-based drugs.⁴³

RNase A catalytic activity was also assessed in the presence of complex 1, although fragments of this compound do not bind to the protein active site. This represents a convincing negative control for the experiments. As expected, the protein retains significant enzymatic activity even in the presence of high concentrations of complex 1 (Figure 7B).

CONCLUSIONS

Organopalladium compounds are a promising class of metal-based compounds with significant medicinal interest and potential. The reactivity of the palladium center can be finely and favorably tuned through the selection of appropriate ligands, and this can be exploited for pharmaceutical purposes. Although they are prodrugs, organopalladium derivatives exhibit a distinct mode of action from platinum compounds.¹ They tend to interact weakly with DNA but strongly with proteins. In light of these findings, it is becoming increasingly important to describe the interaction between organopalladium compounds and target proteins at the molecular level. We have therefore characterized the reactions of organopalladium compounds of medicinal interest with RNase A, using an investigative protocol based on combined ESI MS and XRD approaches that we recently described.^{24–27} These two techniques provide complementary information, since ESI MS can establish the type of adduct formed and the number of metal-containing fragments bound to the protein, while XRD can provide the exact location of metal-binding sites. This combined approach allows us to properly define the protein metalation processes at a molecular level.

ESI-MS studies revealed that both organopalladium compounds (complexes 1 and 2) were able to react with the protein to form stable adducts through coordination of Pd-containing fragments to protein side chains. However, the reactivity of the two complexes toward the protein differs significantly: complex 1 produces a single adduct with high selectivity, whereas complex 2 forms a variety of adducts with different metal-containing fragments bound to the protein at multiple sites. We then successfully determined the crystal structures of the adducts. The crystallographic data indicate that the primary binding site for complex 1 is a histidine side chain on the protein surface. In contrast, complex 2 can metalate the protein at different sites, including the catalytically important residues His12 and His119. Notably, ESI-MS and XRD, despite their different natures, provide a comprehensive description of the RNase A metalation process by these organopalladium complexes. We believe that the observed protein metalation patterns may have more general relevance.

Beyond its immediate significance to palladium drug design, this work also has broader implications for bio-organometallic chemistry. Previous studies on palladium complexes—most notably the [PdCl(allyl)]₂ dimer, whose intrinsic antitumor activity is negligible—have shown that large protein scaffolds such as ferritin can be used to construct artificial or hybrid metalloenzymes based on Pd–C chemistry.⁴⁴ Building on this framework, the well-defined Pd–protein adducts described here extend this paradigm into a biologically active context by embedding organopalladium reactivity within structured, chiral

protein environments that are functionally engaged in cellular processes, at the interface of medicinal chemistry, structural biology and catalysis.

EXPERIMENTAL SECTION

Preparation of Complexes 1 and 2

Complexes 1 and 2 were available in our laboratories, having been previously synthesized by our group according to the procedures reported in the literature.^{9,15} The biochemical studies described herein were performed using the same batches employed in the original synthetic studies. Their preparation, isolation in high yields, and comprehensive analytical characterization have been described in detail in those reports. The characterization included clean NMR spectra and elemental analyses in excellent agreement with the calculated values, confirming the high purity of the compounds.

ESI MS Interaction Experiments with RNase A

Adducts Formation. Adducts were prepared by incubating the protein directly with the different metallodrugs at a 1:3 protein-to-metal ratio. The mixtures were incubated in ultrapure water for 24 h at 37 °C.

Time-Course Experiments. Adducts were prepared by incubating the protein directly with the different metallodrugs at a 1:3 protein-to-metal ratio. The mixtures were incubated in ultrapure water for 5 min, 1 h, 3 h, 8 h, 12 h, and 24 h at 37 °C.

Titration Experiments. Adducts were prepared by incubating the protein directly with different ratios of metallodrugs (1:0.5, 1:1, 1:3, or 1:10 protein-to-metal ratio). The mixtures were incubated in ultrapure water for 3 and 24 h at 37 °C.

The protein solutions were further diluted with water to a final protein concentration of 5×10^{-7} M. Just before analysis, 0.1% (v/v) LC-MS grade formic acid was added. The ESI mass spectra were acquired through direct injection at a flow rate of 7 μ L/min in a TripleTOF 5600+ high-resolution mass spectrometer (Sciex, Framingham, MA, U.S.A.), equipped with a DuoSpray interface operating with an ESI probe. The ESI source parameters were set as follows for all samples. Positive polarity, Ion Spray Voltage Floating 5500 V, Temperature 0, Ion source Gas 1 (GS1) 40 L/min; Ion source Gas 2 (GS2) 0 L/min; Curtain Gas (CUR) 15 L/min, Declustering Potential (DP) 100 V, Collision Energy (CE) 10 V, acquisition range 1000–2600 *m/z*.

X-ray Crystallography

RNase A was crystallized by the hanging drop vapor diffusion method in 22% PEG 4K, 10 mM sodium citrate buffer pH 5.1, using a protein concentration of 20 mg mL^{−1} (1.4 mM). Crystals of metal-free RNase A were soaked for 1 week in a drop containing the crystallization condition saturated with the powder of the Pd complexes 1 or 2 to form the crystals of the respective adducts. After 7 days, the soaked protein crystals were cryo-protected in a solution containing the mother liquor and 30% glycerol and then flash-cooled in liquid nitrogen for X-ray diffraction data collection. Data collection was performed remotely on the ID23–2 beamline of the European Synchrotron of Grenoble (ESRF), France, using a wavelength of 0.87 Å. Crystals were maintained at 100 K during the data collection. Data processing and scaling were performed using Global Phasing autoPROC pipelines.⁴⁵ Data collection statistics are reported in Table S1. The structures were solved by molecular replacement using Phaser,⁴⁶ and the structure of RNase A was deposited in the PDB under the accession code 1JVT³⁸ as a starting model. Model building was carried out with Coot.⁴⁷ The structures were isotropically refined with REFMAC5⁴⁸ using the CCP4 suite.⁴⁹ Refinement statistics are reported in Table S1. Both structures were validated on the PDB validation server and then deposited in the PDB under the accession codes 30UF and 30UG, respectively.

Ribonucleolytic Activity Assays

RNase A catalytic activity was qualitatively measured according to the Kunitz method³⁹ by monitoring cleavage of yeast RNA via UV–vis absorption spectroscopy in the absence and presence of the

organopalladium complexes in 0.050 M sodium acetate at pH 5.0 at room temperature. The Pd–protein adducts were obtained by incubating RNase A with complexes **1** and **2** for 24 h using 1:0.5, 1:1, 1:3, and 1:5 protein-to-metal molar ratios. The experiments were carried out in triplicate with an RNA concentration of 0.5 mg mL^{−1} and an RNase A concentration of 10 μM.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.inorgchem.6c03237>.

ESI-MS titration of the RNase A-complex **2** adduct at 3 h (Figure S1); ESI-MS titration of the RNase A-complex **2** adduct at 24 h (Figure S2); ESI-MS time-course analysis of RNase A incubated with complex **2** for 24 h (Figure S3); and data collection and refinement statistics (Table S1) (PDF)

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Notes

The authors declare no competing financial interest.

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