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Communication

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Investigating the Ruthenium Metalation of Proteins: X-ray Structure and Raman Microspectroscopy of the Complex between RNase A and AziRu

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Supporting Information

ABSTRACT: A Raman-assisted crystallographic study on the adduct between AziRu, a Ru(III) complex with high antiproliferative activity, and RNase A is presented. The protein structure is not perturbed significantly by the Ru label. The metal coordinates to ND atoms of His105 or of His119 imidazole rings, losing all of its original ligands but retaining octahedral, although distorted, coordination geometry. The AziRu binding inactivates the enzyme, suggesting that its antitumor action can be exerted by a mechanism of competitive inhibition.

A number of Ru(II) and -(III) coordination compounds are considered potential anticancer agents because of their relevant antiproliferative activity.[1,2] Although the therapeutic properties of these complexes have been largely recognized, their mechanisms of action are still far from being understood. Current hypotheses comprise cellular uptake, extracellular aquation, hydrolysis, and protein target binding.[3,4] It has been proven that when Ru-based anticancer compounds are administered intravenously, most of the ruthenium in blood plasma is accumulated in a protein-bound form (>97%),[3,4] but the nature and amount of the adducts formed are currently unknown. Thus, in order to understand the mechanism of action of these compounds, it is crucial to identify the way by which the metal interacts with proteins, including what chemical motifs are able to bind its complexes, contributing to their specificity and affinity.[3−5] Our knowledge at the molecular level of the binding properties to proteins of Ru-based compounds is scarce: interactions between Ru-containing drugs and proteins have not been systematically studied, and only 16 structures of protein−Ru adducts have been deposited in the protein data bank (Table S1 in the Supporting Information, SI). Important information about Ru−protein interactions have been obtained by solving (a) the X-ray structure of the complexes between NAMI-A (Figure 1A), a Ru(III) complex in phase II clinical trials,[6,7] and carbonic anhydrase,[8] and (b) the structure of three Ru complexes containing imidazolium or indazolium ligands with lactoferrin.[9] Additional information obtained from X-ray absorption spectroscopy (XAS) studies have provided strong evidence that the antimetastatic activity of NAMI-A arises from its serum albumin adducts, despite the fact that such binding completely changes

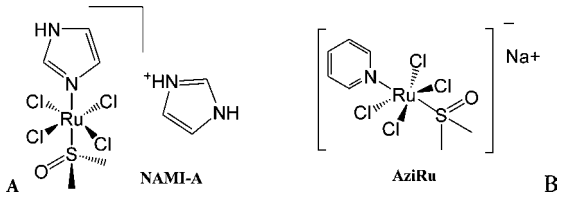

Figure 1. Structures of (A) NAMI-A and (B) AziRu.

the coordination environment of Ru(III).[10,11] XAS data also suggest the presence of Ru(III/IV) clusters and binding of Ru(III) to sulfur-donor, amine, and carboxylato groups of proteins.[11] Analogously, we recently analyzed the metalation of hen egg white lysozyme (HEWL) with AziRu, a NAMI-A analogue where a pyridine (Py) ligand replaces the imidazole of NAMI-A (Figure 1B).[12]

AziRu is among the most promising Ru complexes with antiproliferative properties currently described in the literature.[13,14] The structure of HEWL−AziRu showed that Ru coordinates to His15 and Asp87, losing all its original ligands,[12] and that the last ligand to be exchanged is a Cl^- in the equatorial plane. In the solution used to grow HEWL crystals, AziRu is able to form polyoxo species containing Ru−O−Ru bonds.[12] To gain further insight into the molecular interactions of Ru-containing drugs with proteins and particularly into the AziRu mechanism of action, we have carried out a Raman-assisted crystallographic study on the complex between AziRu and bovine pancreatic ribonuclease (RNase A). RNase A is a small protein (124 residues) that has already been used as a model system to characterize the interactions of metals with proteins.[15] RNase A crystallizes under a range of conditions,[16] one of which is suitable for soaking experiments.[17,18]

Initially, we evaluated the effect of the binding of AziRu on the RNase A catalytic activity, according to the classical enzymatic assay described in the SI. Interestingly, after 24 h of incubation at 25 °C, AziRu almost completely inhibits RNase A (Figure S1 in the SI). Subsequently, crystals of Ru-metalated RNase A suitable for X-ray diffraction studies (PDB 4L55) were obtained via a soaking procedure on protein crystals grown by hanging-drop

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vapor diffusion by mixing 1 μL of a 20 mg/mL RNase A solution (unbuffered) with an equal volume of a reservoir solution containing 20% w/v PEG4K and 50 mM sodium citrate (pH 5.5). After soaking in saturated solutions of AziRu, colorless monoclinic crystals change their color to dark yellow/brown (Figure S2 in the SI). The ruthenated RNase A crystals diffract to 1.65 Å resolution (Table S2 in the SI). The structure is refined up to *R*-factor/*R*-free values of 0.203/0.245. Details on the structure solution, refinement procedure, and statistics are reported in the SI. The analyzed crystal contains two crystallographically independent molecules (molecules A and B; Figure S3 in the SI). The crystal structure shows that the ruthenium label does not significantly perturb the RNase A structure. The Cα root-mean-square deviation of refined models from that of the ligand-free RNase A structure is within the range 0.39−0.60 Å. AziRu binds to two distinct sites in the two molecules with a similar distorted octahedral geometry (Figure 2): in molecule A, Ru

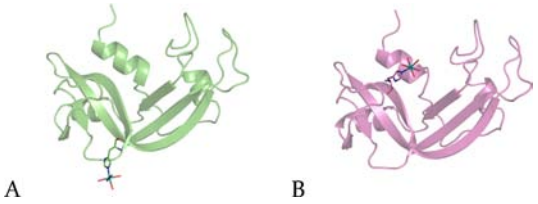

Figure 2. Ribbon representation of the two molecules in the asymmetric unit isoriented to highlight the different AziRu binding sites.

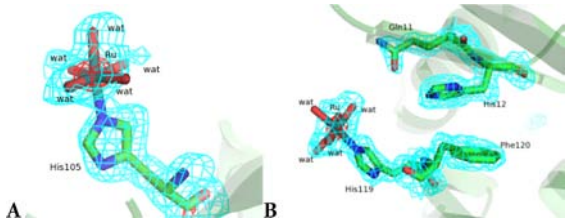

Figure 3. $2F_o - F_c$ electron density maps contoured at the 1σ level (cyan) and the 4σ level (red) showing the Ru ions bound to RNase A. The red peak shows the electron-rich Ru: (A) close to His105; (B) in the active site.

coordinates at the imidazole nitrogen of His105 (Figure 3A); in molecule B, the Ru complex is found at the active site residue His119 (Figure 3B). The presence of Ru has been confirmed by inspection of anomalous electron density maps (Figure S4 in the SI). The binding of AziRu to His119 well explains inhibition of the catalytic activity of the enzyme: the Ru complex binds to the active site and partially occupies the phosphate binding site P1, which is critical for recognition of RNA. The mechanism of RNase A inhibition by AziRu is schematically represented in Figure S5.

His119 can adopt two different conformations, A ($\chi_1 = 160°$) and B ($\chi_1 = 80°$), which interconvert by a 100° rotation around the Cα−Cβ bond and a 180° rotation around the Cβ−Cγ bond.[19] At the Ru binding site, His119 adopts the A conformation.

Although in both Ru binding sites the electron density maps are not excellent (Figure 3), it clearly emerges that the Ru octahedral coordination sphere is completed by small ligands, like water molecules. While phasing crystal structures using heavy-atom derivatives, crystallographers often observe that ligands of the original metal complex are dissociated and that the heavy metal is bound to side chains, with solvent molecules completing its coordination sphere. In the case of the complex RNase A−AziRu, in both sites, the Ru−N distance for the coordinated side chains is close to 2.0 Å, i.e., in line with the values observed in the structures of other protein−ruthenium derivative complexes, which are, on average, equal to 2.1 Å, and lower than those in the adducts lactoferrin−NAMI-A and HEWL−AziRu,[12] where photoreduction to ruthenium(II) could have occurred during X-ray exposure.[20] Among the five (Cs^+, Mg^{2+}, Cu^{2+}, Zn^{2+}, and Ni^{2+}) metal-containing RNase A structures deposited in the PDB, one includes a metal bound to the protein through His105 and His119 (PDB code 1AQP).[15] In this structure, Cu^{2+} is coordinated to the Nε2 atom of His105, to the carbonyl oxygen of Tyr76, and to the nitrogen and oxygen atoms of Glu2 and nitrogen atom of Lys1 of a symmetry-related molecule in one case and to Nε2 of His119, Oε2 of Glu111, and three water molecules in the other. In the latter, His119 adopts the B conformation (Figure S6 in the SI).

A comparison of ruthenium coordination in the HEWL−AziRu and RNase A−AziRu structures reveals its inherent propensity to lose ligands, which might be biologically important in allowing the metal ion to bind proteins and to explicate its biological activity, and definitively demonstrates a preference of this compound to bind His residues. In this respect, it should be noted that, although RNase A possesses four solvent-exposed His (thus eight His in the asymmetric unit), only one Ru−His adduct per molecule forms. This finding suggests that the binding of one ruthenium complex could disfavor the formation of successive adducts. Validation of the RNase A−Ru ligation in the crystal state was performed by collecting Raman spectra of the adduct (Figure 4) using a setup described elsewhere.[21]

The amide I protein Raman band at 1668 cm^{-1} is not significantly affected by the presence of AziRu, consistent with the absence of modifications in the RNase A secondary structure upon binding. In contrast, the Raman bands related to the ruthenium complex in the adduct are significantly changed,

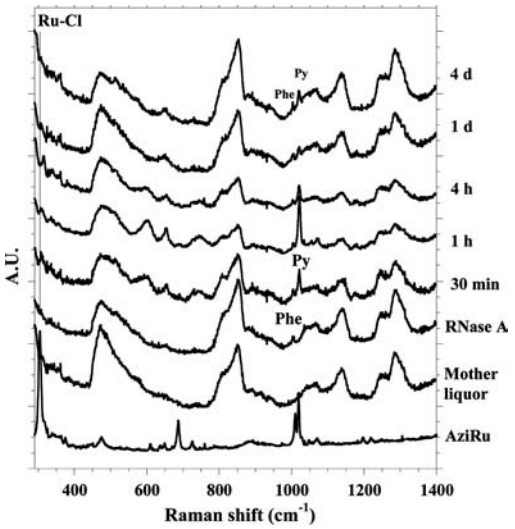

Figure 4. Raman spectra of a RNase A crystal immersed in a 10 mM solution of AziRu for different soaking times (see the SI). Raman spectra of AziRu powder and of mother liquor are reported as references. The vertical line represents the Ru−Cl frequency that is observed only in the AziRu powder. The absence of Ru−Cl bands in the crystals upon soaking indicates a fast exchange of Cl^- ligands.

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confirming the loss of ruthenium ligands in the adduct. Raman spectra of RNase A crystals registered as a function of time upon AziRu soaking suggest that the complex has a faster exchange of Cl^- with water compared to the HEWL−AziRu adduct.[12] This different behavior is in line with kinetic solution studies of Cl^-/water exchange as a function of pH, which reveal that the ruthenium(III) complex has first-order kinetics of ligand exchange at low pH (pH 4.5−5.5), with $t_{1/2}$ of about 65 h in a 10 mM sodium acetate buffer at pH 4.5 and $t_{1/2}$ of just a few hours in a 10 mM sodium citrate buffer at pH 5.5. These findings also agree with previous data collected on NAMI-A.[22] The strong Py signal at 1021 cm^{-1} increases in the first hours and then decreases, suggesting that the whole AziRu enters into the crystal and then binds to the protein, replacing the Py axial position (Figure 4). An evident difference between the formation of HEWL−AziRu and RNase A−AziRu complexes regards the absence of the Ru−O−Ru bands in Raman spectra of RNase A−AziRu crystals. These results agree with the observation that RNase A crystals do not turn color from dark yellow/brown to black, as observed in the case of HEWL.[12] It is possible that, under the experimental conditions used to grow RNase A crystals, the formation of Ru−O−Ru is disfavored. It is plausible that the in vivo formation of polymeric oxo species could be disfavored by the large amount of proteins, like serum albumin and transferrin, that are rich of surface His. In conclusion, a comparison between the results of this work and those previuosly published on the complex between HEWL and AziRu provides conclusive evidence to interpret our results and the mechanism of action of this molecule. The combined use of Raman microscopy and X-ray crystallography provides the advantage of obtaining complementary information and a unique power for elucidating intricate processes such as X-ray-induced damage[23,24] and the formation of protein−metal adducts.[12] Overall, the adopted approach has provided detailed insight into the formation of a AziRu−protein derivative and has suggested a mechanism of enzyme inhibition by Ru binding. The binding does not significantly alter the RNase A structure but inactivates the enzyme. AziRu undergoes dramatic changes in the coordination environment of Ru and is aquated before the covalent binding to His residues of the target protein. At acidic pH and in the presence of a high concentration of Cl^- (i.e., in the conditions used to study the HEWL−AziRu adduct), Cl^-/aqua exchange is slower, and this allows binding of Ru with a Cl^- ligand as the survivor.[12] At pH 5.5 (i.e., in the conditions used in this work), the exchange is so fast that the aquo/hydroxo species is the only one that interacts with the protein. In the formation of the adduct, His side chains are involved. It is likely that, at physiological pH, aquo/hydroxo Ru species are the reactive ones. The activity of many enzymes could be inhibited by the binding of AziRu to the active site. Thus, the compound may interfere with normal function within the tumor microenvironment by acting as a competitive inhibitor of an unknown target. Regarding the formation of possible polynuclear Ru clusters containing Ru−O−Ru bonds, our results suggest that this process critically depends on the solution conditions used. Future studies will be addressed to understand the role, if any, of the oligomers in determining the AziRu biological activity and to identify the possible enzymatic target.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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