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Interaction of Anticancer Ruthenium Compounds with Proteins: High-Resolution X-ray Structures and Raman Microscopy Studies of the Adduct between Hen Egg White Lysozyme and AziRu

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Supporting Information

ABSTRACT: The binding properties of AziRu, a ruthenium(III) complex with high antiproliferative activity, toward a hen egg white lysozyme have been investigated by X-ray crystallography and Raman microscopy. The data provide clear evidence on the mechanism of AziRu–protein adduct formation and of ligand exchange in the crystal state.

The metal-based anticancer agents most commonly used in chemotherapy are platinum compounds like cisplatin, carboplatin, and oxaliplatin.[1−3] However, these platinum complexes show high toxicity and are inactive against some tumors.[4,5] Among the other metal compounds, ruthenium(II/III) complexes have been proposed as efficient alternatives to platinum.[6−9] Among the ruthenium(III)-based drugs, two of them, namely, NAMI-A and KP1019 [Supporting Information (SI), Figure S1], are currently in phase II clinical trials.[10−13] During the past few years, a growing number of studies have been carried out to elucidate the mechanism of action of NAMI-A.[14−16] In particular, it has been shown that this complex is susceptible to various chemical transformations, such as reduction of Ru^{III} to Ru^{II}, exchange of ligands, and formation of polyoxo species,[14] and that it binds to serum albumin and transferrin, suggesting that binding to specific proteins may represent the molecular basis for its biological activity. In the case of NAMI-A, the serum albumin adduct has been established to be the active form.[17] Very interesting data have been obtained examining the crystal structures of the complex between lactoferrin and NAMI-A[18] and of the carbonic anhydrase–NAMI-A adduct.[19] In particular, the latter complex, which is the sole protein–NAMI-A adduct deposited in the Protein Data Bank (PDB), revealed that the Ru atom coordinates to ND of His64 imidazole, losing all of its original ligands but retaining a highly distorted octahedral arrangement completed by H_2O molecules and by carbonyl O atoms of Asn62 and backbone N atoms of His64.[19]

Recently, some of us reported the synthesis and preliminary biological evaluation of a new NAMI-A analogue, called AziRu. In AziRu, a pyridine ligand replaces the imidazole of NAMI-A (SI, Figure S1).[20,21] This compound is one of the most promising ruthenium complexes in terms of anticancer activity currently known, being more cytotoxic than NAMI-A and showing high in vitro antiproliferative activity.[20,21]

In this Communication, the interaction of AziRu with hen egg white lysozyme (HEWL) has been characterized by X-ray crystallography and Raman microscopy. HEWL is a single-chain protein of 129 amino acid residues (MW 14 kDa) proven to be a particularly well-suited scaffold to investigate the fundamental interactions of proteins with metal compounds.[22] HEWL has been used as a model system because it has been shown to bind NAMI-A (in a 1:0.5 ratio),[23] has a well-characterized X-ray structure,[24] and is easy to crystallize, forming crystals (resistant to soaking) in high Cl^- concentration, which should retard the ligand-exchange process of the ruthenium complex. HEWL–AziRu crystals were obtained by both soaking and cocrystallization procedures. In the first approach, tetragonal HEWL crystals, prepared as described in the SI, were soaked in a 5 μL harvesting solution containing a large AziRu excess. During the soaking process, HEWL crystals turned color from transparent to yellow, light green, green, and then black (SI, Figure S2). In the second approach, the protein and ligand were mixed in a 1:1 molar ratio, and then crystals were grown (see the SI for details). The ruthenated HEWL crystals were removed from their mother liquors in a cryoloop and frozen in liquid N_2 with glycerol as a cryoprotectant, and X-ray diffraction data were collected. Here we report the structures of the HEWL–AziRu complex refined using data collected on green and black crystals (SI, Figure S2) and on crystals of the adduct obtained by cocrystallization. Data collection and refinement statistics are reported in the SI, Table S1. The structures have been deposited in the PDB with codes 4J1A and 4J1B. The final models include one HEWL molecule and one AziRu molecule with different occupancies and different ligands. In all cases, the structure of HEWL (Figure 1) is essentially the same as that of the native protein, although various differences were observed in the conformation of some residues when the structures were compared. The Cα root-mean-square deviation of models of the complexes from the native HEWL structure is about 0.20 Å. Local changes induced by adduct formation and a comparison with other metalated

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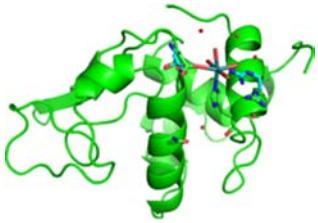

Figure 1. Overall structure of the HEWL–AziRu adduct showing the binding site of ruthenium and its ligands.

HEWL are described in the SI. The electron density map shows that AziRu is bound to the protein, forming an adduct with His15 and Asp87 (Figure 2). There are no other structures of

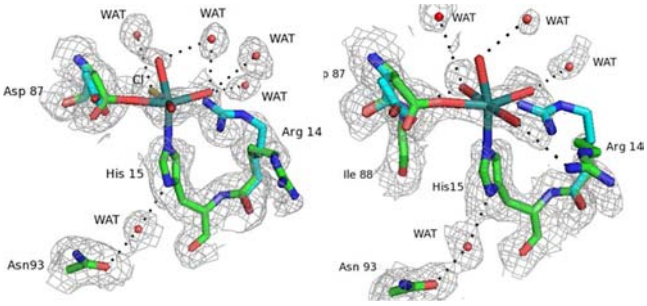

Figure 2. $2F_oF_c$ electron density map contoured at the 1σ level showing the Ru ion bound to HEWL in green (left) and black (right) crystals. Details of the Ru coordination sphere have been shown with O atoms of the aqua ligands directly bound to the ion and Arg14, His15, and Asp87 side chains. Alternative conformations of Arg14 and Asp87 side chains are also reported and colored in cyan. In green crystals, Ru with 0.3 occupancy adopts a distorted octahedral geometry with the NE2 of the His15 side chain, the OD1 of the Asp87, a Cl atom, and three aqua ligands. In black crystals, the occupancy of the same site is 0.5, in excellent agreement with mass spectrometry studies conducted on the HEWL–NAMI-A adduct,[23] and the remaining Cl atom is replaced by a fourth H_2O molecule. The distorted octahedral geometry observed in the crystals has also been confirmed by electron paramagnetic resonance studies performed at room temperature (SI, Figure S3).

protein–ruthenium(III) complexes in the PDB with the metal bound to these two residue side chains at the same time, but this is not surprising because only a very limited number of ruthenium compounds bound to proteins have been described.

The Ru–O and Ru–N distances for the coordinated side chains are close to 2.5 Å, values larger than those previously observed for the structures of other protein–ruthenium derivative complexes that are, on average, equal to 2.1 Å and in the X-ray structures of NAMI-A variants reported in the Crystallography Open Database (codes 4311154 and 4311201),[25] but exactly equal to those found in the adduct between NAMI-A and lactoferrin.[18] The average errors on the bond length calculated using the Cruickshank method are close to 0.2 Å. It should also be mentioned that it is possible that at least some photoreduction to Ru^{II} could happen during the X-ray diffraction data collection, which could increase the bond lengths.[26] The maps clearly indicate the presence of one Cl atom in the green crystals and its absence in black crystals. The presence/absence of chlorine is supported by the Bijovoet difference maps and Raman data (see below); the anomalous maps show the density for the sulfur sites of both methionines and cysteines and allow identification of the position of Cl^- ions already identified in other HEWL structures deposited in the PDB but distant from the Ru atom. Results similar to those described for the black crystals have been obtained in the case of the adduct formed by cocrystallization.

Our crystallographic results are in excellent agreement with the data obtained by Raman microscopy, which has been used to monitor the ligand-exchange process for AziRu in HEWL crystals. Raman spectra during AziRu soaking into HEWL crystals (Figure 3) have been collected using a setup described elsewhere[27] and a 645 nm line (see the SI for details). Raman spectra of AziRu powder have been collected as references as well.

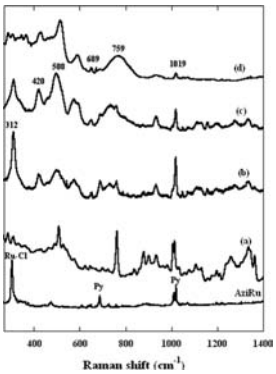

Figure 3. Raman spectra (from 250 to 1350 cm^{-1}) of a HEWL crystal (a) immersed in a saturated solution of AziRu for different times: (b) yellow crystal (a few minutes); (c) green crystal (a few hours); (d) black crystal (7 days). The Raman spectrum of AziRu powder is reported for comparison. Excitation line 645 nm (2 mW at the sample), spectral resolution 4 cm^{-1}, exposure 100 s.

Raman bands related to the protein are not significantly affected by the AziRu soaking. Particularly, amide I and III frequencies are unaffected by the presence of AziRu, consistently with the absence of modifications in the HEWL secondary structure. In contrast, the Raman bands related to the ruthenium complex significantly change. After a few minutes, yellow crystals exhibit Raman spectra (trace b) that are the sum of isolated AziRu and HEWL bands, indicating the entry of the AziRu molecules into the crystal matrix. In a few hours, the exchange of Cl^- with H_2O is evident. This process can be followed from the decrease in the Ru–Cl band at 312 cm^{-1} [28] and the appearance of Ru–H_2O related broad doublets at 420 and 508 cm^{-1} [29] in the green HEWL crystals (trace c), consistent with the aquation process reported for NAMI-A.[14] An increasing and persistent broad band around 759 cm^{-1} indicates Ru–O–Ru formation,[30] dominant in black crystals (trace d). This band has also been observed in a control experiment onto AziRu alone in the same mother liquor, without HEWL crystals (data not shown). The presence of Ru–O–Ru bonds is consistent with recent X-ray absorption spectroscopic data collected on other ruthenium-containing prodrugs and with the finding that crystals change their color to black.[31] Because the Ru–O–Ru oligomers are not observed in the black HEWL crystal structure, polyoxo species might not be well-structured or just adsorbed on the protein crystal. The Ru–N stretching, not observed because it is buried under other bands, should be a shoulder around 300 cm^{-1} [32] for AziRu and around 270 cm^{-1} for the Ru-His adduct (as in NAMI-A[28]). The Ru–S stretching, expected around 1134 cm^{-1},[28] was not observed, suggesting a fast replacement of dimethyl sulfoxide by

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H_2O, in agreement with the crystallographic structures where this ligand is not present.

In conclusion, the combined use of Raman spectroscopy and protein crystallography has provided complementary information that a single technique alone would not produce. For instance, Raman spectroscopy shows not only the ligand exchange that is apparent in the X-ray structures but also the presence of Ru−O−Ru bonds, which are not revealed by crystallography. AziRu is rather labile in aqueous media following a reactivity pattern similar to that previously observed for the therapeutically active NAMI-A.[14] Incubation of AziRu with crystals of HEWL results in the metalation of one site, close to His15 and Asp87 side chains, and in the formation of an adduct where the complex binds to the protein, losing its ligands. The observation that His residues are the main determinants of the ruthenium binding sites of AziRu with HEWL and of NAMI-A with both lactoferrin[18] and carbonic anhydrase[19] allows one to speculate that the same should be true for other proteins, like human serum transferrin and albumin. In both human serum transferrin and albumin, there are numerous exposed His residues that could potentially offer binding sites for ruthenium complexes; furthermore, in human serum transferrin, two His residues are involved in the iron binding site, and in human serum albumin, His residues participate in the recognition of several metals.[32,33] Most remarkably, this "specific" site is solvent-exposed and can still be occupied by a hypothetical AziRu derivative that lacks only one axial ligand. The lability of the ligands, especially the chlorido ligands, in these metal complexes might be biologically important in allowing metal−protein binding. Our data are in very good agreement with the studies of Messori et al. on NAMI-A/HEWL[23] and NAMI-A/carbonic anhydrase,[19] suggesting that NAMI-A and AziRu behave as "extreme prodrugs", whose ultimate function is to provide Ru^{III} ions to its final target after a sequential loss of ligands. Thus, as suggested also by Levina et al.,[34] a modulation of ligand exchange can be important to enhance the cytotoxicity of these compounds, which seems to be related to their peculiar chemical and physical properties, determining their in vivo vehiculation. The comprehension of these effects may significantly contribute to the design of new, more effective ruthenium derivatives.

■ 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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