Reactions of Arsenoplatin-1 with Protein Targets: A Combined Experimental and Theoretical Study

Iogann Tolbatov, Damiano Cirri, Matteo Tarchi, Tiziano Marzo, Cecilia Coletti, Alessandro Marrone, Luigi Messori, * Nazzareno Re, * and Lara Massai

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ABSTRACT: Arsenoplatin-1 (AP-1) is a dual-action anticancer metallodrug with a promising pharmacological profile that features the simultaneous presence of a cisplatin-like center and an arsenite center. We investigated its interactions with proteins through a joint experimental and theoretical approach. The reactivity of AP-1 with a variety of proteins, including carbonic anhydrase (CA), superoxide dismutase (SOD), myoglobin (Mb), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and human serum albumin (HSA), was analyzed by means of electrospray ionization mass spectrometry (ESI MS) measurements. In accordance with previous observations, ESI MS experiments revealed that the obtained metallodrug−protein adducts originated from the binding of the [AP-1-Cl]+ fragment to accessible protein residues. Remarkably, in two cases, i.e., Mb and GAPDH, the formation of a bound metallic fragment that lacked the arsenic center was highlighted. The reactions of AP-1 with various nucleophiles side chains of neutral histidine, methionine, cysteine, and selenocysteine, in neutral form as well as cysteine and selenocysteine in anionic form, were subsequently analyzed through a computational approach. We found that the aquation of AP-1 is energetically disfavored, with a reaction free energy of +19.2 kcal/mol demonstrating that AP-1 presumably attacks its biological targets through the exchange of the chloride ligand. The theoretical analysis of thermodynamics and kinetics for the ligand-exchange processes of AP-1 with His, Met, Cys, Sec, Cys−, and Sec− side chain models unveils that only neutral histidine and deprotonated cysteine and selenocysteine are able to effectively replace the chloride ligand in AP-1.

1. INTRODUCTION

Transition-metal complexes are widely used in medicinal chemistry,1−4 in the case of cisplatin as an anticancer agent being the most representative.5 As a matter of fact, there is a continuous interest in the development of cisplatin derivatives with the objective of ameliorating their antitumor potency while decreasing systemic toxicity.6−8 The toxicity of cisplatin originates from the relatively easy in vivo replacement of the chloride ligands by donor atoms of endogenous targets; actually, the testing of a plethora of less reactive Pt ligands in the place of chloride has permitted the production of metallodrugs with lower systemic toxicity and a higher therapeutic index.9−13 This proves that the design of new active Pt(II)-based compounds should involve the structure-based control of the substitution reaction.14,15

Arsenoplatin-1 (AP-1) is a novel dual-action metallodrug characterized by an antitumor effect based on the synergetic interplay of a square planar Pt(II) center and the coordinated arsenic trioxide moiety16 (Figure 1), resulting in a superior antitumor activity in a majority of cancer cell lines.16,17

The mechanism of action of AP-1 is not yet completely comprehended at the molecular level, although several experimental16−19 and computational20−22 investigations have been reported so far. AP-1 binding to DNA was studied by inductively coupled plasma mass spectrometry on AP-1-DNA adducts extracted from triple-negative breast MDA-MB-231 cancer cells, revealing that gradual and continuous release of the As(OH)2 moiety inside the cell results in the augmented toxicity of arsenoplatin-1 juxtaposed to cisplatin.16 Density-functional theory (DFT) calculations unveiled that guanine is a more favored binding site than adenine for AP-1 as well as for other platinum-containing compounds.20 It was also shown that the hydrolysis of AP-1 necessitates a higher energy barrier than that for DNA platination, although the barrier for aquation is lower than that of cisplatin because of the trans effect of the arsenic moiety.20 A detailed computational study
of the metalation of the bovine pancreatic ribonuclease (RNase A) by AP-1 revealed the binding of His to platinum(II), retaining the Pt–As bond. The computations evidenced that the metalation is more advantageous in water than in the protein milieu, consistent with the character of the protein binding pocket residues.22

Interestingly, the replacement of chloride in AP-1 with iodide did not hamper its cytotoxicity, thus proving that the Pt–As core is the “true” cytotoxic metal scaffold.23

Given the differences in the mechanism of action of arsenoplatin compared to cisplatin, it is plausible to assume that the interactions with proteins may play a prominent part in the action mode of AP-1. To the best of our knowledge, there are only two investigations of the reactivity of AP-1 with proteins. The first study focuses on the interactions of AP-1 with the small model proteins hen egg-white lysozyme (HEWL) and bovine pancreatic ribonuclease (RNase A). The corresponding crystal structures of AP-1-protein adducts revealed that the preferred binding sites for AP-1 are the His side chains in both proteins.16 Unlike cisplatin and carboplatin, which target the sulfurs of Met side chains of RNase A,24 AP-1 does not show any preference for Met side chains. Another evidence of AP-1 targeting His residue was offered by a recent study, in which AP-1 was placed into the apoferritin (Af) nanocage; the resulting X-ray structure revealed the coordination of the AP-1 fragment to the side chain of a His residue.19

The present study has a twofold objective. On one hand, we aim to expand the knowledge of the reactivity of AP-1 with proteins by considering a larger and more representative group of proteins including human carbonic anhydrase 1 (hCA1), bovine superoxide dismutase (SOD), horse heart myoglobin (Mb), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from rabbit muscle, and human serum albumin (HSA). These reactions and the associated adduct formation were examined through the classical ESI MS strategy developed in our laboratory.

On the other hand, the reactions of the neutral AP-1 with water and the histidine, methionine, cysteine, and selenocysteine side chains, which are the main candidates for protein metalation by AP-1, are investigated by means of DFT approaches to shed light on the observed binding preferences of protein residues for AP-1. More specifically, we have employed simple (short) models of these residues where each side chain is modeled by the nucleophilic group, i.e., imidazole, CH₃S⁺, HS⁻, and HSe⁻ for His, Met, Cys, and Sec residues, respectively, whereas the remainder of the chain is rendered by an ethyl group (Scheme 1).

Moreover, we assume that the reaction of AP-1 with protein targets occurs through the nucleophilic substitution on the Pt(II) center of the labile chloride ligand by the entering X ligand via an associative interchange mechanism as depicted in Scheme 2.

To gain a broader comprehension of the binding mechanism of AP-1 with protein targets as well as of their binding preference, we have evaluated both the thermodynamics and kinetics of the proposed mechanistic hypotheses. Indeed, computational studies were frequently and auspiciously employed for the characterization of the reactivity of metals and metallo-drugs with proteins.25–28 Understanding the binding preference of AP-1 would be very beneficial to completely understand its mechanism of action in vivo and may be advantageous to design more efficacious anticancer drugs.

2. RESULTS

2.1. Reactions of AP-1 with a Few Representative Proteins Analyzed by ESI MS. The reactions of AP-1 with the model proteins HEWL and RNase A were studied by ESI MS measurements in our previous study.16 Those results clearly showed that AP-1 binds both proteins through coordination of an [(AP-1)-Cl]⁺ fragment after the release of the Cl⁻ ligand. Complementary X-ray diffraction studies revealed that this metallic fragment was coordinated at the level of His15 of HEWL and His105 and His119 residues in the case of RNase A.

Here, we have extended this type of approach to a larger number of proteins some of them also being of a considerably greater size. Specifically, the following proteins were employed for this new study: hCA1, SOD, Mb, GAPDH, and HSA.

The interactions of these proteins with AP-1 were investigated according to a standard experimental setup including preparation of the protein solution in 2 × 10⁻³ M...
ammonium acetate at pH 6.8; addition of a threefold excess of AP-1; incubation delay; recording of the ESI MS spectra. The resulting deconvoluted ESI MS spectra are reported in Figure 2, with a direct comparison of the proteins’ spectra after and before the AP-1 addition. Particularly, the metalation of all tested proteins already took place after 3 h of AP-1 incubation.

The interpretation of these ESI MS spectra is quite straightforward. In most cases, AP-1-protein adducts are formed as witnessed by the appearance of new peaks of a greater mass; however, the amount of the formed adducts is quite limited. In most cases, a peak characterized by a mass shift of +419 is detected. This mass increase well matches the mass of an [(AP-1)-Cl] fragment, or of its dimer (in the case of hCA1), in line with previous observations.

The spectra of the tested proteins, after 24 h incubation, typically show a net decrease in the intensity of the adducts signal, suggesting a progressive instability of the binding. The spectrum of the GAPDH protein is characterized by two major signals; one, at 35,764 Da, assigned to the native protein, and another, at 35,797 Da, probably due to the Cys150 sulfhydration; this double signal is also detected in the spectrum of the AP-1 adducts. Interestingly, in the case of both GAPDH and Mb, a new fragment of a different mass, with a shift of 311 Da, is observed. Notably, this mass shift well corresponds to the Pt(NHC(CH₃)O)₂ fragment. Observation of this fragment offers direct evidence that arsenoplatin-1, upon interaction with certain proteins, may undergo the breaking of the As–Pt bond and the detachment of the As(OH)₂ group, an interesting feature that had not been observed in the previous studies.

In addition, AP-1 has been tested with HSA, the main plasma protein; we found that AP-1 manifests the tendency to react again, forming an adduct with the Pt(NHC(CH₃)O)₂ fragment. In Figure S1, Supporting Information, the mass spectra of HSA before and after the addition of AP-1 are presented. The deconvoluted mass spectrum of metal-free HSA is marked by the signals at 66,438 and 66,557 Da, corresponding to the protein in its native and its cysteinylated forms, respectively, i.e., the protein with a Cys residue bound to the Cys34. Interestingly, AP-1 upon reacting with HSA produces adducts with both the native and the cysteinylated proteins, primarily with the native protein as evidenced by the lower intensity of the native protein signal with respect to the cysteinylated protein signal.

2.2. Computational Studies. A preliminary investigation on the AP-SCN and AP-1 complexes has shown that the range-corrected CAM-B3LYP density functional with the LANL2DZ effective core potential and the 6-31+G* basis set yields the minimized structures well matching the crystallographic data with Pt–S and Pt–As bond distances within 0.04 Å error. We extracted the AP-1-His complex from the X-ray crystallographic data for the AP-1-HEWL adduct and made a comparison of geometrical parameters obtained by experiment, optimization in the gas phase, and optimization in water (Figure 3). We can see that most of the calculated bond distances, including the crucial metal center–ligand bond Pt–N, were estimated within an error of 0.01 Å, whereas the...
calculated Pt–As bond is within 0.1 and 0.08 Å for the optimization in the gas phase and solvated phase, respectively.

The thermodynamics and kinetics of the ligand substitution of chloride by entering nucleophile molecules were analyzed via DFT computations. The choice of modeling the investigated protein residues with the simplified models shown in Scheme 1, instead of capped or free amino acids, requires some further consideration. On one hand, the capped forms of amino acids are linked to the nucleophilic groups of side chains via hydrocarbon chains of different lengths, which leads to considerable variation in the size of the ligand interacting with metal complex and thus substantially affecting the computation of solvation free energies. On the other hand, free amino acids contain terminus carboxylic acid and amine groups in ionized zwitterionic form, which do not exist in proteins. We assume all residues to exist in their most stable protonation state at pH = 7.2: histidine and methionine are neutral, whereas selenocysteine is anionic (however, we included the neutral form of selenocysteine for completeness). Both neutral and anionic forms of cysteine were considered since both are present at neutral pH, although the anionic form can be found only in low concentrations. Moreover, the anionic form of cysteine might be stabilized in the vicinity of histidine and other basic residues.

We assume that the ligand-exchange reactions on AP-1 undergo an associative interchange mechanism, with reactants and products forming stable noncovalent adducts before and after the reaction. Thus, the geometries of the reactants (R), reactant adducts (RA), transition states (TS), product adducts (PA), and products (P) were calculated. The activation enthalpies and free energies were calculated as the difference between TS and the lowest between reactants and reactant adducts, whereas the reaction enthalpies and free energies were calculated as the difference between reagents and products infinitely apart.

Initially, we analyzed the hydrolysis reaction with the exchange of chloride by a water molecule to test the stability of AP-1 in biological fluids and consequently determine if the chlorido or the aquo form of AP-1 might be the reactive species with protein targets. Indeed, it is well known that cisplatin and other biologically active metal complexes often go through hydrolytic activation, at which the labile aquo ligand replaces at least one ligand of the metal center. The ligand-exchange reactions on AP-1 under an associative interchange mechanism, with reactants and products forming stable noncovalent adducts before and after the reaction. Thus, the geometries of the reactants (R), reactant adducts (RA), transition states (TS), product adducts (PA), and products (P) were calculated. The activation enthalpies and free energies were calculated as the difference between TS and the lowest between reactants and reactant adducts, whereas the reaction enthalpies and free energies were calculated as the difference between reagents and products infinitely apart.

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calculated reaction free energy value for the aquation of AP-1 is 19.2 kcal/mol, a similar value to that calculated in ref 20. This value shows that this reaction is thermodynamically unfavorable and is improbable to take place at physiological temperature, indicating that AP-1 is expected to attack the biomolecular targets with the metal center in its chlorido form.

The reaction of AP-1 with models of the His, Met, Cys, and Sec side chains was consequently investigated assuming the associative interchange mechanism in each case. The optimized geometries of transition state structures allow us to obtain interesting insights into the reactivity disclosed by the analyzed protein residues. For example, the Pt–As bond in all calculated transition states is 2.28–2.30 Å, suggesting that the trans effect is not observed in the geometrical structure with a trigonal bipyramidal or square pyramidal configuration. The transition state geometries for the reaction of AP-1 with the neutral side chain models are characterized by an approximately trigonal bipyramidal coordination of the Pt center (Figure 4) with all entering ligand Pt–N distances of 2.52 and 2.55 Å are observed for 4-His and 5-His, respectively, and the same Pt–Cl distance of 2.56 Å, which suggests that both tautomers disclose almost the same reactivity. The Pt–S/Se bond lengths of 2.67–2.72 Å correspond well with the 2.67–2.71 Å lengths of Pt–Cl bonds for the transition states of neutral Cys, Sec, and Met, indicating that these transition states are neither early nor late. Deprotonated cysteine and selenocysteine, the only anionic nucleophiles in this study, are only 15.1 and 15.4 kcal/mol, respectively, whereas the corresponding values for the reaction free energy are −16.2 and −15.5 kcal/mol.

An overall insight into both thermodynamics and kinetics of the ligand substitution reactions investigated in the present study is provided in Figure 5. The results suggest that AP is expected to preferentially bind at His residues unless deprotonated cysteine or selenocysteine are available.

On the other hand, the reaction of AP-1 with cysteine and selenocysteine in their anionic forms results to be both thermodynamically and kinetically the most favorable. Indeed, the computed activation free energy values for the exchange of chloride ligand with Cys− and Sec− are only 15.1 and 15.4 kcal/mol, respectively, whereas the corresponding values for the reaction free energy are −16.2 and −15.5 kcal/mol.

The binding preference in the AP-1 protein targeting obtained by our computational models implicitly assumes the same steric accessibility of these residues. However, protein side chains characterized by a high solvent exposure are more reachable to the AP complex and thus expected to be more reactive.

To better assess the targetability of specific protein systems by AP-1, the regioselectivity based on the nucleophilic substitution must be paralleled by a study on the solvent exposure of the considered protein residues. Hence, solvent-accessible surface (SAS) analyses (Figure 6) were performed on the X-ray structures of the hen egg-white lysozyme (HEWL) and the bovine pancreatic ribonuclease (RNase A) proteins (pdb ids 5nj1 and 5n76), showing that the His residues that form adducts with AP-1 are closer to the surface. Indeed, the residues His12 and His48 in the bovine pancreatic ribonuclease are not metallated and, after a thorough investigation of the X-ray structure, we might conclude that
and AP-1 at protein-to-metal ratio 1:3 were prepared and diluted with established procedures.\textsuperscript{13,14} of Chemistry, University of Florence in accordance with already established procedures.\textsuperscript{2} AP-1 at the protein-to-metal ratio of 1:0.9 or 1:3 was prepared and diluted with ammonium acetate solution (pH 6.8). The general ESI source parameters optimized for each protein and peptide analysis were as follows.

**SOD parameters** are positive polarity, ion spray voltage 5500 V, temperature 0, ion source gas 1 (GS1) 25 L/min, ion source gas 2 (GS2) 0, curtain gas (CUR) 20 L/min, collision energy (CE) 10 V, declustering potential (DP) 200 V, and range 3000–3400 m/z.

**Mb parameters** are positive polarity, ion spray voltage floating 5500 V, temperature 0, ion source gas 1 (GS1) 40 L/min, ion source gas 2 (GS2) 0, curtain gas (CUR) 15 L/min, collision energy (CE) 10 V, declustering potential (DP) 100 V, and range 700–2200 m/z.

**GAPDH parameters** are positive polarity, ion spray voltage floating 5500 V, temperature 0, ion source gas 1 (GS1) 20 L/min, ion source gas 2 (GS2) 0, curtain gas (CUR) 15 L/min, collision energy (CE) 10 V, declustering potential (DP) 100 V, and acquisition range 600–2000 m/z.

**hCA I parameters** are positive polarity, ion spray voltage floating 5500 V, temperature 0, ion source gas 1 (GS1) 25 L/min, ion source gas 2 (GS2) 0, curtain gas (CUR) 20 L/min, collision energy (CE) 10 V, declustering potential (DP) 200 V, and range 3000–3400 m/z.

**HSA parameters** are positive polarity, ion spray voltage floating 5500 V, temperature 0, ion source gas 1 (GS1) 40 L/min, ion source gas 2 (GS2) 0, curtain gas (CUR) 20 L/min, collision energy (CE) 10 V, declustering potential (DP) 200 V, and range 900–2600 m/z.

For acquisition, Analyst TF software 1.7.1 (Sciex) was employed, and deconvoluted spectra were attained by utilizing the Bio Tool Kit with single-point electronic and solvation energies were performed in solvated phase (C-PCM)\textsuperscript{35,36} and by employing the density functionals as described below.

All geometrical optimizations were performed with the LANL2dz effective core potential for Pt atom\textsuperscript{42} and the 6-31G* basis set for other elements,\textsuperscript{36,38} while single-point electronic and solvation energy computations were performed with the LANL08\textsuperscript{(f)} effective core potential for platinum\textsuperscript{37,41} and the 6-311++G** basis set for other elements.\textsuperscript{41--44} We used the range-corrected DFT functional CAM-
B3LYP\textsuperscript{44} for geometrical optimization and electronic and solvation energies calculations. As we have shown elsewhere,\textsuperscript{21} the CAM-B3LYP/LANL08(\textit{f})/6-311++G**/CAM-B3LYP/LANLDZ/6-31+G* functional-basis set combination yields the best results for geometry and energy computations for the aquation of arsenoplatin-1. DFT functionals are recognized to produce adequate geometries and reaction profiles for transition-metal-containing compounds\textsuperscript{45–48} including Pt-based anticancer compounds.\textsuperscript{49–51}

Frequency calculations were carried out to confirm the convergence to the stationary points and to evaluate zero-point energy (ZPE) and thermal corrections to thermodynamic properties. Intrinsic reaction coordinate (IRC) computations were utilized to determine reactants and products minima connected with the transition states for each examined reaction step.

Single-point electronic energy computations were performed on the geometries optimized in the solution. The C-PCM continuum solvent methodology was employed to account for solvation.\textsuperscript{35} It was demonstrated to yield significantly smaller discrepancies than other continuum models for aqueous free energies of solvation for cations, anions, and neutrals and to be especially efficacious for the calculations of solution properties necessitating an enhanced accuracy of solution free energies.\textsuperscript{37} Free energies of solvation, considered as the difference between the solution energies and the gas phase energies, were added to the gas phase enthalpies and free energies values to have the corresponding values in the aqueous solution.

The solvent-accessible surface (SAS) of each examined amino acid was calculated by employing the SAS option available in Gromacs software.\textsuperscript{53}

4. CONCLUSIONS

This study includes a combined experimental and theoretical investigation of arsenoplatin-1 interactions with protein targets. The analysis of the biomolecular interactions of AP-1 grounded on ESI MS measurements was extended here to a larger number of proteins than in the past, including carbonic anhydrase, superoxide dismutase, myoglobin, glyceraldehyde 3-phosphate dehydrogenase, and human serum albumin. The ESI MS results reveal that AP-1 generates in most cases tight adducts with the studied proteins containing the \([\text{[AP-1]-Cl]}\) fragment, in nice agreement with previous observations made on HEWL and RNase A, and with the computational analysis carried out here. More in detail, the computational studies have considered the reactions of AP-1 with various nucleophiles, which mimic the side chains of neutral histidine, methionine, cysteine, and selenocysteine in neutral form as well as cysteine and selenocysteine in anionic form. The aquation of AP-1 is energetically disfavored with the reaction free energy of 19.2 kcal/mol, thus indicating that AP-1 presumably attacks its biomolecular targets by the direct substitution of the chloride ligand. The theoretical examination of thermodynamics and kinetics for the ligand substitution processes of AP with His, Met, Cys, Sec, Cys\textsuperscript{−}, and Sec\textsuperscript{−} side chain models revealed that only neutral histidine and deprotonated cysteine and selenocysteine can effectively replace the chloride ligand in AP-1.

Moreover, a different and innovative result has been achieved here through the ESI MS experiments when reacting AP-1 with GAPDH and Mb. Indeed, in these latter cases, the adducts just contained a smaller fragment where the \([\text{As-(OH)}\text{₂}]\) moiety is lost. This result is of particular interest as it provides direct evidence that arsenoplatin-1 may undergo degradation in the biological milieu, with the cleavage of the As–Pt bond giving rise to a protein-bonded platinum-containing fragment while releasing an arsenic-containing fragment.

Although the mechanistic details of the \([\text{As(OH)}\text{₂}]\) detachment from AP-1 were not expressly addressed in the present study, our calculations showed that the Pt–As distance is not significantly affected when replacing chloride by a nucleophilic protein ligand. This computational outcome suggests that the \([\text{As(OH)}\text{₂}]\) release is probably subsequent to protein metalation and may be kinetically influenced by the protein environment surrounding the Pt(II) binding site.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.inorgchem.1c03732.

S1: Deconvoluted mass spectrum of human serum albumin and incubated with AP-1. S2: Enthalpy and Gibbs free energy values for the reaction of AP-1 with the investigated protein residue models in solution (PDF)

AUTHOR INFORMATION

Corresponding Authors
Luigi Messori – Department of Chemistry, University of Florence, 50019 Sesto Fiorentino, Italy; orcid.org/0000-0002-9490-8014; Email: luigi.messori@unifi.it

Nazzareno Re – Dipartimento di Farmacia, Università “G d’Annunzio” di Chieti-Pescara, 66013 Chieti, Italy; orcid.org/0000-0002-0957-4049; Email: nre@unich.it

Authors
Iogann Tolbatov – Institut de Chimie Moléculaire de l’Université de Bourgogne (ICMUB), Université de Bourgogne Franche-Comté (UBFC), 21078 Dijon, France; orcid.org/0000-0001-9700-5331

Damiano Cirri – Department of Chemistry and Industrial Chemistry, University of Pisa, 56124 Pisa, Italy

Matteo Tarchi – Department of Chemistry, University of Florence, 50019 Sesto Fiorentino, Italy

Tiziano Marzo – Department of Pharmacy, University of Pisa, 56126 Pisa, Italy; CISUP - Centre for Instrumentation Sharing (Centro per l’Integrazione della Strumentazione Scientifica), University of Pisa, 56126 Pisa, Italy; University Consortium for Research in the Chemistry of Metal ions in Biological Systems (CIRCSMB), 70126 Bari, Italy; orcid.org/0000-0002-2567-3637

Cecilia Coletti – Dipartimento di Farmacia, Università “G d’Annunzio” di Chieti-Pescara, 66013 Chieti, Italy; orcid.org/0000-0002-3609-290X

Alessandro Marrone – Dipartimento di Farmacia, Università “G d’Annunzio” di Chieti-Pescara, 66013 Chieti, Italy; orcid.org/0000-0002-8311-8172

Lara Massai – Department of Chemistry, University of Florence, 50019 Sesto Fiorentino, Italy

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.inorgchem.1c03732

Author Contributions
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