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Platinated oligomers of bovine pancreatic ribonuclease: Structure and stability

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ABSTRACT

The reaction between *cis*-diamminedichloroplatinum(II) (CDDP), cisplatin, a common anticancer drug, and bovine pancreatic ribonuclease (RNase A), induces extensive protein aggregation, leading to the formation of one dimer, one trimer and higher oligomers whose yields depend on cisplatin/protein ratio. Structural and functional properties of the purified platinated species, together with their spontaneous dissociation and thermally induced denaturation, have been characterized. Platinated species preserve a significant, although reduced, ribonuclease activity. The high resistance of the dimers against dissociation and the different thermal unfolding profiles suggest a quaternary structure different from those of the well-known swapped dimers of RNase A.

1. Introduction

Platinum complexes represent one of the most successful families of clinically used anticancer drugs. Cisplatin (*cis*-$PtCl_2(NH_3)_2$, CDDP) is the best known Pt-based anticancer agent. It is generally accepted that its mechanism of action is based on the formation of stable DNA adducts [1]. The cisplatin–DNA adducts unwind and bend the DNA duplex, facilitating the binding by high-mobility group proteins that recognize the cisplatin binding as a DNA damaging event that, ultimately, results in cell death by apoptosis [2–4].

Nevertheless, nucleobases are not the only biological targets of Pt-containing drugs [5]. Platinum can efficiently interact with sulfur atoms of cysteine [6] or methionine residues [7] or even with nitrogen atom of histidine [8,9] or oxygen atoms of aspartic/glutamic side chain residues [10]. While the formation of Pt–DNA adducts has been extensively investigated in relation to cytotoxicity of Pt drugs, less attention has been paid to the formation of Pt–protein adducts [11,12], although they likely play active roles in the mechanisms of action of Pt-based drugs [13–15].

Previous studies have shown that Pt metalation can modify the oligomerization state of proteins, can alter their hydrodynamic properties and/or can cause fine structural variations [16,17]. In order to obtain further information on the formation of protein oligomers induced by the cisplatin binding, we have here characterized the products of the reaction between this drug and the model protein bovine pancreatic ribonuclease (RNase A). We have chosen to study this protein since a) it interacts with cisplatin [7], and b) it has been used as a model system in many fields of protein chemistry [18], including protein metalation [19–21] and protein aggregation [22,23]. In fact, RNase A self-associates through the interchange of its termini (3D-domain swapping) [23]. In particular, it forms an N-terminal end swapped [24] and a C-terminal end swapped dimer [25] (Figure S1), called N-Dimer and C-Dimer, respectively [26,27], two trimers characterized by swapping of both N- and C-termini, and higher domain swapped oligomers [28], including tetramers, pentamers and hexamers [28,29]. Interestingly, RNase A aggregates acquire selective cytotoxicity towards cancer cells, although this feature seems to be limited only to non-solid tumors [30].

The results of our work reveal that 24 h RNase A incubation in the presence of an excess of cisplatin induces the formation of a platinated monomer (Pt-M), whose structure has been solved by X-ray crystallography at 1.95 Å resolution, a platinated dimer (Pt-D), a platinated trimer (Pt-T) and higher oligomers with structural and

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functional properties distinct from those of the previously characterized oligomers of the same protein [23].

2. Materials and method

2.1. Production and purification of platinated oligomers

Cisplatin and RNase A (type XII-A) were purchased from Sigma-Aldrich. RNase A was further purified as described in [28]. Platinated RNase A forms for analytical purpose were obtained by incubating the lyophilized protein with cisplatin at 37 °C in a protein:metallodrug molar ratio 1:2.5, 1:5, 1:10 and 1:15. After 24 h incubation with a mild stirring, aliquots from the reaction mixture were analyzed by denaturing SDS-PAGE, as well as by non-denaturing PAGE. Gels were stained with Coomassie Brilliant Blue R-250, preceded in native PAGE by fixing the bands with 12.5% trichloroacetic acid.

To assess the feature(s) of each platinated oligomer formed, the reaction mixture was gel-filtered on a Superdex 75 HR 10/300 column attached to an ÄKTA Purifier fast protein liquid chromatography (FPLC) system (GE-Healthcare), and equilibrated with 0.40 M sodium phosphate at pH 6.7, at a flow rate of 0.10 mL × min^{-1}. Aliquots corresponding to 3–5 μg of protein were withdrawn from the fractions corresponding to the maximum of the peaks of Pt-M, Pt-D and Pt-T of RNase A and loaded on SDS (i.e., denaturing) polyacrylamide gels. To assess sample homogeneity, identical aliquots were also loaded on 10% polyacrylamide native (i.e., non-denaturing) gel-electrophoresis [28]. In this latter case, the native gels were run at 4 °C to avoid spurious effects due to possible aggregation and/or dissociation during the experiment.

For a preparative experiment, an aliquot of 12 mg of RNase A was dissolved in 2.2 mL of 0.20 M sodium phosphate pH 6.7 at a final concentration of 5 mg × mL^{-1} and incubated for 24 h at 37 °C with a mild stirring in the presence of 2.6 mg of cisplatin (1:10 molar ratio). Platinated RNase A and oligomers were purified from the reaction mixture by gel filtration chromatography on a Sephadex G-75 (1.5 × 72 cm) column equilibrated with 0.2 M sodium phosphate, pH 6.7, at a flow rate of 0.13 mL × min^{-1}.

The concentration of all RNase A species was spectrophotometrically measured at 280 nm, using the extinction coefficient of RNase A ($\varepsilon_{1\%}$ at 280 nm = 0.695).

2.2. Circular dichroism spectroscopy

Circular dichroism (CD) experiments were performed on a Jasco J-810 spectropolarimeter (JASCO Corp., Milan, Italy) at 25 °C. Quartz cells with path length of 0.1 cm were used in the far-UV region from 200 to 250 nm. Each spectrum was obtained averaging three scans, subtracting contributions from the corresponding references and converting the signal to mean residue ellipticity in units of deg × cm^2 × $dmol^{-1}$. Pt-M, Pt-D and Pt-T were analyzed with far-UV CD spectroscopy, at the same concentration of 0.1 mg × mL^{-1}. Spectra have been collected using protein samples obtained upon incubation of the protein with cisplatin at a 1:10 molar ratio, fractioned by gel-filtration on Sephadex G75 eluted in 0.20 M phosphate buffer, pH 6.7, and stored at 4 °C to avoid oligomer dissociation. Other experimental settings were: 20 nm × min^{-1} scan speed, 2.0 nm band width, 0.2 nm resolution, 50 mdeg sensitivity, and 4 s response. Thermal unfolding profiles were obtained by monitoring the CD signal at 222 nm as function of temperature, in the range 20–100 °C, with a heating rate of 1.0 °C × min^{-1}.

2.3. Assays for enzymatic activity

Enzymatic activity of platinated species was measured by monitoring cleavage of yeast RNA via UV–vis spectroscopy, using the Kunitz method [31]. In a typical experiment, the ribonuclease activity on yeast RNA was determined at 25 °C in 0.050 M sodium acetate pH 5.0, using 0.5 mg × mL^{-1} of RNA and enzyme concentration of 0.5 μg × mL^{-1}. The activity of the platinated proteins was compared to that obtained in the same experimental conditions for native RNase A, used as a standard, and for Onconase [32], produced in our lab.

Assays were performed also against dsRNA poly(A):poly(U): the Abs_{260} increase induced by the different enzyme species was monitored following the procedure described by Libonati and Floridi [33].

2.4. Dissociation experiments

The stability of Pt-D and Pt-T, obtained upon mixing protein and cisplatin at 1:10 protein:metallodrug ratio, was assessed by incubating at 4 and 37 °C aliquots of the fraction corresponding to the maximum of each form, without any further modification, i.e., at a concentration ranging from 0.15 to 0.35 mg × mL^{-1} in 0.20 M sodium phosphate buffer, pH 6.7, for time intervals settled between 1 and 21 days. An aliquot of the fraction corresponding to Pt-M (at a protein concentration of 0.75 mg × mL^{-1}) was also incubated in the same conditions for comparison. Samples were analyzed by non-denaturing gel electrophoresis run at 4 °C and at 37 °C.

2.5. Crystallization and X-ray diffraction data collection

Purified Pt-M, obtained upon RNase A incubation in 1:10 protein to metallodrug ratio, was crystallized at 25 °C using the hanging drop vapor diffusion method, protein concentration 15 mg × mL^{-1} and a precipitant solution containing 30% ammonium sulfate and 3.0 M sodium chloride. Crystals appeared within 7 days.

These crystals were fished with nylon loops and flash-cooled at 100 K using nitrogen gas produced by an Oxford Cryosystem without cryoprotectant. This recently developed dehydration procedure [34] has been used to collect X-ray diffraction data of many adducts between protein and metallodrugs (see for example [21]).

A complete data set for Pt-M was collected at 100 K at the CNR Institute of Biostructures and Bioimages, Naples, Italy, using a Saturn944 CCD detector equipped with CuKα X-ray radiation from a Rigaku Micromax 007 HF generator. The crystals belong to space group $P3_221$ and diffract at 1.95 Å resolution; data were processed and scaled using HKL2000 [35]. Details of data collection statistics are reported in Table S1.

2.6. Structure resolution and refinement

The structure of the platinated monomer was determined by molecular replacement, using Phaser [36] and the PDB file 1RNX [37], without water molecules and ions, as search model. Refinement was carried out with REFMAC5 [38]; model building and electron density map fitting were performed using WinCoot [39]. Refinement statistics are reported in Table S2. Structure validation was carried out using Procheck [40]. Coordinates and structure factors were deposited in the Protein Data Bank under the accession code 4RTE.

3. Results and discussion

3.1. Incubation with cisplatin induces the formation of SDS resistant RNase A oligomers

Previous studies have shown that Pt metalation of proteins can induce the formation of protein oligomers and/or cause subtle structural variations [12]. The aim of this work is to monitor the formation of possible aggregates produced by incubation of RNase A with cisplatin and to characterize these oligomers from a structural point of view.

First, we have analyzed the protein incubated for 24 h at 37 °C in the presence of cisplatin (in a 1:10 protein:cisplatin ratio) by gel-electrophoresis run under native conditions (Fig. 1A). Two different