Drug–Protein Adducts

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Structural Investigation of Cisplatin–Protein Interactions: Selective Platination of His19 in a Cuprozinc Superoxide Dismutase**

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Cisplatin (*cis*-$[PtCl_2(NH_3)_2]$) is a leading anticancer drug.[1] Its biological activity is strictly associated with platination of nuclear DNA at guanine sites, the formation of intrastrand cross-links, and the consequent induction of DNA bending.[2] Damage to DNA eventually causes apoptotic cell death through a complex cascade of biochemical processes that are being progressively unraveled.[3] The fact that DNA represents the probable primary target for this metallodrug has prompted a huge number of studies on cisplatin–DNA interactions.[2] In contrast, the interactions of anticancer platinum drugs with proteins have received, until now, far less attention.

Only a very modest fraction of the administered cisplatin reacts with nuclear DNA and produces the critical cytotoxic lesions; the largest amount of cisplatin is reported to bind proteins, both extracellularly and intracellularly.[4] Surprisingly, these latter interactions have been explored only marginally, although they probably constitute the biochemical basis for the relevant systemic toxic effects caused by platinum drugs, and might also play some role in the anticancer mechanism. Thus, it is of great interest, in our opinion, to determine the nature of the platinum-containing molecular fragments that are bound to proteins, their exact location, their strength of binding, and the reversibility of the interactions. A few solution studies on the interactions of cisplatin and analogues with selected proteins have appeared over the years (e.g., ubiquitin,[5] albumin,[6] hemoglobin,[7] transferrin[8]), but we noticed a substantial lack of crystallographic information for protein adducts with platinum drugs. The only kind of structural information available for platinum–protein adducts is that resulting from the use of platinum salts and platinum complexes in the preparation of heavy-atom derivatives of proteins.[9]

These arguments led us to perform new crystallographic investigations of cisplatin derivatives of proteins, to give a detailed structural description of the nature of platinum–protein interactions. Bovine erythrocyte copper–zinc superoxide dismutase (beSOD; EC 1.15.1.1) was selected as the model protein for our study on cisplatin for a number of reasons:[10] the crystal structure of beSOD is available at high resolution;[10] beSOD is a medium-sized protein with several potential binding sites for platinum; it is highly water soluble; it is commercially available; it is known to crystallize easily; and it exhibits great stability under physiological-type conditions.

Crystals of cisplatin-treated beSOD, suitable for X-ray diffraction analysis, were obtained after incubation of the protein with a tenfold molar excess of cisplatin for two weeks. X-ray diffraction data were collected at low temperature and the structure was solved through standard methods to 1.8-Å resolution. To the best of our knowledge, the results presented herein provide the first example of a high-resolution crystal structure for a cisplatin–protein derivative.

The overall structure of cisplatin-treated beSOD (Figure 1) clearly resembles that of the native beSOD used as the starting model for the molecular replacement. The Cα root-mean-square deviation (rmsd) between the two proteins is 0.19 Å and there are no regions where the deviations are significantly high. Also, the physiological copper and zinc atoms are well-defined in the electron-density map of the active site of the enzyme as are the disulfide bridges. The structure seems to be quite rigid as the average *B* factor is 7.3 $Å^2$. In other words, the reaction with cisplatin would cause neither general nor local structural changes of the protein,

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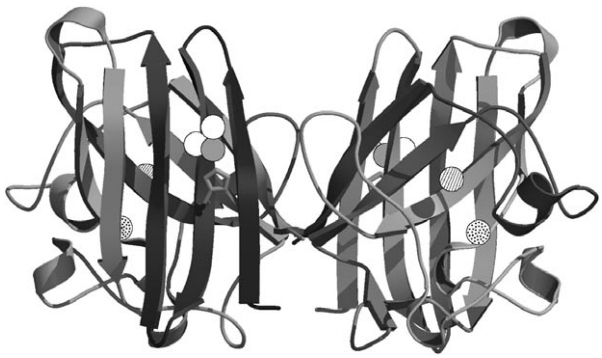

Figure 1. Schematic representation of the asymmetric unit containing the physiological dimer; the side chain of His19 is shown along with Cu (hatched), Zn (dotted), Pt (gray), and Cl (white).

with just monodentate platinum binding occurring on the protein surface.

A detail of the electron-density map around the protein-bound platinum center is shown in Figure 2. The map clearly reveals specific binding of the platinum atom to the NE2

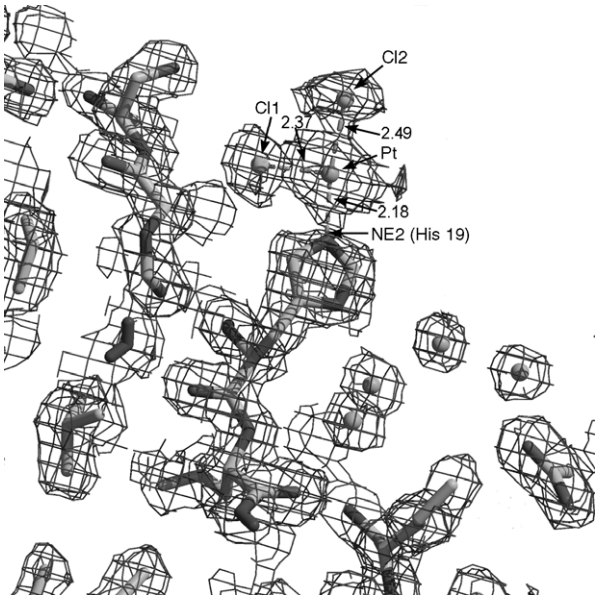

Figure 2. Detail of the $2F_o-F_c$ electron-density map contoured at 1σ showing cisplatin interacting with His19 and the relative bond lengths.

nitrogen atom of His19, in both monomers. The occupancy of the platinum atom is slightly different for the two monomers; the refinement shows values of about 0.7 and 0.6, respectively, which confirm a good binding selectivity. Notably, the electron-density map does not show any presence of additional platinum atoms bound to other residues of either monomer.

Sulfur-containing side chains are known to represent preferred binding sites for platinum compounds; competitive binding of platinum(II) drugs to either nitrogen or sulfur donors is a matter of intense debate.[11] Our structural data on platinated beSOD unambiguously reveal a preference for platinum binding to His compared to Met or Cys residues. This preference may be, at least partially, ascribed to the fact that access to either Met115 or Cys6, the only "free" sulfur-containing groups of beSOD, is somehow sterically hindered. Conversely, Cys55 and Cys144, which are solvent-accessible, are engaged in the formation of an internal disulfide bridge and are thus less prone to react with platinum(II).

Notably, both platinum(II) ions bind the protein in a slightly distorted square-planar arrangement, which is typical for platinum(II) complexes; the coordination environments of the two platinum atoms are very similar but not identical. One of the four ligands is the NE2 nitrogen atom of His19, which anchors the platinum ion to the protein in a monodentate fashion. The adjacent position shows a tiny electron density, which probably results from a very weakly bound water molecule. The remaining coordination positions (one of which is in the *trans* configuration with respect to the histidine nitrogen atom) show a very clear and prominent electron density that was assumed, at first, to arise from the two amino groups of cisplatin.

However, upon careful analysis of the thermal factors of the platinum ligands and of their distances to platinum, it is possible to state quite unambiguously that cisplatin, upon protein binding, releases its two ammonia ligands rather than losing the two chlorine atoms, as might be expected on the grounds of the well-described solution behavior of cisplatin.[1] In fact, if the ligand is refined with the assumption that both adjacent coordination positions (or one of them) are occupied by a nitrogen atom, the *B* factors go down to unrealistic values, whereas, if the two positions are refined assuming the presence of two chlorine atoms, the *B*-factor values obtained are comparable to those of the copper and zinc atoms in the active site of the protein (around 15 Å^2). Also, the interaction distances strongly support this interpretation, as the distances between the platinum atom and these two coordination positions are too large (2.55(12) Å in the case of the *trans* position with respect to the histidine nitrogen atom, and 2.28(12) Å for the other one) compared to the usual Pt–N distances (which should be around 2.1–2.2 Å).[12] A careful inspection of the crystal structure revealed tight contact between a chlorine atom and the main-chain carbonyl group of Thr17 (the distance is 3.23(12) Å).

The proposed release of the ammonia ligands is a quite remarkable and unexpected finding in relation to the classical solution chemistry of cisplatin. However, recent theoretical studies by Deubel and co-workers[13] have established that loss of the ammonia ligands is indeed feasible within the specific protein microenvironment, and may represent a possible mechanism of cisplatin inactivation as both ammonia groups are normally required for its biological activity.[2] These authors showed that the less-polarizable environment, which is likely present when amino acid residues of proteins react with the drug, greatly reduces the *trans* influence and enhances the *trans* effect at the platinum center.[13] Also, the different interactions that can occur between cisplatin and the protein surface might well favor alternative reactivity pathways for the metal center over the time that is needed for crystal formation and growth.

Overall, the present results are, in our opinion, of significant interest as they represent the first high-resolution crystal structure for a cisplatin–protein adduct. Unambiguous evidence is provided for His19 as the primary anchoring site for cisplatin. Binding is shown to be highly selective: occupation of the His site is relevant (> 60–70 %), while no other binding sites could be detected on the protein surface even at very low occupation percentages. Remarkably, clear evidence for ammonia release from cisplatin is provided. In addition, our study proposes a general X-ray-based strategy for investigating metallodrug–protein interactions, which relies on cocrystallization of adducts of metallodrugs with selected model proteins.

Experimental Section

Crystals of platinated beSOD were obtained by using the hanging-drop method for cocrystallizing the protein (5 mg mL^{-1}) with cisplatin at a platinum/protein molar ratio of 10:1. Cisplatin from Sigma–Aldrich was used (code P4394, lot 124F-0474). The precipitant buffer in the reservoir solution consisted of Tris-HCl (50 mM, pH 7.5), PEG 4000 (18 %), and 2-propanol (20 %). Small crystals appeared within 3 days at 4 °C, and were allowed to grow at room temperature for 2 weeks.

A monochromatic experiment at the Cu_α wavelength was performed on a crystal of beSOD grown in the presence of cisplatin by the rotation method on a PX-Ultra sealed-tube diffractometer (Oxford Diffraction) at 100 K. The crystal diffracted up to 1.7-Å resolution (resolution: 52.0–1.8 Å), with 32303 (3880) unique reflections out of 154261 (8429) reflections, and belonged to space group $P2_12_12_1$ ($a = 47.21$, $b = 50.92$, $c = 146.74$ Å; $\alpha = \beta = \gamma = 90°$) with two molecules (a complete physiological dimer) in the asymmetric unit and a solvent content of about 52 %. The overall completeness was 96.1 (81.4) %, $R_{sym} = 17.5$ (42.6) %, multiplicity: 4.8 (2.2), $I/(\sigma I) = 4.1$ (1.7). The numbers in parentheses refer to the values in the highest resolution shell (1.9–1.8 Å).

The dataset was processed by using the program MOSFLM[14a] and scaled by the program SCALA,[14b] with the TAILS and SECONDARY corrections (the latter restrained with a TIE SURFACE command) to achieve an empirical absorption correction.

The structure of the cisplatin adduct was solved by the molecular replacement technique; the model used was that of a monomer of beSOD (PDB-ID 1SXS) from which all the water molecules and ions were omitted. The correct orientation and translation of the dimer within the crystallographic unit cell were determined with standard Patterson search techniques,[14c,d] as implemented in the program MOLREP.[14e,f] The isotropic refinement was carried out using REFMAC5[14g] for all atoms except Cu, Zn, and Pt, which were refined anisotropically. In between the refinement cycles the model was subjected to manual rebuilding using XtalView.[14h] The same program was used to model the cisplatin adduct into the electron-density map.

Water molecules were added by the standard procedures within the ARP/wARP suite.[14i] The final structure containing 287 water molecules, 2184 protein atoms (two molecules), and 10 ions was refined (resolution range: 37.0–1.8 Å) to a final crystallographic R factor (R_{cryst}) of 0.202 and an R_{free} value of 0.236. The stereochemical quality of the refined models was assessed by using the program Procheck[14j] and is within the standard tolerance, with rmsd values of 0.014 Å for bond lengths and 1.6° for bond angles. The Ramachandran plot is of very good quality, with 88.6 % of residues in the most-favored regions, 11.4 % in the additionally allowed ones, and no residues in generously allowed or in disallowed regions.

The coordinates for the cisplatin–beSOD complex have been deposited in the Protein Data Bank under the accession 2AEO.

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