Structural dynamics of cisplatin binding to histidine in a protein

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The platinum anti-cancer agents cisplatin and carboplatin bind to the histidine 15 residue in the model protein hen egg white lysozyme. By using temperatures either side of the protein glass transition state (~180 K), several platinum binding modes are seen and show that not all these platinum modes are stable. In particular, the mean square displacement vibration amplitudes of the cisplatin and of the histidine to which it is bound are analysed in detail. As well as the multiple platinum peaks, the electron density for the His-15 side chain is weak to absent at 150 K and 200 K, which points to the imidazole ring of the His side chain sampling multiple positions. Most interestingly, the His-15 imidazole becomes more ordered at room temperature. © 2014 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution 3.0 Unported License.

I. INTRODUCTION

Cisplatin and carboplatin are important platinum anti-cancer agents, used to treat a variety of different cancers. They exert their anti-cancer effect by targeting the DNA major groove, forming intrastrand and interstrand cross-links between guanine bases via their N7 atoms causing distortion of the DNA helix that leads to inhibition of DNA replication and transcription and triggers apoptotic cell death. However, cisplatin and carboplatin are known to bind to a variety of plasma proteins including: a copper transporter, cytochrome C, human albumin, ubiquitin, glutathione, Na+/K+-ATPase, and prion peptides. Cisplatin and carboplatin binding to hen egg white lysozyme (HEWL) as a model protein has been extensively studied using X-ray crystallography using the standard tetragonal HEWL crystallisation conditions (NaCl and NaAc) with the addition of dimethyl sulfoxide (DMSO) and cisplatin/carboplatin, the platinum anti-cancer agents bound to the Nδ and Nz atoms of His-15 even without DMSO i.e. in aqueous medium but requires prolonged chemical exposure. A plausible role of the chloride ions is to extract the hydrogens from the histidine to create an imidazolyl ion. Alternatively, a tautomeric form for the histidine is a possibility considered in detail in our previous study. The impetus for our new study looked at growing triclinic HEWL crystals co-crystallised with cisplatin and carboplatin to study their binding at atomic resolution. HEWL has been previously studied at 0.65 Å (Ref. 22) in a triclinic form as well as with neutrons. Thus, crystallising cisplatin or carboplatin under triclinic HEWL crystallisation conditions could lead to obtaining the desired atomic resolution details. By studying this crystal form at multiple temperatures would, in addition to obtaining atomic or better resolution, allow the structural dynamics to be investigated by separating any static disorder from dynamical variations. Proteins are not static objects, they sample ensembles of conformations to achieve dynamic structures adapted to their function. Studying the dynamics of ligands binding to amino acid residues in proteins is important to see how the binding is different as amino acids are highly dynamic and can sample many conformations.
are mainly static but the diffraction data can contain information about alternative conformations of the amino acid or ligand, representing the dynamics of the system studied.

Our study confirms the binding of cisplatin and of carboplatin to His-15 in this triclinic crystal form and 0.98 Å resolution has been obtained using a home source Cu Kα alpha CCD diffractometer. The results reveal a versatile binding of the cisplatin and carboplatin to the His-15 residue, and more versatile than seen in our previous studies in the tetragonal and orthorhombic crystal forms. The use of multiple temperatures either side of the protein glass transition for cisplatin (anticipated to be 180 K), revealed that not all these platinum cisplatin binding modes are stable, which is again a different result to our results in the tetragonal and orthorhombic crystal forms. The order of the His-15 side chain is clearest at room temperature (RT) as evidenced by the visibility of the electron density.

II. METHODS

A. Crystallisation conditions

Triclinic HEWL crystals were grown using a similar method to Wang et al. (2007); however, the batch method was used with 40 mg HEWL (2.7 mM) co-crystallised with 2.6 mg cisplatin (8.1 mM) or 3 mg carboplatin (8.1 mM). 460 µl of a 0.02M NaAc solution along with 460 µl of a 0.5M NaNO₃ solution was used with 75 µl DMSO added. The crystallisation was undertaken at 295 K.

B. X-ray data collection, crystal structure solution, and refinement

X-ray diffraction data from three separate cisplatin HEWL triclinic crystals were collected at data collection temperatures of 150 K, 200 K, and RT (i.e., approximately 295 K) see Table I. Paratone was used as the cryoprotectant for the crystal sample datasets collected at 150 and 200 K, with a 0.4–0.5 mm loop used to mount the crystals. For the RT dataset, a 0.7 mm quartz capillary was used to mount the crystal. The carboplatin HEWL crystal X-ray diffraction data were collected at a temperature of 200 K with paratone again used as the cryoprotectant. Each of the X-ray diffraction data sets was collected on a Bruker APEX II home source CCD diffractometer, with the detector placed 40 mm away from the crystal using an X-ray wavelength of 1.5418 Å. The diffractometer data collection strategy for triclinic necessarily involved multiple passes used with different swing angles, sample rotation ranges, and exposure times. The diffraction datasets were processed using the internal Bruker APEXII software.

Each crystal structure was solved using molecular replacement with Phaser in CCP4i, using the lysozyme structure 2w1y as the molecular search model. Model building, adjustment, and refinement were carried out using the Coot molecular-graphics program and REFMAC5, refining individual anisotropic B factors in CCP4i for the datasets at 150 and 200 K and isotropic B factors for the dataset at RT. Cisplatin and carboplatin ligand binding occupancies were calculated using SHELX. Crystallographic and refinement parameters are summarized in Table I.

III. RESULTS

A. Cisplatin and carboplatin binding to His15 of triclinic HEWL

Cisplatin and carboplatin are, in their respective studies, both seen bound to the Nd and Ne atoms of His-15 in triclinic HEWL. For the cisplatin datasets collected at the three different data collection temperatures (150 K, 200 K, and RT), and the carboplatin dataset at 200 K there is evidence for split-occupancy binding (Figure S1) as evidenced by the presence of multiple anomalous difference density peaks in close proximity to one another as well as equivalent peaks in the 2Fo-Fc density maps (Table II). Each individual assigned platinum atom showed a lower binding occupancy (Table III) than previously observed. However, the summed occupancies of the multiple platinum atoms for the cisplatin case in the Ne binding sites is larger for the 150 K and 200 K datasets than we have seen before. The cisplatin Nd binding
However, the Ne binding site at each temperature shows multiple platinum positions (Figures S1(a)–S1(d)).

Due to previously seeing slight differences in the electron density maps from using different diffraction data processing programs, the 200 K diffraction data, as a test, were reprocessed using X-ray Detector Software (XDS). In particular, this was used to check the cisplatin electron density around the His-15. Figure S2 shows the XDS electron density around the His-15 binding sites for the cisplatin 200 K structure. The electron density maps are indeed very similar between the two processing programs, whereby three platinum atoms are also seen for the XDS processed dataset and moreover gave the same resolution cut off limit.

### TABLE I. X-ray crystallographic data and protein model refinement statistics.

| Protein          | Cisplatin | Cisplatin | Cisplatin | Carboplatin |
|------------------|-----------|-----------|-----------|-------------|
| Unit cell parameters (Å)/(deg) | a = 26.99 | a = 26.77 | A = 27.34 | a = 26.96 |
|                  | b = 31.81 | b = 31.38 | B = 32.13 | b = 31.79 |
|                  | c = 34.07 | c = 33.86 | C = 34.29 | c = 34.05 |
|                  | x = 89.08 | x = 88.90 | A = 88.04 | x = 88.76 |
|                  | β = 72.00 | β = 72.31 | B = 71.17 | β = 71.99 |
|                  | γ = 67.81 | γ = 68.46 | γ = 68.35 | γ = 68.33 |
| PDB id’s         | 4mwk      | 4mwm      | 4mwn      | 4oxe |
| Data collection temperature (K) | 150       | 200       | 295       | 200 |
| Crystal size (mm) | 0.6       | 0.3       | 0.25      | 0.2 |
| Total absorbed X-ray dose (MGy) | 0.31      | 0.37      | 0.48      | 0.31 |
| Crystal growth time | 5 weeks   | 8 days    | 6 weeks   | 11 days |
| Observed reflections | 202732    | 118846    | 112029    | 156662 |
| Unique reflections | 51605     | 37288     | 19160     | 35817 |
| Resolution (Å)   | 29.28–0.98 | 32.09–1.12 | 32.31–1.42 | 32.21–1.13 |
|                  | (1.02–0.98) | (1.15–1.12) | (1.51–1.42) | (1.16–1.13) |
| Completeness (%) | 90.6 (51.7) | 95.1 (84.6) | 99.2 (98.4) | 94.1 (77.0) |
| Rmerge (%)       | 0.045 (0.209) | 0.087 (0.179) | 0.145 (0.554) | 0.086 (0.510) |
| Mean I/σ(I)      | 15.6 (3.2) | 5.9 (2.1) | 6.8 (1.1) | 7.2 (2.0) |
| Redundancy       | 3.5 (0.6) | 2.2 (0.9) | 5.7 (2.0) | 2.9 (1.1) |
| Cruickshank diffraction precision index (Å) for coordinate error | 0.022 | 0.037 | 0.084 | 0.046 |
| Number of protein atoms | 1007 | 998 | 992 | 998 |
| Average B factor (Å²) for protein atoms | 7.2 | 11.4 | 14.7 | 9.4 |
| Number of water molecules | 142 | 94 | 41 | 98 |
| Average B factor (Å²) for water molecules | 16.4 | 19.7 | 22.6 | 30.2 |
| Number of cisplatin/carboplatin atoms | 15 | 11 | 4 | 13 |
| Average B factor (Å²) for cisplatin and carboplatin atoms | 33.5 | 38.8 | 27.1 | 36.3 |
| Number of other bound atoms | 85 | 72 | 28 | 54 |
| Average B factor (Å²) for other bound atoms | 18.9 | 35.4 | 29.3 | 32.0 |
| R factor/R free (%) | 11.7/14.5 | 14.7/18.7 | 20.8/23.5 | 17.7/22.1 |
| R factor all | 11.9 | 14.9 | 21.0 | 17.9 |
| root mean square deviation bond lengths (Å)/Angles (deg) | 0.039/2.907 | 0.023/2.323 | 0.021/2.247 | 0.031/2.592 |
| Ramachandran favoured | 97.5 | 97.5 | 96.0 | 95.8 |
| Ramachandran allowed | 2.5 | 2.5 | 3.2 | 4.2 |
| Ramachandran disallowed | 0 | 0 | 0.8 | 0 |

*aThe PDB validation reports for each structure are available in the PDB at the 4mwk, 4mwm, 4mwn and 4oxe entries.

*bTable 1 of the supplementary material shows the calculation used to estimate the total absorbed dose per dataset.

*cThe geometric edge of the detector set the resolution to be at 0.98 Å resolution.

*dResolution where |I/σ(I)| crosses 2 = 1.61 Å.

*eThe residue in the disallowed region of the Ramachandran plot is Asn-103, and which is part of a turn region in the protein.

Site only shows a split occupied platinum at 150 K data collection temperature (Figure S1(a)). However, the Nε binding site at each temperature shows multiple platinum positions (Figures S1(a)–S1(d)). Due to previously seeing slight differences in the electron density maps from using different diffraction data processing programs, the 200 K diffraction data, as a test, were reprocessed using X-ray Detector Software (XDS). In particular, this was used to check the cisplatin electron density around the His-15. Figure S2 shows the XDS electron density around the His-15 binding sites for the cisplatin 200 K structure. The electron density maps are indeed very similar between the two processing programs, whereby three platinum atoms are also seen for the XDS processed dataset and moreover gave the same resolution cut off limit.
TABLE II. Anomalous difference density and 2Fo-Fc density peak heights (as a multiple of σ or rms, respectively) for each platinum atom per dataset at the Nδ and Nε binding sites.

|                  | Nδ binding site |            |            | Nε binding site |            |            |
|------------------|-----------------|------------|------------|-----------------|------------|------------|
|                  | Pt 1            | Pt 2       | Pt 1       | Pt 2            | Pt 3       | Pt 4       |
|                  | Anom 2Fo-Fc     | Anom 2Fo-Fc| Anom 2Fo-Fc| Anom 2Fo-Fc     | Anom 2Fo-Fc| Anom 2Fo-Fc|
| Cisplatin HEWL crystal at 150 K | 17             | 14         | 6.1        | 5.6             | 7.1        | 4.4        |
| Cisplatin HEWL crystal at 200 K | 15             | 22         | 6.1        | 11              | 9.1        | 16         |
| Cisplatin HEWL crystal at RT   | 4.6            | 9.0        | 4.6        | 7.5             | 4.6        | 7.5        |
| Carboplatin HEWL crystal at 200 K | 3.9           | 4.6        |            | 8.5             |            | 6.9        |
As well as the multiple platinum binding sites, the electron density for the His side chain although weak (Figure 1) at each temperature improves with temperature; in the Protein Data Bank (PDB) validation reports for each structure it is only the His residue from the 150 K structure which shows up as an outlier. Weak electron density is due to a structure sampling multiple positions. In the His-15 case, due to these being relatively light atoms, the electron density will become weak in such a situation. By contrast, with the platinum atoms being heavier, the split-occupancy platinum binding sites will be seen more readily in the electron density.

Carboplatin was studied as a cross check of the cisplatin behaviour; the 200 K temperature was the one selected for study. For this carboplatin dataset (Figure S1(d) 31), the B factor for one of the platinums is large at 95 Å². Also, in the 2Fo-Fc electron density map in the Ne binding site there is extra detail for the atoms bound to the platinum atom, which in this case is

TABLE III. Occupancy values (%) of each platinum atom at the Nδ and Ne binding sites of His-15. Occupancies were calculated using SHELX12 and the errors on the occupancies were calculated using the full matrix inversion in SHELXL.

|                  | Nδ binding site | Ne binding site |
|------------------|----------------|-----------------|
|                  |Pt 1       |Pt 2       |Pt 1       |Pt 2       |Pt 3       |Pt 4       |
| Cisplatin HEWL crystal at 150 K | 22 +/- 5 | 13 +/- 12 | 7 +/- 9 | 60 +/- 2 | 22 +/- 9 | 25 +/- 8 |
| Cisplatin HEWL crystal at 200 K | 29 +/- 10 | 14 +/- 9 | 16 +/- 12 | 44 +/- 13 |
| Cisplatin HEWL crystal at RT | 36 +/- 2 | | 29 +/- 2 | 33 +/- 2 |
| Carboplatin HEWL crystal at 200 K | 20 +/- 5 | | 32 +/- 7 | 23 +/- 8 |

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FIG. 1. Face on view of the 2Fo-Fc electron density map around the His-15 side chain. (a) 150 K, (b) 200 K, and (c) RT. The 2Fo-Fc electron density map is shown in purple at a contour level of 1.5σ. The steadily improving electron density for the His 15 imidazole at increasing temperature is clearly visible. Figure S1 of the supplementary material shows the edge on view.
assigned to be a portion of the cyclobutanedicarboxylate (CBDC) moiety of the carboplatin molecule, as there is naturally no anomalous difference electron density at that position. There are fewer carboplatin split occupancy platinum atoms than is the case for cisplatin at the same data collection temperature; the reason for this is not known.

B. Structural dynamics of the cisplatin Pt atoms at the multiple temperatures

Due to the platinum atoms showing versatile binding across all three temperatures studied (Figure S1), the mean u square displacement values ($\langle u^2 \rangle$) of each platinum atom’s split occupancy were calculated based on their B factors using the formula $B = 8\pi^2\langle u^2 \rangle$. Figure 2 shows the plot of their $\langle u^2 \rangle$ values against temperature for each platinum atom in the binding sites, which is of the same form as Figure 2 in Doster et al. $^34$ The plot of mean square displacement values against temperature should ideally follow a quadratic curve. This is seen for Ne platinum 3 (green triangles). The other platinum atom sites do not show this exact trend, but do steadily increase with temperature, and some of the platinum atom sites, Nδ platinum 1 and Ne platinum 1 at 150 K and 200 K, respectively, have a lower mean $\langle u^2 \rangle$ displacement value compared to the average protein atom (orange stars) at the same temperature. One of the platinum atom sites at the data collection temperature of 150 K (Ne platinum 4) has an unusually high B factor (138 Å$^2$) and hence high $\langle u^2 \rangle$ (1.82 Å$^2$) and so it is omitted from the graph.

IV. DISCUSSION

The results show a split-occupancy binding for cisplatin and for carboplatin at the nitrogen binding sites of His-15 (Figure S1), evidenced by the presence of multiple anomalous difference electron density peaks and equivalent 2Fo-Fc density peaks (Table II). Thus, multiple binding modes (up to four at the Ne binding site and up to two at the Nδ binding site for cisplatin) are seen. As well as the multiple platinum peaks, the electron density for the His-15 side chains (Figure 1) are weak, which also points to the imidazole ring of the His side chain sampling multiple positions. This obviously correlates with the multiple platinum peaks as the His 15 takes up different positions so the nitrogen atoms can bind to the platinum at each of its sites. This could arise from a rotation about the Cβ imidazole bond, which would place the Nδ and Ne atoms in multiple positions near the Pt atoms, which is supported by the fact that no electron density is observed for the His-15 side chain. The occupancy values for the platinum atoms are individually lower (Table III), compared to those previously seen in the tetragonal HEWL crystal cases apart from platinum 2 in the Ne binding site of the 150 K crystal with an occupancy of 60%, which is similar. This platinum site, however, has a very large B factor.
(144 Å²). During the refinement procedure in Refmac, this platinum atom is somewhat unstable in position, whereas the Ne platinum 4 atom at 200 K has a B factor of 138 Å² and is ever present in the electron density after refinement. For a B factor of 144 Å² one can readily calculate that such a platinum atom’s X-ray scattering at 4 Å resolution, say, is 1% of that at zero scattering angle. This is then probably adequate for it to be resolved as a separate atom from its neighbours, which is indeed the case in the 2Fo-Fc and anomalous difference electron density maps. Whether it is an adequate scattering for Refmac to return a fairly well estimated B factor value of 144 Å², and a stable position for it remains a good question. The protein crystallography research community view of the question “what is the largest light atom B factor deemed to be reasonable?” is a value of ~80 Å². To accept a larger maximum value for a relatively heavier atom, albeit split-occupancy, is also thereby probably reasonable; but what then finally is a tolerable, physically meaningful, maximum B factor value? This is a significant question for the study of structural dynamics by crystallography to be considered in future in more detail.

The summed occupancies for each of the two cisplatin dataset’s platinum sites studied at 150 K and 200 K at the Ne of His15 is larger than has been seen previously. By using multiple temperatures in our study either side of the protein glass transition state, anticipated to be 180 K, this revealed two things: one is that not all these platinum cisplatin binding modes are stable, i.e., with high B factors developing in the Ne platinum 2 case. Another observation is that the mean square displacement vibration amplitudes of the most ordered platinum sites increased more than the average protein atom (Figure 2). Might this arise from X-ray absorbed dose effects? In a previous study, we showed that cisplatin binding was stable up to a dose of 1.7 MGy. The total absorbed X-ray doses of each dataset at the three temperatures in this study were 0.31 MGy, 0.37 MGy, 0.48 MGy, and 0.31 MGy (Table I and Table S1), and show that the cisplatin and carboplatin should be stable at these absorbed X-ray doses.

V. CONCLUSIONS

These studies show a split-occupancy binding of cisplatin to a histidine in the triclinic form of HEWL, and therefore more versatile than seen in our previous studies in the tetragonal and orthorhombic crystal forms. In particular, multiple cisplatin binding modes (up to four at the Ne binding site and up to two at the Nd binding site) were seen. The carboplatin showed a similar behaviour of split occupancy binding, although with two fewer sites than cisplatin. Second, by use of temperatures either side of the protein glass transition state, anticipated to be 180 K, this revealed two things: one was that not all these platinum cisplatin binding modes were stable, i.e., with very high B factors developing in two sites and another observation was that the mean u² displacement vibration amplitudes of the most ordered platinum sites increased more than the average protein atom. The electron density variation of the His-15 imidazole becoming more ordered at room temperature, although counter intuitive in terms of the physics, is obviously driven by the cisplatin binding chemistry but whose precise details of why there are multiple binding sites are difficult to discern; this system may well interest quantum chemists for detailed theoretical computations including molecular dynamics.

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See supplementary material at http://dx.doi.org/10.1063/1.4883975 for the further, figure of cisplatin and carboplatin bound to the Nδ and Nε atoms of His-15, the figure of cisplatin binding to the Nδ and Nε atoms of His-15 as seen with the X-ray diffraction data at 200 K processed via XDVS and a table of total X-ray absorbed Dose for each crystal.

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