Platination of superoxide dismutase with cisplatin: tracking the ammonia ligands using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS)†

Stefan K. Weidt, C. Logan Mackay, Pat R. R. Langridge-Smith and Peter J. Sadler*

Received (in Cambridge, UK) 7th February 2007, Accepted 23rd March 2007
First published as an Advance Article on the web 5th April 2007
DOI: 10.1039/b701903c

The high mass accuracy of FT-ICR MS combined with ^{15}N-labelling shows that mono- and di- platinated products from the reaction of erythrocyte superoxide dismutase with the anticancer drug cisplatin in solution retain their ammine ligands, in contrast to a recent X-ray crystallographic study.

Cisplatin, *cis*-[$PtCl_2(NH_3)_2$], is a widely used anticancer drug. There is interest in understanding not only the mechanism of its cytotoxicity, but also its side-effects and cellular resistance mechanisms. Although the major target site is thought to be DNA,[1] interactions with proteins are likely to play important biological roles.[2–7]

In aqueous media, cisplatin is usually activated by aquation, the substitution of its chlorido ligands by water. Aqua adducts are more reactive than cisplatin itself. Platinum(II) has a high affinity for sulfur ligands,[8] and strong binding sites on proteins are usually at the sulfur atoms of methionine (thioether) and cysteine (thiol). The nitrogen atoms in the imidazole side-chain of histidine are also potential binding sites. Although methionine S is normally the kinetically-preferred binding site, transfer to the more thermodynamically-stable Pt–N coordination has been observed.[9,10]

A recent paper by Calderone *et al.* reported[11] some remarkable and unexpected results. Their X-ray crystal structure of bovine superoxide dismutase (beSOD) after reaction with cisplatin showed platination of His19 but with both chlorido ligands still bound to Pt and both ammine ligands absent (Fig. 1). Apart from the Nε of His19, the other ligand(s) could not be defined in the electron density map. Such an observation is intriguing and raises the possibility that reactions of cisplatin with this enzyme can follow an unusual pathway in which ammonia release from platinum is readily promoted, perhaps induced by the high *trans* effect of protein S-ligands. We report here solution studies of the platination of bovine erythrocyte superoxide dismutase by cisplatin using FT-ICR MS. The high resolving power has allowed us to define the coordination sphere of bound Pt and to compare our findings with the reported X-ray crystal structure.

First we recorded the FT-ICR MS mass spectrum of beSOD (M_w 31432 Da).‡ The instrument was tuned so that the source region could transfer the ions as gently as possible into the high vacuum system. The gentle conditions from both the buffered solution and the desolvation enabled the beSOD non-covalent dimer to be observed and the bound copper and zinc ions to be retained. Fig. S1a shows the isotopic modelling of the beSOD 11+ ion, which has a very close match over all the isotopomers giving peaks above the noise level. The mass error from the isotopic fit is

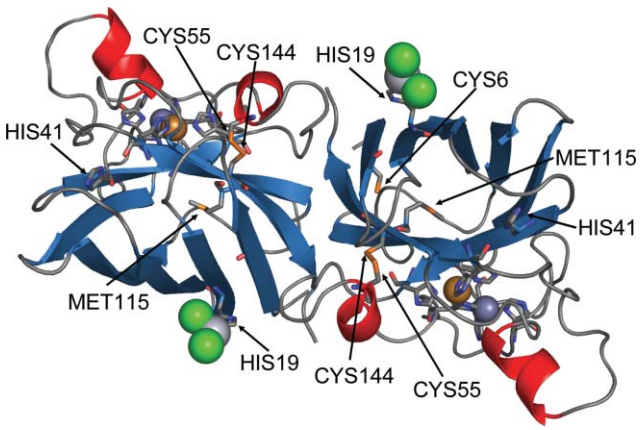

Fig. 1 X-ray structure of dimer of beSOD after platination with cisplatin showing the unexpected retention of Cl ligands and loss of NH_3 ligands after platination of His19 (PDB 2AEO).[11] Possible sites for further platinum binding are indicated and labelled: Met115, His41, Cys6, Cys55 and Cys144. Colour code: Pt silver, Cl green, Cu bronze, Zn grey-blue.

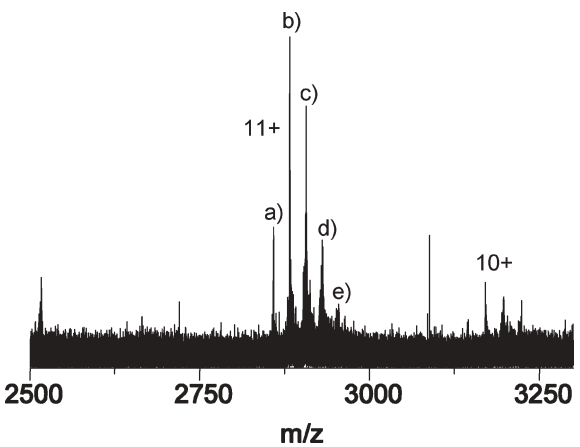

Fig. 2 The mass spectrum of beSOD after reaction with cisplatin for 16 h. Labelled peaks refer to 11+ ions of $(beSOD)_2$ (a) unmodified, (b) + $\{PtCl(NH_3)_2\}$, (c) + $\{PtCl(NH_3)_2\}_2$, (d) + $\{PtCl(NH_3)_2\}_3$, (e) + $\{PtCl(NH_3)_2\}_4$.

School of Chemistry, University of Edinburgh, West Mains Road, Edinburgh, UK EH9 3JJ. E-mail: p.j.sadler@ed.ac.uk; Fax: +44 (0)131 650 6452; Tel: +44 131 650 4729

† Electronic supplementary information (ESI) available: FT-ICR MS of $(beSOD)_2$ and of (beSOD)-$\{PtCl(NH_3)_2\}$ produced by collisional dissociation. Links to PDB visualizations are included in the online supplementary data. See DOI: 10.1039/b701903c

0.7 ppm, indicative of a good fit to the expected elemental composition. Fig. S1b shows that only three charge-states are seen for the dimer, the predominant charges being 11+ along with 10+, while the 12+ ion is of a very low abundance.

Copper-zinc superoxide dismutase (133 μM) was then incubated with a 10-fold molar excess of cisplatin or ^{15}N-labelled cisplatin (>98% enrichment) in aqueous solution at 310 K for 16 h. The samples were then stored for up to 32 days at 253 K until they were ready to be thawed, injected onto the gel filtration column as previously described, and analysed.

After reaction of beSOD with cisplatin,§ the predominant ion observed by FTMS was a monoplatinated 11+ ion (Fig. 2). Peaks for four platinated adducts can be seen, with the fourth being of very low abundance, just above the noise. The 10+ ion is very weak, but does appear to show the same binding pattern with the monoplatinated species being the most abundant. The mass spectrum shows that up to two $\{PtCl(NH_3)_2\}^+$ cisplatin fragments can bind per monomer. The observed mass is consistent with one of the chlorido ligands on each platinum being displaced upon binding, while the two ammine ligands are still present (Fig. 3a).

Increasing the capillary voltage allowed collisonally-induced dissociation within the source region of the homo-dimer, while maintaining the bound platinum complex. This increased the signal-to-noise ratio and reduced the charge on the molecular species making it easier to resolve the isotope pattern. The dissociation of the dimer led to the appearance of peaks from the unmodified beSOD monomer along with peaks from the mono- and di-platinum adducts (Fig. S2b). The increased internal energy also appears to promote dissociation of chlorine from platinum. Fig. S2a shows the isotopic modelling of a beSOD monomer with an increased mass of PtN_2H_6Cl, which is consistent with the binding of a $\{PtCl(NH_3)_2\}^+$ fragment to the protein.

Use of ^{15}N-labelled cisplatin in the reaction led to a clear mass shift of up to two atomic mass units per platinum bound to the protein, further supporting the conclusion that the ammine ligands have not been displaced during the binding of cisplatin to the protein (Fig. 3b).

A summary of the observed masses for the most abundant isotopomers using native ESI of beSOD along with the dimer and monomer peaks arising from reaction with cisplatin is shown in Table 1. The proposed elemental formulae are also listed along with the calculated most abundant mass at the same charge. It can be seen that the mass errors between calculated and observed values (based on external calibration) are all <10 ppm. The peak resolving powers are over 100,000, which meant that isotopic resolution was easily achieved, and this facilitated isotopic modelling for comparison with the proposed elemental formulae. Therefore our MS data suggest that in solution one or two $\{PtCl(NH_3)_2\}^+$ units readily bind to beSOD *via* loss of one chlorido ligand from cisplatin, but with retention of the two ammine ligands.

Consideration of space-filling models of beSOD (PDB 1Q0E or 2AEO) suggest that the likely binding sites are His19 and His41. The sulfurs of Cys6 (the only free thiol in the protein) and Met115 are buried in the core of the protein and are likely to be inaccessible to cisplatin. Cys55 and Cys144 form a disulfide bond, usually a very weak binding site. Hence the course of the reaction between cisplatin and beSOD in solution over a period of reaction (16 h) at 310 K (body temperature) studied here is more in line with the expected chemical reactivity of cisplatin,[12] based on an initial aquation and targeting of imidazole nitrogen in the absence of access to sulfur ligands. The crystals used in the reported study[11] of beSOD were obtained from a sample of protein which had reacted with 10 mol equiv. of cisplatin for 2 weeks (under unspecified conditions). Although a single binding site (His19) was detected in the X-ray structure, in solution there is clearly at least

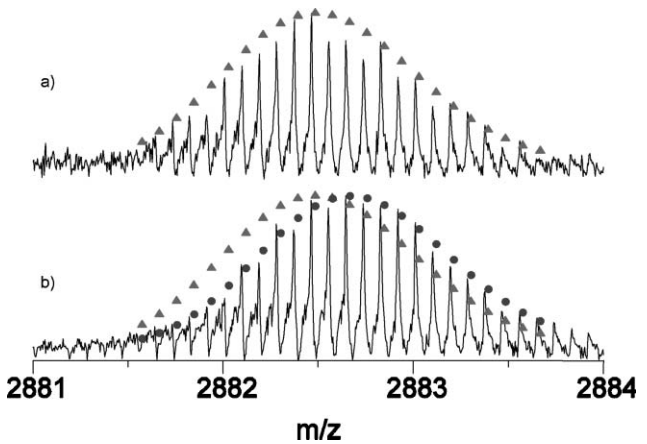

Fig. 3 The mass spectrum of the 11+ ion of $(beSOD)_2$-$\{PtCl(NH_3)_2\}$ obtained from reactions using (a) natural abundance cisplatin and (b) ^{15}N-labelled cisplatin. The 2 Dalton shift in isotopic pattern is consistent with the retention of the $^{15}NH_3$ ligands after the platination. Key: $C_{1344}H_{2157}N_{398}O_{442}Pt_1S_8Cl_1Cu_2Zn_2$ ▲ $C_{1344}H_{2157}N_{396}{}^{15}N_2O_{442}Pt_1S_8Cl_1Cu_2Zn_2$ ●.

Table 1 The masses of the observed most abundant isotopomer of each major ion and the proposed formula and the calculated masses. The mass error and resolving power of the most abundant mass are also listed

beSOD	Charge	Observed m/z^a	Formula	Calculated m/z^a	Error/ppm	Resolving power
Dimer, Fig. S1	11+	2858.487	$C_{1344}H_{2152}N_{396}O_{442}S_8Cu_2Zn_2$	2858.485	0.7	150,000
Monomer	6+	2620.281	$C_{672}H_{1076}N_{198}O_{221}S_4CuZn$	2620.277	1.5	220,000
Dimer + Pt, Fig. 2b	11+	2882.464	$C_{1344}H_{2157}N_{398}O_{442}Pt_1S_8ClCu_2Zn_2$	2882.483	6.8	209 000
Dimer + 2 Pt, Fig. 2c	11+	2906.565	$C_{1344}H_{2163}N_{400}O_{442}Pt_2S_8Cl_2Cu_2Zn_2$	2906.573	2.7	156 000
Dimer + 3 Pt, Fig. 2d	11+	2930.563	$C_{1344}H_{2169}N_{402}O_{442}Pt_3S_8Cl_3Cu_2Zn_2$	2930.572	3.0	114 000
Dimer + 4 Pt, Fig. 2e	11+	2954.666	$C_{1344}H_{2175}N_{404}O_{442}Pt_4S_8Cl_4Cu_2Zn_2$	2954.661	1.5	N/A
Monomer + 1 Pt–Cl	6+	2658.262	$C_{672}H_{1081}N_{200}O_{221}PtS_4CuZn$	2658.280	6.5	160 000
Monomer + 1 Pt, Fig. S2	6+	2664.427	$C_{672}H_{1082}N_{200}O_{221}PtS_4ClCuZn$	2664.443	5.9	151 000
Monomer + 2 Pt–Cl	6+	2702.266	$C_{672}H_{1086}N_{202}O_{221}Pt_2S_4ClCuZn$	2702.276	3.6	136 000
Monomer + 2 Pt	6+	2708.257	$C_{672}H_{1087}N_{202}O_{221}Pt_2S_4Cl_2CuZn$	2708.272	5.7	160 000

[a] Most abundant isotopomer.

one other strong Pt binding site, possibly His41. It is possible that monoplatinated beSOD crystallized preferentially.

Our work demonstrates the potential of high resolution FT-ICR MS for defining the coordination spheres of protein adducts of metallodrugs in solution. Such adducts can play important roles in mechanisms of action and, in the case of cisplatin, especially in toxic side-effects.

We thank RCUK (Interdisciplinary Research Collaboration in Proteomic Technologies, RASOR), BBSRC (studentship for SW) and SHEFC for support, members of the EC COST Action D39 for stimulating discussions, and Dr Vivienne Munk (Edinburgh), for her gift of natural and ^{15}N-labelled cisplatin.

Notes and references

‡ To prepare the samples for mass spectrometric analysis and to remove unbound cisplatin and thus prevent formation of further adducts during the electrospray desolvation process, a gel filtration column was used with 20 mM ammonium acetate pH 7 as mobile phase. An aliquot of the sample (20 μL) was injected onto a Tosoh Bioscience TSKgel SuperSW 2000 gel filtration column (4.6 × 30 cm) and eluted with the ammonium acetate buffer. An Agilent 1100 series HPLC was used at a flow rate of 350 μL min^{-1} and the UV detector monitored the absorbance at 214 nm. The major fraction with an elution time of *ca.* 8 to 12 min with a volume of *ca.* 1.5 mL was collected (*ca.* 75× dilution of the sample).

The gel filtration fraction was analysed using electrospray ionisation at a flow rate of 100 μL h^{-1} and detected by a Bruker Daltonics 9.4 T Apex III FT-ICR MS modified with a heated metal capillary built in-house. 500 scans were accummulated to produce a signal-to-noise ratio of 4 : 1.

§ Use of ammonium-based buffers, although they are compatible with electrospray mass spectrometry, was avoided during the reaction of beSOD with cisplatin to prevent possible exchange with ligands on platinum during this reaction period. Under the conditions used in this study, Fig. 2 clearly shows multiple binding sites. The isotope fitting and ^{15}N data show that it is the ammine ligands that are retained after the usual activation of cisplatin *via* aquation.

1 E. R. Jamieson and S. J. Lippard, *Chem. Rev.*, 1999, **99**, 2467–2498.
2 M. C. Cox, K. J. Barnham, T. A. Frenkiel, J. D. Hoeschele, A. B. Mason, Q. Y. He, R. C. Woodworth and P. J. Sadler, *J. Biol. Inorg. Chem.*, 1999, **4**, 621–631.
3 B. P. Esposito and R. Najjar, *Coord. Chem. Rev.*, 2002, **232**, 137–149.
4 (*a*) D. Gibson and C. E. Costello, *Eur. J. Mass Spectrom.*, 1999, **5**, 501–510; (*b*) T. Peleg-Shulman, Y. Najajreh and D. Gibson, *J. Inorg. Biochem.*, 2002, **91**, 306–311.
5 R. Mandal, R. Kalke and X. F. Li, *Chem. Res. Toxicol.*, 2004, **17**, 1391–1397.
6 I. Khalaila, C. S. Allardyce, C. S. Verma and P. J. Dyson, *ChemBioChem*, 2005, **6**, 1788–1795.
7 A. I. Ivanov, J. Christodoulou, J. A. Parkinson, K. J. Barnham, A. Tucker, J. Woodrow and P. J. Sadler, *J. Biol. Chem.*, 1998, **273**, 14721–14730.
8 (*a*) J. Reedijk, *Chem. Rev.*, 1999, **99**, 2499–2510; (*b*) J. Reedijk, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 3611–3616.
9 S. S. G. E. van Boom and J. Reedijk, *J. Chem. Soc., Chem. Commun.*, 1993, 1397–1398.
10 K. J. Barnham, M. I. Djuran, P. de S. Murdoch and P. J. Sadler, *J. Chem. Soc., Chem. Commun.*, 1994, 65–66.
11 V. Calderone, A. Casini, S. Mangani, L. Messori and P. L. Orioli, *Angew. Chem., Int. Ed.*, 2006, **45**, 1267–1269.
12 In a recent study of lysozyme platinated with cisplatin the ammine ligands were also retained on bound Pt: A. Casini, G. Mastrobuoni, C. Temperini, C. Gabbiani, S. Francese, G. Moneti, C. T. Supuran, A. Scozzafava and L. Messori, *Chem. Commun.*, 2007, 156–158.