Copper chaperone Atox1 interacts with the metal-binding domain of Wilson’s disease protein in cisplatin detoxification

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Human copper transporters ATP7B (Wilson’s disease protein) and ATP7A (Menkes’ disease protein) have been implicated in tumour resistance to cisplatin, a widely used anticancer drug. Cisplatin binds to the copper-binding sites in the N-terminal domain of ATP7B, and this binding may be an essential step of cisplatin detoxification involving copper ATPases. In the present study, we demonstrate that cisplatin and a related platinum drug carboplatin produce the same adduct following reaction with MBD2 [metal-binding domain (repeat) 2], where platinum is bound to the side chains of the cysteine residues in the CxxC copper-binding motif. This suggests the same mechanism for detoxification of both drugs by ATP7B. Platinum can also be transferred to MBD2 from copper chaperone Atox1, which was shown previously to bind cisplatin. Binding of the free cisplatin and reaction with the cisplatin-loaded Atox1 produce the same protein-bound platinum intermediate. Transfer of platinum along the copper-transport pathways in the cell may serve as a mechanism of drug delivery to its target in the cell nucleus, and explain tumour-cell resistance to cisplatin associated with the overexpression of copper transporters ATP7B and ATP7A.

Key words: ATPase, Cu(I) transport, Wilson disease protein (ATP7B), cisplatin, drug resistance, metal transport.

INTRODUCTION

Cisplatin has been one of the most potent and widely used anticancer drugs for 40 years. It is effective against testicular, ovarian, cervical, bladder, head and neck cancers, melanoma and lymphomas. Despite severe side effects, intrinsic and acquired resistance of tumours to cisplatin, and evaluation of thousands of alternative platinum-based compounds, cisplatin remains a mainstay of anticancer chemotherapy [1,2]. So far, only very few other platinum compounds, including carboplatin and oxaliplatin, have been introduced into the clinical practice alongside cisplatin (Supplementary Figure S1 at http://www.biochemj.org/bj/454/bj4540147add.htm). The anticancer effect of cisplatin is caused primarily by the impaired DNA replication in rapidly dividing cells due to the formation of intra- and inter-strand cross-links that cause cell apoptosis. The primary site of platinum reaction with DNA is believed to be N7 of guanine [3]. Cisplatin may also cause apoptosis through the endoplasmic reticulum stress pathway [4]. En route to its target in the cell nucleus, cisplatin undergoes complex chemical transformations and interacts with various proteins and small molecules. Many of these interactions modulate cisplatin toxicity and lead to tumour resistance to the drug [5].

Copper-transport proteins have emerged as the major factors affecting biological activity of cisplatin [6–8]. Cisplatin entry into the cell is facilitated by the passive copper carrier Ctrl [9] and possibly Ctr2 [10]. Metal chaperone Atox1, which physiologically delivers copper to the active membrane transporters ATP7B (Wilson’s disease protein) and ATP7A (Menkes’ disease protein), was found to bind cisplatin [11–13] and regulate cisplatin accumulation in the cells [14,15]. Both Wilson’s disease ATPase (ATP7B) [7,16] and Menkes’ disease ATPase (ATP7A) [17] have been linked to tumour resistance to cisplatin.

ATP7B and ATP7A are structurally similar P-type ATPases, which catalyse copper transport across the membranes of human cells. ATP7B delivers copper into the trans-Golgi network for incorporation into the copper-dependent enzymes, and transports excess copper out of the cell [18]. The protein has eight transmembrane helices and three functionally distinct cytoplasmic domains. ATP hydrolysis, which powers copper transport, occurs in the ATP-binding domain and is assisted by the A-domain (actuator domain). Unique to the mammalian copper-transporting ATPases is the large N-terminal domain, which contains six homologous MBDs (metal-binding domains (repeats)) of approximately 70 amino acid residues in length. Each repeat contains a conserved GM(T/H)CxxC motif, where Cu(I) binds to the thiolate groups of the two cysteine residues in a linear repeat, which catalyses copper transport across the membranes of human cells. ATP7B delivers copper into the cell nucleus, and explain tumour-cell resistance to cisplatin associated with the overexpression of copper transporters ATP7B and ATP7A.

A positive correlation between the expression level of ATP7B and cell resistance to cisplatin has been well documented for several different types of tumours, and reviewed in previous papers [6,16]. Active extrusion of platinum derivatives was proposed to explain the increased cell resistance to cisplatin associated with ATP7B [25]. Alternatively, cisplatin could be trapped and detoxified by the MBD of ATP7B without active transport across the membrane. We have shown that cisplatin...
indeed binds to the copper-binding sites in the N-terminal domain of ATP7B [26].

In addition to DNA, cisplatin reacts with many other abundant cellular components including proteins, phospholipids, glutathione and individual amino acids [3]. Most of the platinum in cisplatin-treated cells was found to be associated with biopolymers [27] or glutathione [28]. The reaction with glutathione was proposed to be one of the major routes of cisplatin detoxification in the cells [28,29]. Even in the cells overexpressing ATP7B, its abundance is very low compared with glutathione, which is present at millimolar concentrations, or even compared with the potential protein acceptors of cisplatin such as metallothionein [28] and thioredoxin [30]. How then can a moderate increase in the ATP7B expression level confer significant resistance to cisplatin? To address this question and characterize cisplatin interaction with the MBD of ATP7B at the molecular level, we investigated the reaction of cisplatin with the second metal-binding repeat (MBD2).

EXPERIMENTAL

Protein expression and purification

The unlabelled and uniformly 15N-labelled Atox1, and the wild-type and mutant variants of MBD2 were expressed as fusions with an intein and the chitin-binding domain under wild-type and mutant variants of MBD2 were expressed as fusions with an intein and the chitin-binding domain under control of the IPTG-inducible lac promoter using pTYB12 vector (New England Biolabs) in Escherichia coli BL21(DE3) cells, and purified essentially as described previously [31]. The purified protein was dialysed against two changes of 50 mM Hepes (pH 7.4) and 50 mM NaCl, the second time with 0.6 mM TCEP [tris-(2-carboxyethyl)phosphine] added. A typical protein yield was 3 mg/l of cell culture. The protein concentration was determined by the Cu-BCA assay (Pierce). Before the treatment with cisplatin or CuCl, MBD2 was incubated on ice for 30 min with 10 mM EDTA at 50 μM protein concentration, and then dialysed against metal-free 50 mM Hepes, pH 7.4, 50 mM NaCl and 0.6 mM TCEP to remove the bound metals and reduce the cysteine residues.

Analysis of cisplatin binding by MS

MBD2 was incubated with cisplatin (STREM Chemicals) for 2 h at 25°C at a 1:1 molar ratio in 50 mM Hepes, pH 7.4, 50 mM NaCl and 5 mM TCEP. Then the samples were buffer-exchanged on the Sephadex G-25 columns against 20 mM ammonium acetate (pH 7.5) to remove free cisplatin, buffer and salt. Samples were diluted 2.5 times in 67% methanol and directly infused into Q-TOF Ultima Global mass spectrometer (Waters) with Nanospray source option in the positive ion electrospray mode. Data were processed using MassLynx 4.1 and MaxEnt deconvolution algorithm to transform multiply charged peaks to the single charged positive ion spectra. Ubiquitin was used as a standard to calibrate and confirm MaxEnt transformations.

NMR

Samples for NMR contained 50 mM Hepes, pH 7.4, 50 mM NaCl, 5 mM TCEP, 1 mM DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate), 5% (v/v) 2H2O, and either 50 μM (cisplatin and copper-binding studies) or 0.5–1.0 mM (chemical shift assignments and structure determination) MBD2. Cisplatin, carboplatin or copper was added at a 1:1 molar ratio. For chemical shift assignments of the cisplatin-bound MBD2, the protein was treated with cisplatin at 50 μM protein concentration at a 1:1 molar ratio, and then concentrated to 0.5 mM by ultrafiltration. All NMR sample tubes were flushed with argon and then flame-sealed. NMR experiments were performed on a 600 MHz Bruker spectrometer equipped with a Cryoprobe with z-axis pulse field gradients. Combined chemical shift change was calculated as \( \left( \Delta \delta_{\text{NH}} + \Delta \delta_{z} \right) / 2 \), where \( \Delta \delta_{\text{NH}} \) and \( \Delta \delta_{z} \) are the chemical shifts changes of the amide proton and nitrogen respectively. Backbone chemical shift assignments were made from HNCO, HNCA, HN(CO)CACB and HN(CA)CO data. Side-chain chemical shift assignments were made from 3D H(CHO)NH, CC(ON)H and \( ^{1}H,^{15}N\)-TOCSY experiments. Distance restraints were derived from the 3D \( ^{1}H,^{15}N\)-NOEYS 3D \( ^{1}H,^{13}C\)-NOEYS experiments. Data were processed and analysed using NMRPipe [32]. The structure was calculated using CYANA 3.0 [33], validated using PSVS [34] (Supplementary Table S1 at http://www.biochemj.org/bj/454/bj4540147add.htm) and deposited in the Protein Data Bank as PDB code 2LQB. Chemical shift assignments have been deposited in the Biological Magnetic Resonance Bank as BMRB code 18301.

Atox1–MBD2 and MBD2–GSH cisplatin transfer experiments

To measure cisplatin transfer from Atox1 to MBD2, purified unlabelled Atox1 was treated with cisplatin as described above for MBD2 at a cisplatin/protein molar ratio of 1:1. Absence of cross-linking was confirmed by SDS/PAGE. Atox1 was concentrated to 0.5 mM, and added to 15N-labelled MBD2 at a molar ratio of 1:1 before recording the \( ^{1}H,^{15}N\)-HSQC spectra. For the reverse transfer experiment, unlabelled unmodified Atox1 was added at 2:1 ratio to 15N-labelled MBD2 pre-treated with cisplatin by the standard procedure. To monitor the return of MBD2–cisplatin complex to the apo form in the presence of GSH, the protein was reacted with cisplatin under standard conditions for 12 h, then subjected to gel filtration on a Superdex-200 column in the buffer containing 50 mM Hepes (pH 7.4) and 50 mM NaCl, and concentrated to 50 μM. Then GSH was added to the protein to a final concentration of 5 mM.

XAS (X-ray absorption spectroscopy)

Purified MBD2 was treated with cisplatin for 2 h or carboplatin for 8 h at 1:1 molar ratio and protein concentration of 50 μM in 50 mM Hepes, pH 7.4, 50 mM NaCl and 5 mM TCEP. A control sample of cisplatin was prepared in the same way, but without the addition of protein. Non-bound platinum compounds were removed by passing the MBD2 sample through a Sephadex G-25 column. The protein was concentrated to 0.2–0.5 mM, supplemented with 15% (v/v) glycerol, loaded into a 2 mm×3 mm×25 mm acrylic cuvette with a thin Mylar window and frozen in liquid nitrogen. The reference platinum compounds were finely ground and diluted with boron nitride to achieve absorption of 2 units at the platinum L\( _{\text{m}} \) edge. The mixtures were packed into the aluminium holders with a 1-mm pathlength with thin Mylar windows.

XAS measurements used beamline 7–3 at the Stanford Synchrotron Radiation Lightsource with SPEAR storage ring operating at 3 GeV using a Si(220) double-crystal monochromator. X-ray absorption spectra were measured at the platinum L\( _{\text{m}} \) absorption edge and the samples were maintained at 10 K. X-ray absorption was measured by monitoring the X-ray fluorescence excitation spectrum using a 30-element Ge array detector. To avoid non-linearity of the detector due to the high count rates, six absorption-length zinc oxide X-ray
filters were used to preferentially absorb scattered radiation. Soller slits were placed between the sample and the detector to reduce filter fluorescence. X-ray transmittance was measured using nitrogen-filled ionization chambers. The monochromator was energy-calibrated with reference to the lowest energy LII-emission peak of the platinum metal foil, which was assumed to be 11.563.0 eV. The EXAFS (extended X-ray absorption fine structure) oscillations \( \chi(k) \) were analysed by curve-fitting using the EXAFSPAK suite of computer programs (http://ssrl.slac.stanford.edu/exafspak.html) employing the EXAFSPAK suite of computer programs (http://ssrl.slac.stanford.edu/exafspak.html) employing \( \chi(k) \) theoretical phase functions generated with the program FEFF 7.0. No smoothing. Fourier filtering or related manipulations were performed on the data. Fourier transforms were phase-corrected for platinum–sulfur scattering for the protein samples, platinum–chlorine for cisplatin solutions and powder, and platinum–oxygen for the carboplatin sample.

RESULTS

We have shown previously that at least three repeats in the N-terminal domain of ATP7B bind cisplatin at or in the immediate proximity of the copper-binding sites. The indications that cisplatin binds at the copper-binding CxxC motif were particularly clear for MBD2 [26], which is also the preferred acceptor from the copper chaperone Atox1 [35]. Therefore we chose MBD2 for a more detailed investigation of cisplatin binding and transfer by NMR and XAS. To analyse cisplatin binding to MBD2 in the context of the protein structure, we have completed backbone chemical shift assignments and determined a 3D structure of the metal-free MBD2 by NMR. The structure of MBD2 (Figure 1) is similar to that of the other MBDs of ATP7B and the MBD2 of Menkes’ disease ATPase (ATP7A) (Supplementary Figure S2A at http://www.biochemj.org/bj/454/bj4540147add.htm) [36], which shares 60% sequence identity with MBD2 from ATP7B. The mean ensemble RMSD between the NMR structures of MBD2 from ATP7B and ATP7A is 1.8 Å (1 Å = 0.1 nm) for the backbone atoms. Similar to ATP7A, we observed conformational disorder in the loop connecting β1-strand to the α1-helix, which houses the copper-binding CxxC motif, in ATP7B (Supplementary Figure S2).

We then mapped the cisplatin-binding site in MBD2 by NMR using chemical shift perturbation analysis (Figure 2). Cisplatin was shown recently to cause denaturation and inter-molecular cross-linking of a functionally related copper chaperone Atox1 [13] and of ATP7B MBD6 [37] when it was present in large excess over the protein. Considering the large number of potential protein and low-molecular-mass reactants in the cytosol, formation of platinum cross-linked homo-oligomers is unlikely to take place in the cell. At an MBD2 concentration of 50 μM and a cisplatin to protein ratio of 1:1, we observed complete (more than 90%) binding of cisplatin to MBD2 without any signs of protein unfolding or intermolecular cross-linking as judged by NMR (Figure 2A) and non-reducing SDS/PAGE (Supplementary Figure S3 at http://www.biochemj.org/bj/454/bj4540147add.htm). Consistent with the earlier observations, cisplatin did cause MBD2 aggregation at higher cisplatin to protein ratios (Supplementary Figure S4 at http://www.biochemj.org/bj/454/bj4540147add.htm).

We have compared the effects of cisplatin and copper binding on the MBD2 spectra (Figures 2A and 2C). Cu(I) binding to MBD2 caused large chemical shifts changes in the CxxC motif, and a few neighbouring residues (Figure 3). Cisplatin binding produced major changes in the CxxC region, but, in addition, cisplatin caused chemical shift changes in a larger region proximal to the CxxC motif in the 3D space, including the α2-helix and parts of the β-sheet region (Figure 3B). Neither cisplatin nor copper caused any changes in the spectrum of the SxxS variant of MBD2, where the cysteine residues in the copper-binding motif were replaced with serine residues (Figures 2B and 2D). Taken together, these data demonstrate that cisplatin reacts with cysteine residues in the copper-binding CxxC motif. A more extensive spread of the secondary chemical shifts caused by cisplatin is consistent with the larger size of the bound drug molecule compared with the copper ion, and may also reflect conformational changes in the protein required to accommodate platinum with four ligands in a square planar co-ordination. We also investigated binding of carboplatin, another common platinum chemotherapy drug, to MBD2. Despite quite different chemical structures of the two drugs, spectral changes caused by carboplatin (Figure 2E) were identical with those caused by cisplatin indicating that the platinum derivative bound to the protein is the same in both cases. The result suggests that ATP7B can confer resistance to both drugs through the same molecular mechanism. Binding of both cisplatin and carboplatin to MBD2 occurred in the slow exchange regime, but the rates of reaction of MBD2 with cisplatin and carboplatin were quite different. The binding of cisplatin was complete within 4 h, whereas a complete protein modification with carboplatin was achieved only after several days (Figure 4).

We then investigated the nature of the MBD2 (see Figure 2F for amino acid sequence) adduct with cisplatin. Electrospray–MS of cisplatin-modified MBD2 showed a single prominent peak at 9109 Da in addition to a smaller peak at 8414 Da, the molecular mass of the adduct and the product of its aquation (Supplementary Figure S4 at http://www.biochemj.org/bj/454/bj4540147add.htm). Consistent with the earlier observations, cisplatin reacted with cysteine residues in the copper-binding CxxC motif. A more extensive spread of the secondary chemical shifts caused by cisplatin is consistent with the larger size of the bound drug molecule compared with the copper ion, and may also reflect conformational changes in the protein required to accommodate platinum with four ligands in a square planar co-ordination. We also investigated binding of carboplatin, another common platinum chemotherapy drug, to MBD2. Despite quite different chemical structures of the two drugs, spectral changes caused by carboplatin (Figure 2E) were identical with those caused by cisplatin indicating that the platinum derivative bound to the protein is the same in both cases. The result suggests that ATP7B can confer resistance to both drugs through the same molecular mechanism. Binding of both cisplatin and carboplatin to MBD2 occurred in the slow exchange regime, but the rates of reaction of MBD2 with cisplatin and carboplatin were quite different. The binding of cisplatin was complete within 4 h, whereas a complete protein modification with carboplatin was achieved only after several days (Figure 4).

We then investigated the nature of the MBD2 (see Figure 2F for amino acid sequence) adduct with cisplatin. Electrospray–MS of cisplatin-modified MBD2 showed a single prominent peak at 9109 Da in addition to a smaller peak at 8414 Da, the latter corresponding to the unmodified MBD2 with a theoretical molecular mass of 8416 Da (Figure 5). A product with the same molecular mass was observed after reaction of MBD2 with carboplatin (Supplementary Figure S5 at http://www.biochemj.org/bj/454/bj4540147add.htm). As expected, no reaction products were detected by MS of the SxxS variant of MBD2 following incubation with cisplatin (Supplementary Figure S6 at http://www.biochemj.org/bj/454/bj4540147add.htm).

The difference of 695 Da between the adduct and the unmodified MBD2 is much larger than the molecular mass of cisplatin (300 Da) or the product of its aquation (Supplementary Figure S1), which is believed to be the active form of the drug. The possibility of two cisplatin molecules bound to the protein can be also excluded, because the reaction was conducted at a 1:1 molar ratio of drug to protein, and resulted in a complete protein modification without formation of the cross-linked products. This led us to the conclusion that platinum bound to MBD2 must contain new bulky ligands that would account for the observed molecular mass of the adduct.
Figure 2  Cisplatin, carboplatin and copper binding to MBD2 analysed by $^1$H,$^{15}$N-HSQC spectroscopy

(A) Overlay of apo (black) and cisplatin-bound (red) wild-type MBD2 spectra; (B) overlay of SxxS-MBD2 spectra recorded with (red) and without (black) cisplatin; (C) overlay of apo (black) and Cu(I)-bound (red) wild-type MBD2 spectra; (D) overlay of SxxS-MBD2 spectra recorded with (red) and without (black) copper; (E) overlay of MBD2 spectra after reaction with cisplatin (black) and carboplatin (red); (F) sequence of MBD2 with elements of secondary structure shown. Assignments of peaks showing significant metal-dependent secondary shifts are shown by corresponding sequence numbers. In the MBD2 protein constructs, Q5 (Gln5) corresponds to Gln141 of the full-length ATP7B. The first four residues are cloning artefact.

We then used XAS to determine the chemical form of platinum bound to MBD2. In general, EXAFS can readily discriminate between the backscatterers with significantly different atomic numbers, but not so if they are similar. Thus nitrogen and sulfur are trivial to distinguish, whereas phosphorus, sulfur and chlorine are hard to tell apart. The best fit of the platinum LIII EXAFS spectra of MBD2 reacted with either cisplatin or carboplatin (Figure 6) consistently shows four ligands with sulfur, chlorine or phosphorus atoms in the first shell (Table 1). This result is in marked contrast with the spectra of pure cisplatin in solution, which show an average of $2.0 \pm 0.7$ oxygen or nitrogen ligands, and $1.9 \pm 0.4$ sulfur, chlorine or phosphorus ligands in the first shell, which is consistent both with the chemical structure of cisplatin and its partial aquation in solution, where the two ammine groups remain in place, and the two chlorines are gradually replaced by the water molecules (Supplementary Figure S1).

As our data clearly indicate involvement of the cysteine residues in the CxxC motif in the reaction with cisplatin, two of the four phosphorus, sulfur and chlorine platinum ligands in the MBD2–platinum complex are likely to be side-chain sulfur atoms of cysteine residues. Previous studies of cisplatin interaction with cysteine residues in proteins showed that platinum usually loses chlorine ligands in the course of the reaction [12,28,39], whereas carboplatin does not contain chlorine at all. Thus it was unlikely that the remaining two ligands in the MBD2-platinum adducts were chlorine atoms. One of the platinum ligands in the crystal structure of Atox1 complex with cisplatin [12], along with the two cysteine side chains and a backbone amide group, was found to be TCEP, a common reducing agent, which was also used in our experiments. By analogy, we considered the possibility that two TCEP molecules are bound to platinum in the MBD2–platinum adducts.
Atox1 interacts with ATP7B in cisplatin detoxification

Figure 3  Cisplatin and copper bind to the same region of MBD2

(A) Combined chemical shift changes caused by copper and cisplatin binding as a function of amino acid residue number. (B) Regions of the MBD2 structure affected by cisplatin and copper binding. C18 (Cy530) and C21 (Cy531) were not assigned in cisplatin-bound form (*cyan).

Figure 4  Time course of cisplatin and carboplatin reaction with MBD2, and of the return of the MBD2–cisplatin adduct to the apo form in the presence of glutathione

Concentration of the MBD2–cisplatin complex (MBD2–Pt) was calculated as [MBD2–Pt] = [MBD2total]V27–Pt/(V27–apo + V27–Pt), where [MBD2total] is the total MBD2 concentration in the NMR sample, and V27–apo and V27–Pt are peak volumes of the G27 backbone amide signal in the cisplatin bound form and apo form respectively measured in the 1H,15N-HSQC spectrum (compare with Figure 2A) recorded at a given time after cisplatin or carboplatin addition to MBD2, or glutathione addition to the MBD2–cisplatin complex. Cisplatin (○); carboplatin (□); glutathione (△).

Comparison of the XAS spectra of MBD2–cisplatin with the model compound cis-dichlorobis(triethylphosphine)platinum(II) provided support for this interpretation. The L_{3} edge spectra of Pt(II) bound to two phosphine ligands have a unique shape and characteristically shifted energy threshold compared with the spectra of the other platinum compounds. Unlike pure cisplatin, the spectra of MBD2–cisplatin, dichlorobis(triethylphosphine)platinum(II), and free cisplatin solution prepared with TCEP all displayed these characteristic features (Figure 7, and Supplementary Figure S8 at http://www.biochemj.org/bj/454/bj4540147add.htm). On the basis of the XAS data, we conclude that Pt(II) is co-ordinated by two sulfur and two phosphorus ligands in the product of the reaction between MBD2 and cisplatin (Figure 7). Consistent with this interpretation, the molecular mass of 9109 Da observed by MS for the MBD2 modified with cisplatin corresponds to the protein with one atom of platinum and two TCEP molecules (calculated molecular mass 9112.21 Da).

The emerging role of copper ATPases in cell resistance to cisplatin is confounded by an apparent paradox. ATP7A and ATP7B are low-abundance proteins. As a useful estimate, ATP7A at normal expression levels is present at approximately 500 molecules per cell [40]. At the same time, the concentration of the other potential cisplatin scavengers, such as glutathione can reach millimolar levels. How then can a modest increase in ATP7B expression level result in a significant increase in cell resistance to cisplatin? A possible explanation is that cisplatin hijacks copper chaperone Atox1, which hands over platinum derivatives directly to the MBDs of ATP7B and, by extension, ATP7A. To test this hypothesis, we investigated platinum transfer from Atox1 to MBD2 by NMR.
The addition of unlabelled Atox1 pre-loaded with cisplatin to 15N-labelled MBD2 produced chemical shift changes that were almost identical with those caused by the free cisplatin (Figure 8). This demonstrates transfer of the cisplatin derivative from Atox1 to MBD2 in the same chemical form that results from the reaction of MBD2 with free cisplatin. When this experiment was performed with 15N-labelled Atox1 and unlabelled MBD2, all of the peaks in the Atox1 spectrum returned to the apo form chemical shift values (Supplementary Figure S9 at http://www.biochemj.org/bj/454/bj4540147add.htm), although some peaks corresponding to the residues in the region around the CxxC motif showed loss of intensity, which may reflect chemical or conformational exchange. Taken together, data from the present study show that the cisplatin derivative was indeed fully transferred to MBD2 and was not bridging an Atox1–MBD2 heterodimer. Transfer of platinum from Atox1 to MBD2 was unidirectional. No transfer of platinum from MBD2 pre-treated with cisplatin to the free Atox1 was observed (Supplementary Figure S10 at http://www.biochemj.org/bj/454/bj4540147add.htm).

We have proposed previously that the MBDs of ATP7B serve as a kinetic trap for cisplatin, rapidly binding the drug with high affinity and then releasing it to a more abundant terminal acceptor in the cytosol, such as glutathione. Consistent with this idea, we have observed gradual return of MBD2 reacted with cisplatin to the apo form in the presence of GSH, indicating that glutathione can indeed serve as a platinum acceptor from MBD2. Although the rate of this process was low, it proceeded to a complete restoration of the MBD2 apo form (Figure 4).

**Table 1 Platinum LIII edge EXAFS curve-fitting parameters for cisplatin and carboplatin in the buffer and bound to MBD2**

Values in parentheses are the estimated S.D. obtained from the diagonal elements of the covariance matrix. We note that these are precisions and are distinct from the accuracies which are expected to be larger (≈ ± 0.02 Å for R, and ± 20% for N and σ²). Threshold energy shift ΔE₀ (eV) was calculated for model compounds and protein samples and its average value of −9.8 eV was used as fixed parameter for all subsequent refinements. The amplitude scale factor, otherwise known as the many-body amplitude reduction factor, or S₀², was defined by fitting data from a number of model fit-error compound species as 1.0. The function F is defined as \[ F = \sqrt{\sum_{i} \frac{x(k)_{\text{calc}} - x(k)_{\text{expt}}}{x(k)_{\text{expt}}}^2} \]

where the summations are over all data points included in the refinement. In all cases the k-range of the data fitted was from 1.0 to 14.2 Å⁻¹. N, co-ordination numbers; R, interatomic distances (Å); σ², Debye–Waller factors (Å²).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nitrogen or oxygen ligand</th>
<th>Sulfur, chlorine or phosphorus ligand</th>
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<tr>
<td>MBD2 plus cisplatin</td>
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<tr>
<td>MBD2 plus carboplatin</td>
<td>–</td>
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<tr>
<td>Cisplatin*</td>
<td>2.0(7)†</td>
<td>2.040(3)</td>
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<tr>
<td>Cisplatin plus TCEP*</td>
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†EXAFS spectra are included in Supplementary Figure S7 at http://www.biochemj.org/bj/454/bj4540147add.htm. +In this refinement the co-ordination number was constrained to total four, with ratio between the individual components being allowed to vary.
DISCUSSION

We have shown previously that the MBDs in the N-terminal domain of ATP7B react with and detoxify cisplatin. In the present study we investigated the molecular mechanism of cisplatin’s interaction with MBD2. In this protein, cisplatin reacts with the cysteine residues in the CxxC copper-binding motif. The other MBDs in ATP7B and ATP7A are similar in sequence and structure to MBD2 and are likely to react with cisplatin by the same mechanism, although it is not clear whether all of them are accessible to cisplatin in the full-length N-terminal domain in the native enzyme. MBD6’s reaction with cisplatin has been described recently [37].

All of the cisplatin ligands can be replaced by nucleophiles, depending on their type and steric accessibility in the protein [41]. We have demonstrated that following the reaction of cisplatin with MBD2, platinum loses all of its ligands and is co-ordinated by the sulfur atoms in the side chains of the two proximal cysteine residues. The two other ligands were identified as substituted phosphine groups of the reducing agent TCEP present in the reaction medium. Sulfur ligands have a strong trans-labilization effect that induces fast release of the ammine ligands in the trans-position [27,42], and although we also observed the TCEP reaction with cisplatin in the absence of protein, the exact reaction route for cisplatin binding to MBD2 is not clear. Although TCEP is not a physiological compound, the reaction of cisplatin with MBD2 in the presence of TCEP is similar to the other reported cases of cisplatin binding to proteins and peptides, where a reaction with cysteine or methionine residues leads to the exchange of ammine and chlorine ligands by two sulfur atoms, and two other ligands, which can vary depending on the protein structure and experimental conditions [41,43]. Biphosphane derivatives of platinum, where the metal is co-ordinated by two phosphorus and two sulfur or chlorine atoms, are under active investigation as potential chemotherapy agents, and some of them show antiproliferative activity similar to that of cisplatin [44,45].

In the cell, the reaction between cisplatin and the CxxC motif in MBD2 probably produces an adduct where platinum is co-ordinated by two sulfur atoms from the cysteine side chains, and, possibly, by the backbone amide groups of the neighbouring amino acid residues. Because the ammine ligands are believed to be important for binding of cisplatin to DNA [42], removal of these ligands as observed in our experiments may provide a mechanism for cisplatin detoxification by the MBDs. Interestingly, NMR, EXAFS and MS data consistently show the same type of platinum co-ordination for cisplatin and carboplatin after binding to MBD2 despite notably different chemical structures of the two compounds. This indicates that the mechanism of ATP7B-mediated resistance should be the same for both drugs.

Cisplatin toxicity to the tumour cells is believed to be mainly on the basis of its binding to DNA, which causes formation of intra- and inter-strand cross-links and eventually leads to apoptosis [46]. At the same time, cisplatin readily reacts with the highly abundant thiol groups of proteins and glutathione in the cytoplasm, and this reaction competes with a slower aquation reaction (Supplementary Figure S1), which produces the active form of platinum that preferentially reacts with DNA. Reaction with cysteine residues, which often leads to the loss of ammine groups [12,28,41] and produces much less reactive forms of Pt(II), has been long recognized as a likely mechanism for cisplatin detoxification in the cell. However, cisplatin, which is present in the cell in micromolar concentrations during chemotherapy, does reach its target DNA in sufficient amounts to kill the cells despite these competing reactions in the cell cytosol. This suggests more complex pathways than a simple competition between the detoxification by indiscriminate reactions with cysteine and methionine residues on the one hand, and diffusion of the activated form of cisplatin to reach DNA in the cell nucleus on the other.

A puzzling aspect of tumour resistance to cisplatin is by now a well-established strong positive correlation between the expression level of copper-transporting ATPases, ATP7B and ATP7A, and tumour resistance to cisplatin observed both in the experimental setting in cell culture and in tumours from the patients undergoing chemotherapy with cisplatin. Silencing of ATP7B expression by siRNA significantly decreased cell resistance to cisplatin [47,48], providing perhaps the strongest argument for the direct involvement of ATP7B in cisplatin detoxification in the cell.

Active extrusion of platinum derivatives has been proposed to explain the resistance to cisplatin associated with overexpression of ATP7B and ATP7A. However, it is unclear whether the experimentally observed rate of platinum transport [25] is sufficient to confer cell resistance in vivo. As an alternative, we proposed that cisplatin is detoxified by sequestration in the multiple MBDs in the N-terminal domain of ATP7B [26]. Expression of a soluble fragment of the N-terminal domain containing copper-binding repeats 1–4 (MBD1–4) in E. coli
protected the cells from the toxic effects of cisplatin [26]. Thus sequestration in the MBDs of ATP7B can, in principle, increase cell resistance to cisplatin without active extrusion of the drug across the cell membrane.

The low abundance of ATP7B, even under moderate overexpression conditions observed in tumour isolates, raises two further questions. Why does cisplatin preferentially react with ATP7B rather than with much more abundant alternative cysteine-containing acceptors in the cytosol? How can a limited binding capacity of ATP7B account for the effective detoxification of cisplatin under the conditions of chemotherapy?

A possible answer to these questions is suggested by our observations of platinum transfer from Atox1 pre-loaded with cisplatin to MBD2. As MBD2 and the other MBDs were shown previously to accept copper from Atox1 [21,35,49], this process appears to parallel the route of physiological copper relay in the cell. However, unlike copper transfer between Atox1 and MBD2, which is readily reversible, cisplatin transfer from Atox1 to MBD2 is unidirectional. That result strongly supports the concept of cisplatin transfer along the copper-transport pathways in the cell [8]. Atox1 may accept cisplatin at the entry point into the cell, possibly copper channel Ctrl, and deliver it directly to the MBDS of ATP7B (or ATP7A) avoiding interception by glutathione or other low-affinity acceptors in the cytosol. Platinum is then gradually released from ATP7B to a terminal acceptor in the cytosol, such as glutathione, in a less reactive form, whereas Atox1 is recycled in the apo form ready to bind another cisplatin molecule.

The number of ATP7A, and probably ATP7B, molecules in the normal cell is estimated to be approximately 500 [40], compared with approximately 10⁶ Atox1 molecules. This raises a question of whether the rate of platinum transfer from MBD2 to glutathione observed in our experiments would be sufficient to recycle MBDS fast enough to effectively detoxify cisplatin in the cell. Assuming that every MBD can bind platinum, and ATP7B/ATP7A overexpression levels can reach 10-fold, the maximum total platinum binding capacity of copper ATPases in a resistant cell would be approximately 3×10⁶ molecules. Also, the other CxxC-containing proteins, such as thioredoxin or glutaredoxin, or possibly metallothionein, may react with MBD–platinum faster than glutathione, and serve as intermediates in the platinum relay. The abundance of each of these proteins is on the order of 10⁶ molecules per cell [40]. Interestingly, glutaredoxin was indeed shown to interact with MBDS of ATP7B and ATP7A, and mediate their reaction with glutathione [50]. Finally, it should be noted that such quantitative estimates are subject to a large uncertainty, because the protein abundance numbers used above were obtained with a single common cell line, and may be quite different for the particular tumour and tissue types that show ATP7B/ATP7A–related resistance to cisplatin.

If Atox1 delivers cisplatin to the detoxification pathway, could it also be involved in the platinum drug delivery to the target DNA? The possibility that a reactive form of platinum can be transferred from an intermediate acceptor protein to DNA has been considered previously, and some support for this hypothesis was obtained from the experiments with model platinum compounds [42]. On the other hand, experiments with other combinations of sulfur-ligand platinum donors and nucleotide acceptors failed to detect such a transfer [42,51], and, to our knowledge, there are no reports of cisplatin transfer from proteins to DNA. Still, the intriguing possibility of reactive platinum delivery to the nucleus by a specific protein carrier remains open, and metal chaperones appear to be the logical candidates for this role.

A previous study identified Atox1 as a copper-responsive transcription regulation factor that binds to DNA and accumulates in the nucleus [52]. This discovery suggests a Trojan horse mechanism for the efficient delivery of cisplatin to DNA by Atox1. Consistent with this idea, deletion of Atox1 increased cell resistance to cisplatin [14,15] and reduced cisplatin binding to DNA [15], although the magnitude of the reported effect indicates that Atox1 was not the only route of cisplatin delivery to the target. The opposite view, implicating Atox1 in resistance to cisplatin, has also been published [13]. Cisplatin might exploit both the Trojan horse and the frontal attack mechanisms for reaching the DNA target. In the first case, cisplatin binds to Atox1 at the entry point into the cell and is delivered to the nucleus in the Atox1-bound form. Atox1 binding to DNA facilitates reaction of cisplatin with the latter. A fraction of cisplatin-loaded Atox1 interacts with ATP7B (or ATP7A) and transfers cisplatin to MBDS, reducing the amount of the drug reaching DNA. As the expression level of ATP7B/ATP7A in the cell increases, so does the fraction of cisplatin transferred to MBDS, which explains the correlation between the ATP7B/ATP7A expression level and cell resistance to cisplatin. This mechanism may predominate at low intracellular concentrations of cisplatin. At higher concentrations, in addition to Atox1 and ATP7B/ATP7A, cisplatin may directly react with a variety of lower-affinity intracellular acceptors, such as glutathione, thioredoxin and metallothionein, and a small fraction of the drug can reach DNA after various chemical modifications.

In conclusion, we have shown that cisplatin and carboplatin react with the MBDS of Wilson’s disease ATPase (ATP7B) at the conserved CxxC motif in the copper-binding site. Both drugs produce the same adduct with MBD2, where platinum is bound to the protein through the side chains of the proximal cysteine residues. A cisplatin derivative can be also transferred to MBD2 from the copper chaperone Atox1 pre-loaded with cisplatin, producing the same type of adduct with the protein. The Atox1–ATP7B interaction may be a step in the ATP7B-dependent pathway of cisplatin detoxification in the cell, whereas direct delivery of reactive platinum to the nucleus by Atox1 may potentiate cisplatin toxicity. Competition between these two pathways determined by the expression levels of ATP7B and, by extension, ATP7A may affect cancer cell sensitivity to cisplatin and carboplatin.

AUTHOR CONTRIBUTION

Nataliya Dolgova, Sergiy Nokhrin and Corey Yu designed and performed the experiments and analysed the data; Graham George designed the research and analysed the data; Oleg Dmitriev designed the research, analysed the data and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Copper chaperone Atox1 interacts with the metal-binding domain of Wilson’s disease protein in cisplatin detoxification

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Figure S1 Common platinum anticancer drugs

(A) Chemical structure of cisplatin and the aquation reaction, which produces the form of drug highly reactive with DNA. (B) Structures of carboplatin and oxaliplatin, the other two common platinum chemotherapeutic drugs.
Figure S2  Structures of MBD2 from human copper-transporting ATPases

(A) Stereo view of the lowest energy ensembles of MBD2 from ATP7B (PDB code 2LQB; blue) and ATP7A (PDB 1S6O; red) aligned to minimize ensemble rmsd of the backbone atoms. (B) ATP7B MBD2 relaxation times $T_2$ and $T_1$ as a function of residue number. The position of the copper-binding motif (CoxC) is shown.

Figure S3  SDS/PAGE analysis of purified MBD2

Purified MBD2 analysed by SDS/PAGE without added reducing agents (no 2-mercaptoethanol in the sample buffer), without cisplatin (middle lane) and after 12 h of incubation with cisplatin at a 1:1 molar ratio (right-hand lane). Left-hand lane contains molecular mass markers.
Atox1 interacts with ATP7B in cisplatin detoxification

Figure S4  Cross-linking of MBD2 caused by the excess of cisplatin
$\text{^1H,}^\text{15N-HSQC spectra of MBD2 without TCEP before (A) and after incubation with cisplatin at a 2:1 molar ratio (B) and 5:1 molar ratio (C). Note the loss of cross-peak intensity in (B) and (C) compared with (A) as a result of protein aggregation.}$

Figure S5  Deconvoluted ESI–MS spectra of MBD2 and MBD2 treated with carboplatin
MBD2 (A) and MBD2 treated with carboplatin (B).

Figure S6  The SxxS variant of MBD2 does not react with cisplatin
(A) ESI–MS spectrum of MBD2 mutant with cysteine residues in the CxxC motif replaced by serine (SxxS variant) has two distinct peaks. The peak at 8383 Da corresponds to the theoretical molecular mass of the protein. The origin of the peak at 8256 Da is not clear. (B) No new peaks were observed in the spectrum of SxxS-MBD2 after incubation with cisplatin.
Figure S7  EXAFS indicates reaction of free cisplatin with TCEP

Platinum LIII edge EXAFS (A) and corresponding Fourier transforms (B) of cisplatin in the buffer with and without TCEP. The continuous lines show the k^2-weighted experimental EXAFS data, and the broken lines show the theoretical fits.

Figure S8  Model platinum compounds with phosphorus ligands produce distinct XAS spectra

Structures (A) and platinum LIII edge XAS spectra (B) of the model compounds cis-dichlorobis(triethylphosphine)platinum(II) (broken line) and cis-dichlorobis(diethylsulfide)platinum(II) (continuous line) recorded in a powdered form.

Figure S9  The apoAtox1 is regenerated after platinum transfer to MBD2

Overlays of ^1H,^15N-HSQC spectra of Atox1 (A) before (black) and after (red) incubation with cisplatin and (B) before incubation with cisplatin (black) and after (red) addition of unlabelled MBD2 to the cisplatin-loaded Atox1. Sequential assignments for apo-Atox1 (BMRB code 62266) are shown in (A).
Atox1 interacts with ATP7B in cisplatin detoxification

Figure S10  Platinum transfer from Atox1 to MBD2 is unidirectional

$^{1}$H,$^{15}$N-HSQC spectra of MBD2 (A) before (black) and after (red) incubation with cisplatin at a 1:1 molar ratio, and (B) cisplatin-loaded MBD2 before (black) and after (red) addition of unlabelled apo-Atox1 at a 2:1 molar ratio.

Table S1  Statistics for structure calculation of MBD2

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