Vectorial Displacement

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Translocation of Platinum Anticancer Drugs by Human Copper ATPases ATP7A and ATP7B**

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Abstract: *Cisplatin, carboplatin, and oxaliplatin are widely used anticancer drugs. Their efficacy is strongly reduced by development of cell resistance. Down-regulation of CTR1 and up-regulation of the Cu-ATPases, ATP7A and ATP7B, have been associated to augmented drug resistance. To gain information on translocation of Pt drugs by human Cu-ATPases, we performed electrical measurements on the COS-1 cell microsomal fraction, enriched with recombinant ATP7A, ATP7B, and selected mutants, and adsorbed on a solid supported membrane. The experimental results indicate that Pt drugs activate Cu-ATPases and undergo ATP-dependent translocation in a fashion similar to that of Cu. We then used NMR spectroscopy and ESI-MS to determine the binding mode of these drugs to the first N-terminal metal-binding domain of ATP7A (Mnk1).*

ATP7A and ATP7B share 54% sequence identity and are associated, respectively, with Menkes and Wilson's diseases. Both ATPases are 160–170 kDa proteins with eight transmembrane (TM) domains and six cytosolic N-terminal metal-binding domains (NMBD). N- and P-domains are involved in ATP binding and hydrolysis. The actuator (A)-domain interacts with the ATP-binding domain and is required for conformational changes during ATP hydrolysis (Figure 1).[1] For many P-type ATPases, the ion transport requires passing through the membrane portion of the protein.[2] It has been demonstrated that, similarly to Cu, Pt binds to the CXXC motifs of the NMBDs of ATP7B and that such interaction mediates cancer cell resistance to cisplatin.[3,4]

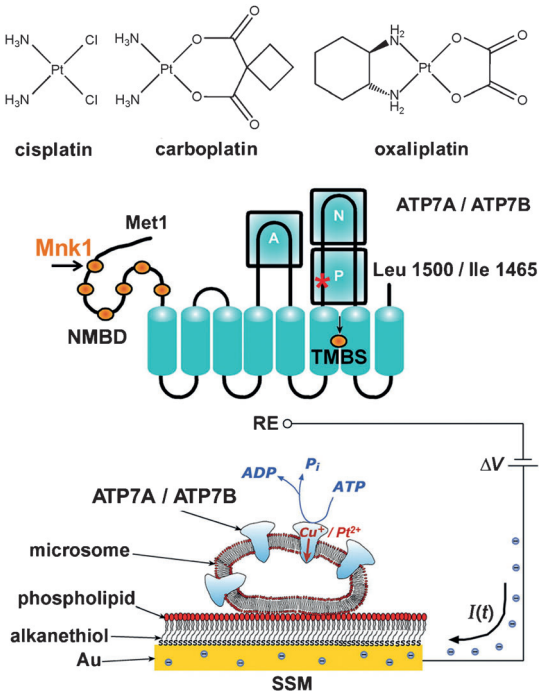

Figure 1. Structures of platinum complexes (top), topology of P-type Cu-ATPases ATP7A/ATP7B (middle), and diagram of a microsome containing ATP7A/ATP7B adsorbed on an SSM (bottom). When charge displacement occurs, a compensating current $I(t)$ flows along the external circuit (the blue spheres represent electrons) to keep constant the potential difference (ΔV) applied across the whole system. RE = reference electrode.

Recently, the solid supported membrane (SSM) technique was employed to investigate charge movements (Cu^+) in recombinant human Cu-ATPases.[5–7] The addition of ATP to microsomes enriched with recombinant ATP7A/B and adsorbed on a SSM is followed by an electrogenic event recorded as a current transient (see Figure 1; see also the Supporting Information). The current transient is due to the flow of electrons along the external circuit toward the electrode surface, and is required to compensate for the potential difference across the vesicular membrane produced by the displacement of positive charges (attributed to vectorial translocation of bound Cu^+) into the microsome upon ATP utilization. The current recorded by the SSM method is a measure of the rate of change of the transmembrane potential, and is not sensitive to stationary currents. Therefore, only electrogenic steps within the first

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catalytic cycle are measured, whereas steady-state events after the first cycle are not detected.

In the present work, we first examined the effect of Zeise's salt ($K[PtCl_3(C_2H_4)]$), which is characterized by very labile chloride ligands owing to the strong labilizing effect of ethylene, and then extended the investigation to the aquated species of cisplatin and oxaliplatin (*cis*-$[Pt(NH_3)_2(H_2O)(SO_4)]$ and *cis*-$[Pt(R,R\text{-dach})(H_2O)(SO_4)]$, dach = 1,2-diaminocyclohexane) pre-incubated for 30 min in KCl (300 mM). In the presence of chloride *cis*-$[Pt(NH_3)_2(H_2O)_xCl_{2-x}]$ and *cis*-$[Pt(R,R\text{-dach})(H_2O)_xCl_{2-x}]$ species are formed, however, for the time of the experiment (< 90 min), the monoaqua and diaqua species are still present in relevant amounts (ca. 50 % of the total) and thus they may represent the active form of the drugs in vivo.

The SSM setup was initially tested for Cu translocation. Microsomes containing ATP7A/B adsorbed on the SSM were incubated in a buffer solution containing $CuCl_2$ (5 μM) for ca. 30 min (in the presence of excess dithiothreitol, Cu^{2+} is reduced to Cu^+). Then the solution was exchanged with an identical buffer solution containing $CuCl_2$ (5 μM) and, in addition, ATP (100 μM), and the current transient measured (Figure 2, black lines and columns). After this preliminary experiment, the system was flushed with a buffer solution containing a Pt complex (5 μM) and then incubated for 30 min. The solution was then exchanged with an identical buffer solution containing a Pt complex (5 μM) and, in addition, ATP (100 μM), and the current transient detected (Figure 2, red lines and columns). A similar overall charge was translocated in all cases (Figure 2, black and red columns), but the current transient detected in the case of Zeise's salt (Figure 2 a,b) exhibits a slower decay than those

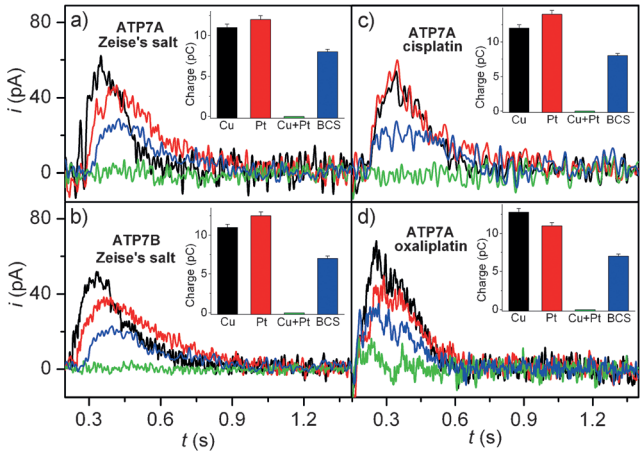

Figure 2. Current measurements on ATP7A (a, c, and d) and ATP7B (b) in the presence of Zeise's salt (a and b), aquated cisplatin (c) and oxaliplatin (d). Representative current transients induced by ATP (100 μM) concentration jumps in the presence of: $CuCl_2$ (5 μM; black lines), Pt complex (5 μM; red lines), a mixture of $CuCl_2$ and Pt complex (5 μM each; green lines), and a mixture of $CuCl_2$ and Pt complex (5 μM each) with BCS (1 mM; blue lines). The insets show the charges related to ATP-induced current transients obtained under the various experimental conditions (the same color code is used for lines and columns). Error bars are a standard deviation of six measurements on the same SSM.

observed in the cases of $CuCl_2$, cisplatin (Figure 2 c), and oxaliplatin (Figure 2 d). Therefore, current measurements point to a comparable movement of Cu- or Pt-related charge through ATP7A/B upon ATP utilization. Importantly, no current transient was detected if the ATP (100 μM) jump was performed with a buffer solution not containing $CuCl_2$ or a Pt complex.

To check if there was interference between the two metallic species, we carried out an ATP concentration jump in the presence of both $CuCl_2$ and Pt complex, with each at 5 μM concentration: no current transient was detected (Figure 2, green lines and columns). A cross-check experiment was performed by the addition of bathocuproine disulfonate (BCS; 1 mM) to the buffer solution containing $CuCl_2$ and the Pt complex. Following an ATP jump, a current transient was recovered (to ca. 60–70 % of the charge measured in the presence of 5 μM Pt complex alone; Figure 2, blue lines and columns). Thus, chelating Cu^+ with BCS allows Pt-related charge translocation by ATP7A/B to be partially restored.

To further investigate Pt-related charge movements upon ATP utilization, analogous experiments were performed using specific mutants of ATP7A and ATP7B. First, the effect of Asp1044 mutation in ATP7A was analyzed. Asp1044 is the residue receiving the ATP γ-phosphate at the catalytic site in the cytosolic P-domain of ATP7A, to form the aspartyl phosphorylated intermediate. Its mutation produces catalytic inactivation of the ATPase. We found that the D1044A mutant yields no current transient upon ATP jump either in the presence of $CuCl_2$ (5 μM) or in the presence of a Pt drug (5 μM; Supporting Information, Figure S1 A). This result indicates that the ATP-induced movement of Cu or Pt-related charge through the ATPase is directly correlated to the formation of the acyl-phosphate intermediate by ATP consumption.

We then considered the effects of site-directed mutations C575A/C578A of the CXXC motif in the 6th NMBD of ATP7B, which was shown to be critical for Cu-related charge displacement.[5] The C575A/C578A double mutant was found to be catalytically inactive,[5] therefore no current transient was detected following an ATP jump in the presence of a Pt drug (5 μM), as well as in the presence of $CuCl_2$ (5 μM; Figure S1 B). Thus, it was concluded that C575A/C578A mutation in the 6th NMBD interferes with both Cu and Pt binding, thereby preventing ATP-dependent charge movements.

Having found that the 6th NMBD of ATP7B was essential for charge movements, we focused our investigation on the interaction of one of these domains with Pt substrates. All NMBDs share similar characteristics and we chose the first domain of ATP7A, Mnk1. Fully ^{15}N-labeled and selectively ^{13}C-cysteine-labeled Mnk1 (^{15}N,^{13}C-Cys Mnk1) was bacterially expressed with a recognition site for the restriction protease Factor Xa and a $(His)_6$ tag at the C-terminus, which is crucial for its purification by metal-affinity chromatography. After cleavage of the tag, four amino acids (IEGR) of the restriction site were left at the C-terminus of the protein. The N-terminal methionine was partially processed in *E. coli*, yielding a 76-amino acid protein (numbering the first codon (Met) as residue 1). Therefore, the ESI-MS spectrum of

*apo*Mnk1 is formed by two series of multiply charged states corresponding to the protein with and without the first Met residue (Figure S2).

When cisplatin is added to *apo*Mnk1 (100 μM) in equimolar amounts under anaerobic conditions, ESI-MS spectra of the reaction mixture show a new set of signals; their intensity increases over time, indicating the formation of adducts between Mnk1 and the Pt complex. The mass difference between the corresponding charged states indicates binding of a $\{Pt(NH_3)_2Cl\}^+$ and a $\{Pt(NH_3)_2\}^{2+}$ fragment to the protein. In the early stage of the reaction, the monodentate adduct $\{Pt(NH_3)_2Cl\}^+$-Mnk1 is by far the major species. After 24 h of incubation, the signals corresponding to the chelate adduct $\{Pt(NH_3)_2\}^{2+}$-Mnk1 become the most intense (Figure 3 a,b).

Similar behavior was observed in the reaction of *apo*Mnk1 with oxaliplatin, however the reaction appears to be slower: after 3 h of incubation only weak signals relative to the monodentate adduct $\{Pt(R,R\text{-dach})(oxalateH)\}^+$-Mnk1 are present, together with those of *apo*Mnk1; after 24 h, the *apo*Mnk1 signals decrease while intense signals relative to the chelate adduct $\{Pt(R,R\text{-dach})\}^{2+}$-Mnk1 appear (Figure 3 c,d).

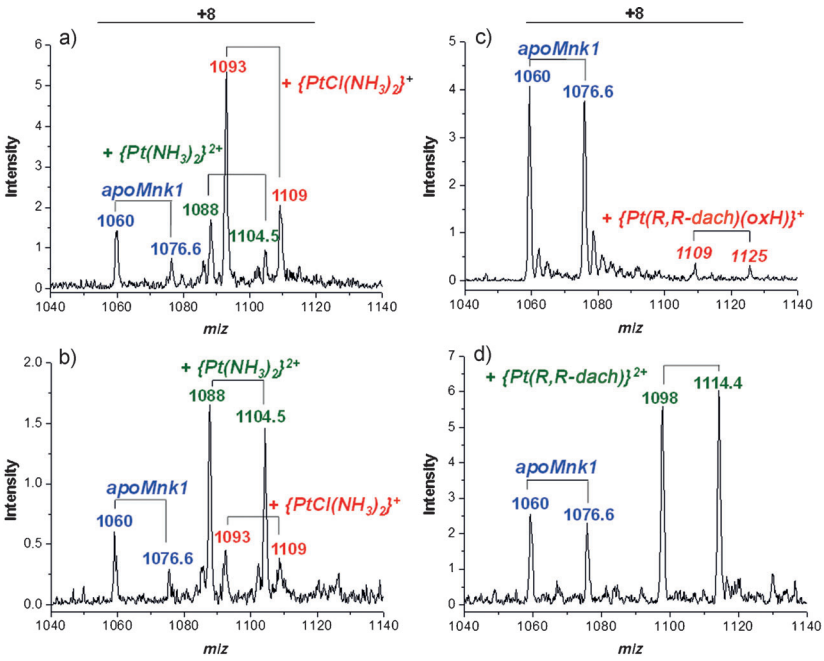

Figure 3. ESI-MS spectra of ^{15}N,^{13}C-Cys Mnk1 treated with 1 molequiv of cisplatin (a and b) or oxaliplatin (c and d) after 3 (a and c) and 24 h (b and d) of incubation. Peaks corresponding to a +8 charged state are shown. Each species comprises two pairs of signals corresponding to Mnk1 with and without Met1 as the first residue.

The starting ^1H,^{15}N-HSQC spectrum of ^{15}N-labeled cisplatin incubated with *apo*Mnk1 shows signals of the dichlorido species (*cis*-$[PtCl_2(^{15}NH_3)_2]$, δ $^{15}N = -67$ ppm) and monoaqua species (*cis*-$[PtCl(^{15}NH_3)_2(H_2O)]^+$, δ $^{15}N = -66$ and -83 ppm for the $^{15}NH_3$ *trans* to Cl and to H_2O, respectively).[8] After 3 h of incubation, the intensity of these cross-peaks decreases simultaneously with the appearance of two cross-peaks relative to the intermediate species characterized by one $^{15}NH_3$ group *trans* to a Cl atom and one $^{15}NH_3$ group *trans* to a S atom (δ ^{15}N ca. -66 and -38 ppm, respectively). After 24 h of incubation, only two cross-peaks are present in the spectrum that are characteristic of $^{15}NH_3$ *trans* to S atoms (δ ^{15}N ca. -43 and -36 ppm, respectively), thus indicating a complete loss of chloride ions from the Pt complex (Figure 4 a–c).

To prove that cysteine residues of the Cu^+-binding motif, CXXC, are involved in Pt coordination, ^1H,^{13}C-HSQC spectra were recorded on ^{15}N,^{13}C-Cys Mnk1 incubated with one equivalent of cisplatin (Figure S3 A–C). After 3 h of incubation, the original cross-peaks of *apo*Mnk1 nearly disappear, with the concomitant appearance of new cross-peaks for α-CH and β-CH_2 groups of Cys15 and Cys18, that correspond to a monodentate adduct with the Pt drug (Figure S3 B). A different set of Cys signals, which correspond to formation of the chelate adduct, is observed after 24 h (Figure S3 C). The ^1H,^{13}C-HSQC spectra also display natural-abundance ε-CH_2 cross-peaks of lysines, which all resonate in the same frequency region. These latter signals do not experience any relevant change upon reaction with cisplatin (Figure S4).

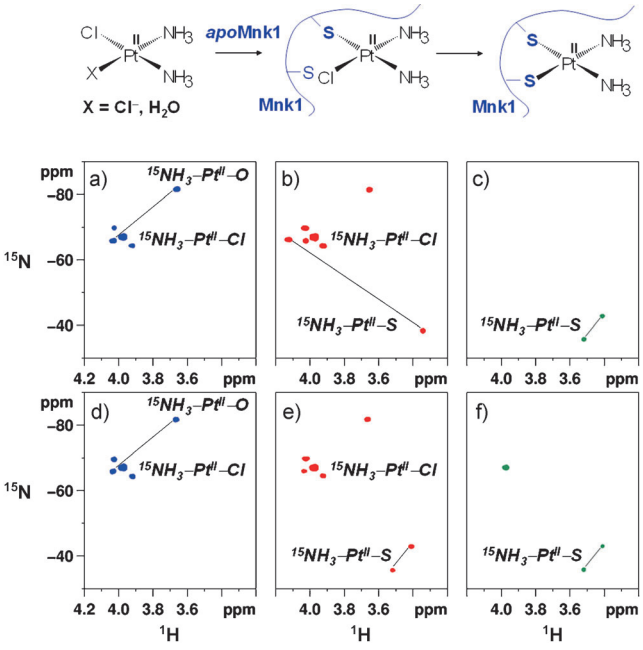

Figure 4. 2D ^1H,^{15}N-HSQC spectra of *cis*-$PtCl_2(^{15}NH_3)_2$ incubated at pH 7.0 with 1 mol equiv of *apo*Mnk1 (a–c) and Cu^+-Mnk1 (d–f) at 0 h (a and d; blue), 3 h (b and e; red), and 24 h (c and f; green) after mixing. Cross-peaks are assigned to $^{15}NH_3$ groups *trans* to O, Cl, or S donor atoms, respectively. Cross-peaks belonging to the same species are connected by a straight line. The reaction of cisplatin with *apo*Mnk1 is shown at the top.

To explore the exchange behavior of the chelate adduct, after 24 h incubation of ^{15}N,^{13}C-Cys Mnk1 with one equivalent

of unlabeled cisplatin, a second equivalent of ^{15}N-labeled cisplatin was added. After an additional 24 h (and up to several days of incubation) there is no evidence of $^{15}NH_3$ signals *trans* to the S atoms, and the ^{1}H,^{13}C-HSQC spectrum of the protein is not further perturbed, which indicates that no exchange occurs between the second equivalent of cisplatin and the Pt already chelated by the two Cys residues of Mnk1 in the chelate adduct ($\{Pt(NH_3)_2\}^{2+}$-Mnk1). Since in the cytosol there is a relevant concentration of glutathione (GSH, ca. 5 mM), we wanted to explore if the reaction between cisplatin and *apo*Mnk1 would take place also in the presence of (10 fold) excess GSH. Results showed that Mnk1 competes successfully with GSH for complexation to platinum (Figure S5).

One equivalent of ^{15}N-labeled cisplatin was added to preformed Cu^+-Mnk1 adduct. Both ESI-MS (Figure S6) and NMR measurements (Figure 4 d–f) indicate that the Pt drug can bind to Cu^+-Mnk1, as found for the *apo*protein, but in the case of the *holo*protein there is no evidence of monodentate adduct formation. Instead, the chelate adduct (both $^{15}NH_3$ *trans* to S atoms) is already formed after 3 h of incubation, thus indicating that Cu^+ can catalyze Pt drug chelation by two Cys residues with a complete loss of chlorido ligands. Notably, the chemical shifts of the ammine ligands of cisplatin are the same as those observed in the former experiment with *apo*Mnk1 after 24 h incubation. Also the ^{1}H,^{13}C-HSQC spectrum of ^{15}N,^{13}C-Cys Cu^+-Mnk1, after 3 h of incubation with cisplatin, shows a great decrease in intensity of the signals of the *holo*protein, while the signals of $\{Pt(NH_3)_2\}^{2+}$-Mnk1 appear (compare Figure S3 E with Figure S3 D,C), clearly indicating that cisplatin can displace Cu^+ bound to CXXC motifs, thus potentially interfering with cellular Cu homeostasis. Furthermore, after 24 h of incubation, a remarkable reduction in the intensity of $^{15}NH_3$ and ^{15}N,^{13}C-Cys Mnk1 signals (Figure 4 f; see also Figure S3 F) is observed, accompanied by the coalescence of amide signals in the middle of the ^{1}H,^{15}N-HSQC spectrum, a typical sign of protein unfolding (Figure S7). Notably, the $\{Pt(NH_3)_2\}^{2+}$-Mnk1 chelate adduct alone unfolds at a much slower rate (Figures S3C and S7C), thus indicating that the Cu^+ ion displaced by cisplatin promotes protein unfolding.

Taken together, our measurements clearly support ATP-induced Pt-related charge translocation in ATP7A/B. The charge translocation occurs with rates analogous to that of Cu in the case of aquated cisplatin or oxaliplatin. Numerical integration of the ATP-induced current transients in the presence of $CuCl_2$ or Pt complex yields similar values for the translocated charge (Figure 2, black and red columns), thus indicating that an equal amount of positive charge is displaced by ATP7A/B upon ATP utilization within a single catalytic cycle. Analysis of bacterial Cu-ATPase, that is CopA from *A. fulgidus*[9] and *L. pneumophila*,[10] suggested that the transport stoichiometry could probably be two Cu^+ ions per hydrolyzed ATP. Thus, assuming the same stoichiometry (2 Cu^+ per ATP) in the case of human ATP7A/B, our measurements indicate that one Pt species bearing two positive charges, or two Pt species bearing a single positive charge, is/are translocated following utilization of one ATP molecule. Based on the stability of the ammine groups, which are not trans-labilized by cysteines, we propose that the complex that translocates bears the ammine groups.

The lack of charge translocation following mutation of the catalytic Asp1044 of ATP7A or the CXXC motif in the 6th NMBD of ATP7B clearly demonstrates that the Pt-related charge movement is dependent on formation of a phosphorylated intermediate, as well as conformational adjustments analogous to those required for Cu-related charge translocation.

The presence of both Cu and Pt in the buffer solution abolishes charge movements, indicating that simultaneous occupancy of activating sites by the two metal ions prevents catalytic activation, as has been previously observed.[11] Interestingly, the use of a Cu chelator (BCS) partially restores charge movement, thus demonstrating direct involvement of the Pt drug.

Copper binding to CXXC motifs of NMBDs triggers protein conformational changes that weaken the interactions between the N-terminus and the ATP-binding domain, and favor catalytic phosphorylation.[12] Based on the NMR experiments performed with ^{15}N-labeled cisplatin, we propose the mechanism depicted at the top of Figure 4 for the reaction of cisplatin with one such NMBD. The drug initially forms a monodentate adduct ($\{Pt(NH_3)_2Cl\}^{+}$-Mnk1) with the protein. This is an early adduct that may be relevant for Pt-related charge translocation. On a longer timescale, Pt releases the second chloride while keeping the two ammines, thus forming the chelate adduct ($\{Pt(NH_3)_2\}^{2+}$-Mnk1). Once the chelate adduct is formed, no Pt exchange can occur at the CXXC motif at a detectable rate.

At variance with what occurs with the MXXM motifs of CTR1,[13] the S atoms of cysteines do not labilize the ammine ligands of the Pt drug that are *trans* to them. This surprising difference was already noted in a previous paper from our group reporting the interaction of Pt drugs with Atox1, which is structurally similar to Mnk1.[14] It is possible that the protein exerts a protective effect, as witnessed by the strong shielding in the ^{1}H dimension of the cross peaks of the cisplatin-Mnk1 adducts. Cisplatin is able to readily displace Cu^+ bound to the CXXC motif and coordinates directly in a chelating fashion, however the displaced Cu ion destabilizes the Pt-Mnk1 structure. An analogous unfolding behavior has previously been observed for the structurally similar Atox1.[15]

In conclusion, our studies show that cisplatin and oxaliplatin can activate the catalytic cycle of ATP7A/B, leading to charge movement. Cisplatin can react with the ATPase N-terminal extension, initially forming quite long-lasting monodentate adducts, which then evolve into stable and unreactive chelate adducts. It is likely that translocation by ATPases (at short incubation times) and sequestration in the N-terminal extension (at long incubation times) may contribute to cisplatin resistance in vivo, with the predominance of either phenomenon depending on dosage and time of administration. Resistance would be certainly higher under conditions of ATPase protein overexpression.

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