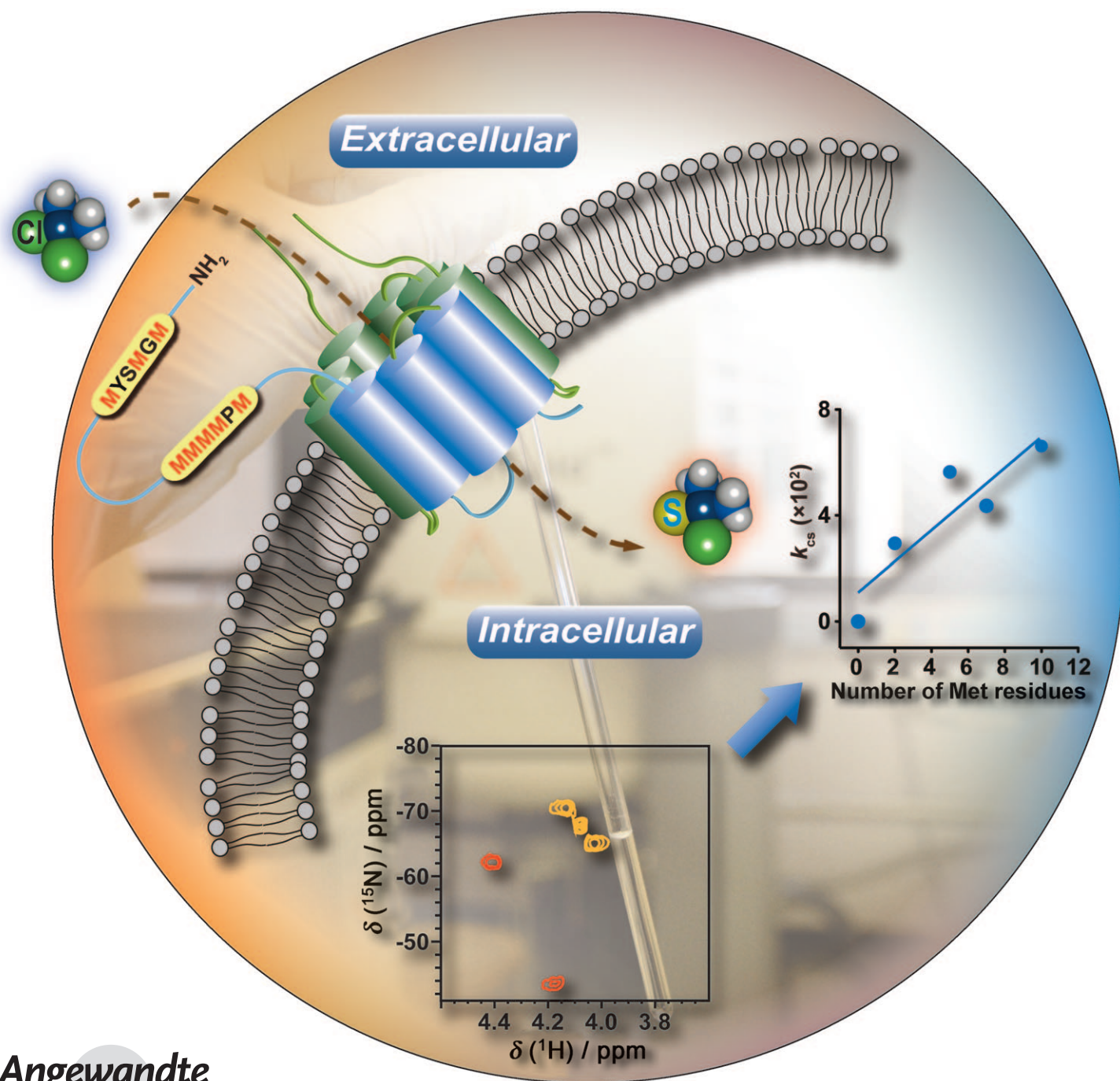


The Effect of the Extracellular Domain of Human Copper Transporter (hCTR1) on Cisplatin Activation**

Xinghao Wang, Xiubo Du, Hongyan Li, Denise So-Bik Chan, and Hongzhe Sun*



Despite their extensive clinical use, the cellular uptake mechanism of Pt anticancer drugs, for example, cisplatin (*cis*-[PtCl₂(NH₃)₂], cDDP) is not fully understood. It was previously believed that Pt drugs enter cells by passive diffusion, followed by intracellular hydrolysis and activation, and subsequent formation of intrastrand cross-linked adducts with DNA, which could inhibit DNA replication and induce cell death.^[1] In the past few years, copper transporter 1 (CTR1), the major plasma-membrane transporter involved in intracellular Cu homeostasis, has been unexpectedly found to play a substantial role in cDDP influx,^[2] although cDDP shares little similarity with Cu^I in terms of physical properties, for example, structures and charges.

Human copper transporter 1 (hCTR1) is a glycosylated membrane protein (190 amino acids) that has three putative transmembrane domains, an extracellular N terminus, and an intracellular C terminus (Figure 1a).^[3] A recent structural study by cryoelectron crystallography revealed that hCTR1 forms a symmetrical homotrimer with a channel-like architecture in order to allow import of Cu^I.^[4] The extracellular N terminus of hCTR1 contains two methionine (Met)-rich and two histidine (His)-rich motifs that are thought to be essential for the function of the transporter.^[3,5] Although considerable progress has been made toward understanding the structure–function aspects of hCTR1-mediated Cu transport, the molecular mechanism of cDDP uptake is still poorly understood. Several synthetic peptides were recently used to mimic the extracellular domain for such studies,^[6] however they might not resemble the actual situation of the intact protein (domain).

We report herein how the N-terminal extracellular domain of hCTR1 (hCTR1_N) was overexpressed and the role of the Met- and His-rich motifs on cDDP binding was studied by either mutagenesis or chemical modification. To improve the solubility and stability of the protein, hCTR1_N with different lengths with various tags was screened and overexpressed, and the hCTR1_N (1–55) with a GB1 tag^[7] gave the best performance. This wild-type hCTR1_N (1–55), known as WT, as well as three related mutants, namely MutA, MutB, and MutC, which were constructed by mutating the methionine residues of the first, second, and both Met-rich motifs of the wild-type hCTR1_N to alanine (Ala) (Figure 1b and Figure S1 in the Supporting Information), were overexpressed and purified to apparent homogeneity, as shown by SDS-PAGE analysis (Figure S2).

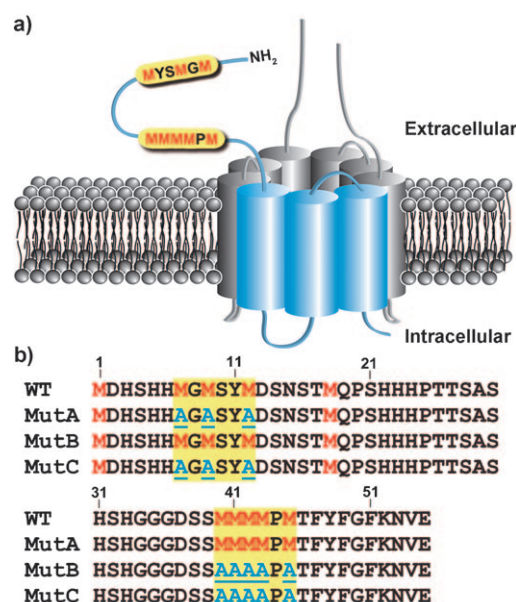


Figure 1. a) hCTR1 assembly on a cell membrane. Met-rich regions in the N terminus are highlighted in yellow. b) Sequences of the wild-type hCTR1_N and its three mutants. Methionine residues are colored in red, and Met-rich domains are highlighted in yellow with the mutation points underlined.

The kinetics of the reaction of [¹⁵N]cDDP (*cis*-[PtCl₂(¹⁵NH₃)₂], **1**) with the WT protein was monitored by ¹H and 2D SOFAST-HMQC NMR experiments.^[8] The latter experiment is a new technique that enables the relatively fast kinetic process for individual species to be followed. A series of time-dependent 2D [¹H,¹⁵N] SOFAST-HMQC spectra of 1.2 mM [¹⁵N]cDDP in the absence and presence of one molar equivalent of the WT protein in 20 mM phosphate buffer containing 100 mM NaCl at pH 7.4 were acquired at 298 K (Figure 2a and S4c). The most intense cross-peak, which appears at −67.9/4.08 ppm, is assignable to cDDP. The intensity of this signal in the presence of the protein decreased more rapidly over time compared with that in the spectrum recorded in the absence of the protein (Figure 3a). This observation is indicative of the acceleration of cDDP consumption that is triggered by hCTR1_N. Simultaneously, two weak cross-peaks at −66.8/4.11 (**2a**) and −81.7/3.71 (**2b**) ppm are assignable to mono-aqua species (*cis*-[PtCl(H₂O)(¹⁵NH₃)₂]⁺, **2**) appeared and their intensities increased within approximately 100 min and then decreased with time (Figure 3b). In contrast, the intensities of the cross-peaks of the mono-aqua species increased steadily and reached a plateau in the absence of the protein under identical conditions; this result is indicative of the possible interaction of mono-aqua species with the WT protein. Notably, two additional cross-peaks at −62.7/4.43 (**3a**) and −44.2/4.17 (**3b**) ppm that are assignable to mono-sulfur species (*cis*-[PtCl(Met)(¹⁵NH₃)₂]⁺, **3**) appeared and could be observed over a period of 7 h (Figures 2a and 3c). Time-dependent ¹H NMR spectra (Figure S3) recorded under the same conditions demonstrated that the signals at 2.0–2.2 ppm assignable to the ε-CH₃ group of the methionine residues were perturbed with incubation time, thus implying that cDDP binds to the

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[**] This work was supported by the RGC of Hong Kong (704306, 704207, HKU107C, 703808, 704909P and N_HKU75209), the Croucher Foundation, and the University of Hong Kong. We thank D. J. Thiele, (University of Michigan) and H. Y. Hu (Shanghai Institute for Biological Sciences) for the hCTR1 gene and pGBTNH vector, and Z. J. Guo (Nanjing University) and K. H. Sze (HKU) for helpful comments.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201006739>.

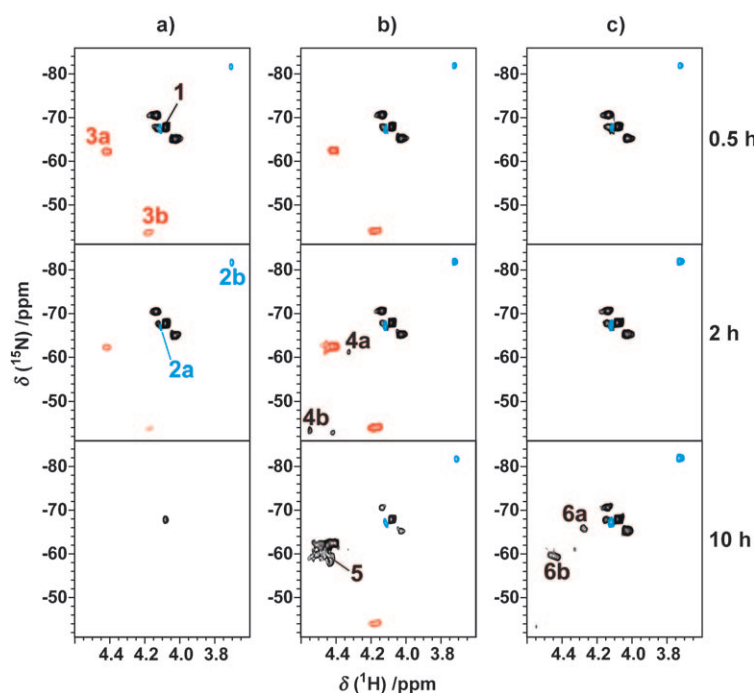


Figure 2. Comparison of the 2D $[^1\text{H}, ^{15}\text{N}]$ SOFAST-HMQC spectra of $[^{15}\text{N}]$ cDDP ($\text{cis-}[\text{PtCl}_2(\text{H}_2\text{O})_2]$). NMR spectra of $[^{15}\text{N}]$ cDDP (1.2 mM) in the presence of one molar equivalent of WT (a), MutC (b) and Met-modified MutC (c) at 0.5 (upper), 2 (center), and 10 h (lower). The peaks were assigned to cDDP (**1**, Cl-Pt-NH₃); monoaqua species ($\text{cis-}[\text{PtCl}(\text{H}_2\text{O})(\text{H}_3\text{N})_2]^+$, **2**; Cl-Pt-NH₃, **2a**; H₂O-Pt-NH₃, **2b**); monosulfur species ($\text{cis-}[\text{PtCl}(\text{Met})(\text{H}_3\text{N})_2]^+$, **3**; Cl-Pt-NH₃, **3a**; S-Pt-NH₃, **3b**); cDDP binding with N-terminal methionine with GB1 tag, $\text{cis-}[\text{Pt}(\text{Met-N,S})(\text{H}_3\text{N})_2]^+$ (**4**; N-Pt-NH₃, **4a**; S-Pt-NH₃, **4b**); cDDP binding to histidine after loss of one ammine ligand, $\text{cis-}[\text{Pt}(\text{His})(\text{Met})(\text{H}_3\text{N})_2\text{X}]$ (N-Pt-NH₃, **5**) and $\text{cis-}[\text{PtCl}(\text{His})(\text{H}_3\text{N})_2]$ (**6**; Cl-Pt-NH₃, **6a**; N-Pt-NH₃, **6b**). Peaks of species **2** and **3** are highlighted in blue and red, respectively.

protein through the thioether group of the methionine residues without apparent preference. No signal was observed after 14 h of reaction because of the release of ammine ligands from cDDP, as confirmed by the observation of free $[^{15}\text{N}]\text{H}_4^+$ at 0.1/7.11 ppm in the 2D $[^1\text{H}, ^{15}\text{N}]$ HSQC spectrum upon lowering the pH to 2.5 (data not shown). The release of ammine ligands from cDDP is likely attributed to the strong *trans*-labilizing effect of the methionine sulfur atom on the bound cDDP species, and is consistent with previous reports.^[9]

The kinetic profiles of these Pt species are shown in Figure 3a–c, and the relative concentrations of species **1**, **2**, and **3** were calculated by integration of peaks **1**, **2b**, and **3b** in the 2D SOFAST-HMQC spectra respectively. Kinetic data were fitted to the proposed reaction scheme (Figure 3e) and the rate constants for each step are summarized in Table 1. Based on these rate constants, we made a further calculation that shows that after 7 h of incubation, approximately 82 % and 9 % cDDP remained in the absence and presence of the WT protein, respectively. When comparing the rate constants of each step for the reaction of cDDP with the WT protein, we found that the apparent rate of substitution of one chloride ligand of cDDP by methionine ($k_{\text{cs(app)}} = k_{\text{cs}} \times P_0 = 7.94 \times 10^{-5} \text{ s}^{-1}$, where P_0 is the initial protein concentration of

0.0012 M) is about 3.6 times of the rate of formation of monoaqua species **2** (k_{ca}) at the beginning of reaction. The amounts of monosulfur species **3** formed by direct binding and prehydrolysis of cDDP in the first hour of reaction were further calculated to be approximately 89 % and 11 %, respectively, thus suggesting that direct binding is likely to be the predominant process. Our result indicated that, in the presence of the WT protein, the replacement of the chloride ligand is much faster than the hydrolysis of cDDP. Such a phenomenon was also observed in previous work when cDDP reacts with methionine,^[10] thus suggesting that hCTR1_N facilitated the activation of the drug through the methionine residue.

The effects of three mutants on the kinetics of cDDP binding under identical conditions were then investigated for comparison. The GB1 tag was found to have a negligible effect on the reaction, in spite of its N-terminal methionine residue (see the Supporting Information). All mutants share similar features with the wild-type protein, as three major Pt^{II} species (**1**, **2**, **3**) are observed, as shown from the 2D SOFAST-HMQC spectra (Figures 2b and S4a). Interestingly, extra cross-peaks that are assignable to binding of Pt^{II} ions with nitrogen ligands ($\text{cis-}[\text{Pt}(\text{His})(\text{Met})(\text{H}_3\text{N})_2\text{X}]$, **5**; Figures 2b and S4a) were observed in the late stage of reaction for these mutant proteins, thus indicating a possible pathway for monosulfur species **3** after loss of the ammine ligand. The kinetic data of the Pt species **1**, **2**, and **3** in the presence of mutant proteins MutA, MutB, and MutC were fitted to the same reaction scheme

Table 1: Rate constants for the formation of different Pt^{II} species in the absence and presence of hCTR1_N and its variants.

Protein applied	$k_{\text{ca}} [\times 10^{-5} \text{ s}^{-1}]$	$k_{\text{cs}} [\times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}]$	$k_{\text{s}} [\times 10^{-4} \text{ s}^{-1}]$
Buffer	1.79 ± 0.09	— ^[a]	— ^[a]
GB1 tag	2.11 ± 0.05	0.24 ± 0.01	1.11 ± 0.17
WT	2.22 ± 0.14	6.62 ± 0.10	10.1 ± 0.40
MutA	2.26 ± 0.16	4.36 ± 0.11	2.50 ± 0.07
MutB	2.70 ± 0.17	5.64 ± 0.15	7.43 ± 0.37
MutC	2.33 ± 0.17	2.94 ± 0.12	1.43 ± 0.07
WT (His-mod)	3.06 ± 0.40	7.88 ± 0.31	4.85 ± 0.24
MutC (His-mod)	2.29 ± 0.08	2.79 ± 0.12	0.30 ± 0.02
MutC (Met-mod)	2.44 ± 0.06	— ^[a]	0.69 ± 0.09

[a] Not applicable.

as the WT protein (Table 1). As expected, the k_{ca} values of the mutants (MutA, MutB, and MutC) are similar to that of the WT protein. In contrast, the k_{cs} values decreased almost by one-third and one-fifth compared with the WT protein for MutA and MutB, respectively, but by one-half for MutC, where only two methionine residues are present. Upon chemical modification of these residues (see the Supporting Information), no monosulfur species were observed (Figure 2c). Surprisingly, two peaks appeared at $-66.0/4.28$ (**6a**) and $-59.3/4.43$ (**6b**) ppm after 3 h of reaction; these signals

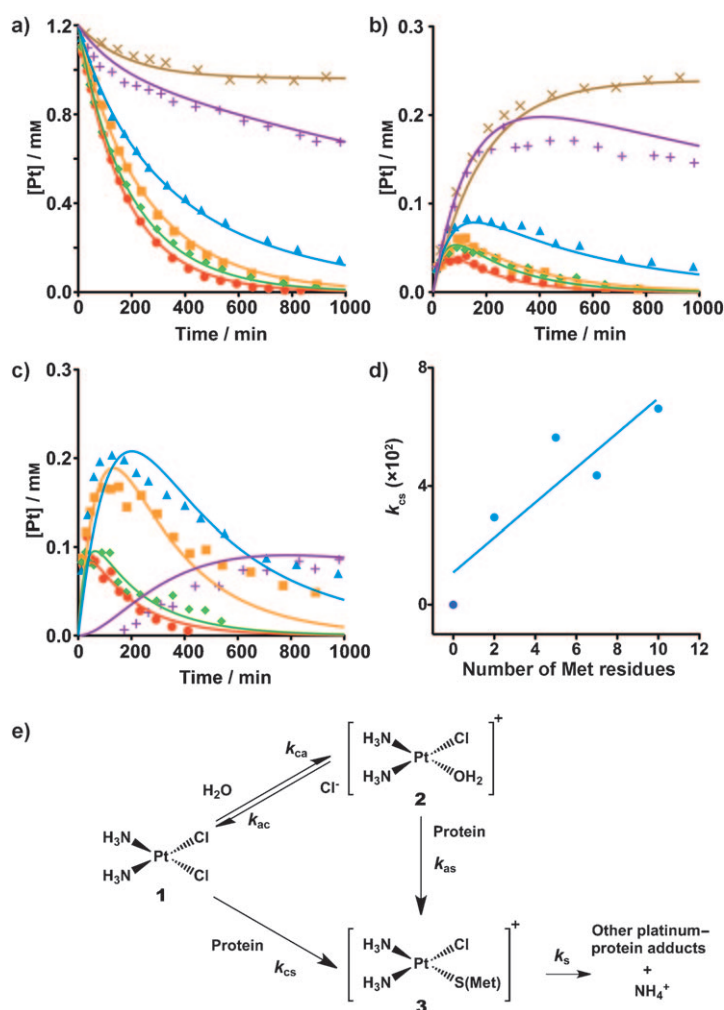


Figure 3. Kinetic profiles of different platinum species and the reaction pathway of cDDP with the protein. Concentrations of a) cDDP, b) mono-aqua, and c) monosulfur (or histidine-binding species for Met-modified MutC) Pt^{II} species versus time in the absence ($\times$) and presence of one molar equivalent of WT ($\bullet$); MutA ($\blacksquare$); MutB ($\blacklozenge$); MutC ($\blacktriangle$); and Met-modified MutC ($+$). The curves represent the best fits using the rate constants listed in Table 1. d) Dependence of the rate constants (k_{cs}) on the number of the methionine residues of the proteins. A linear regression was performed with the coefficient of determination (R^2) of 0.8105. e) Pathway of the reaction between cDDP and the protein. For Met-modified MutC, an alternative reaction scheme (Figure S6) is used because of the different reaction mechanism.

are tentatively assigned to the binding of cDDP to the imidazole ring of the histidine residues (*cis*- $[\text{PtCl}(\text{His})-(^{15}\text{N})\text{H}_3]_2^+$, **6**). This kinetic data was best fitted when direct binding was excluded ($k_{\text{cs}}=0$), thus indicating that the binding of cDDP with histidine requires a prehydrolysis step that resulted in a much lower rate of cDDP binding to Met-modified (Met-mod) MutC than that of the unmodified protein. This result suggests that methionine residues are the key binding sites of cDDP in hCTR1_N. Similar to k_{cs} , the overall consumption rate of the species **3** (k_{s}) in the presence of mutant proteins was lower than that of WT.

According to our kinetic data, the formation and consumption rates of monosulfur species, that is, k_{cs} and k_{s} , revealed a roughly linear correlation with the numbers of

methionine residues of the proteins (Figure 3d). This result is consistent with our ^1H NMR data that indicate that cDDP binds nonpreferentially to the methionine residues of the protein. The methionine residues therefore play a critical role not only in the initial step of cDDP binding to hCTR1_N, but also in the postbinding process. The rates (k_{cs} and k_{s}) in the presence of MutA and WT are slightly lower than expected, and indicate that the first Met-rich motif contributed more to the binding and postbinding process than the second Met-rich motif, probably because the second Met-rich motif clustered, thus leading to the steric hindrance that restricts the accessibility of other molecules (cDDP or nucleophiles in the solvent).

The effects of His-rich motifs on cDDP binding and activation were further examined by chemical modification of histidine residues of both WT and MutC proteins. The 2D SOFAST-HMQC spectra of cDDP in the presence of His-modified proteins (His-mod; Figure S4b) were similar to those of the unmodified proteins, except that no histidine-bound cDDP species (**5**) were observed. According to kinetic profiles, histidine modification significantly reduced the value of k_{s} whereas that of k_{cs} was only slightly affected (Figure S5 and Table 1). These observations indicated that histidine residues are not the primary target for cDDP under the conditions used, instead the histidine residues may interact with Met-bound cDDP species (activated cDDP) and affect their kinetic behavior.

To further identify the cDDP–protein adducts, MALDI-TOF MS was used with both WT and MutC proteins (15 μM) in 0.2 mM phosphate buffer, 100 mM NaCl, pH 6.8 incubated with 5 molar equivalents of ^{15}N cDDP for 9 and 24 h at ambient temperature. Under these conditions, the protein is present as a monomer, as revealed by gel-filtration chromatography (data not shown). Addition of cDDP to apo-WT for 9 h led to the appearance of a new peak at m/z 14333.5, which corresponds to $[M_1+\text{Pt}-\text{H}]^+$ (calcd: 14333.7, M_1 = apo-WT; Figure 4). The intensity of this peak further increased after 24 h of reaction and a new peak at m/z 14526.6, which was assigned to $[M_1+2\text{Pt}-3\text{H}]^+$ (calcd: 14526.8) was observed.

Another weak peak at m/z 14717.0 (the precise m/z value could not be determined because of the low intensity as well as peak broadening) was also observed, which is tentatively assigned to $[M_1+3\text{Pt}-5\text{H}]^+$ (calcd: 14719.9). Similarly, the addition of cDDP to MutC led to the appearance of weak peaks at 13870.6, which could be assigned to $[M_2+\text{Pt}+^{15}\text{N}\text{H}_3-\text{H}]^+$ (calcd: 13870.8, M_2 = apo-MutC; Figure 4). The MALDI-TOF MS data demonstrated that the binding of cDDP to both the WT and MutC protein is a kinetically controlled process and a maximum of three and one Pt atoms per monomer were found for WT and MutC, respectively.

Our combined NMR and MS data demonstrated that cDDP kinetically binds almost nonpreferentially to the methionine sulfur atoms of the protein; this behavior is

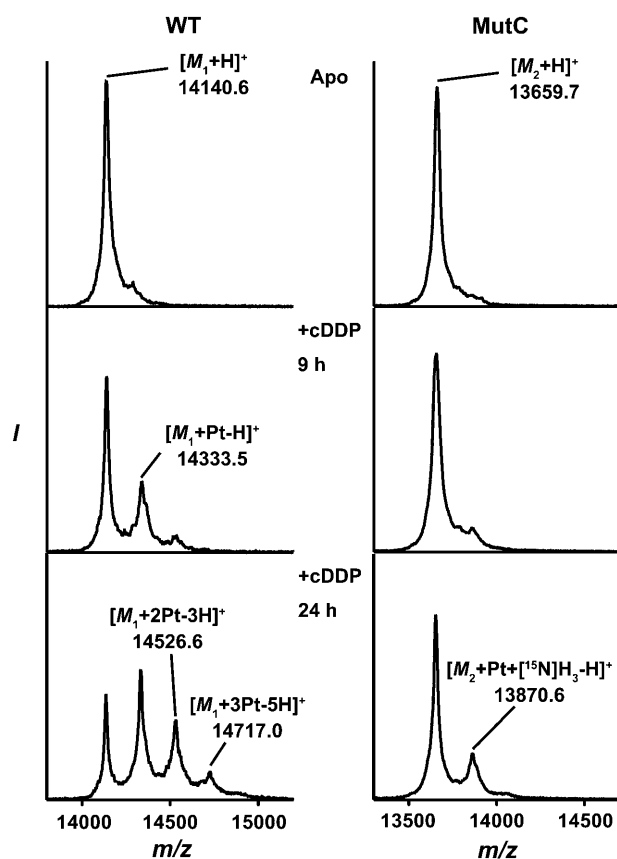


Figure 4. MALDI-TOF spectra of the apo- and cDDP-bound WT (left) and MutC (right). M_1 and M_2 represent apo-WT and apo-MutC respectively (calcd: 14139.6 and 13658.7).

consistent with a previous mutagenesis study showing that none of specific residues was identified for the tight binding of Cu.^[11] This result is not surprising since only one solvent-accessible thioether group is required to allow the formation of the monosulfur species (*cis*-[PtCl(Met)(NH₃)₂]⁺, **3**). Significantly, cDDP binds to hCTR1_N predominately by a direct binding process, in contrast to DNA binding,^[12] which required hydrolysis prior to the interaction. Such binding profoundly facilitates the activation of the drug.

Given the different charges and radii of Pt^{II} and Cu^I ions, cDDP is unlikely to be transported in the naked form (Pt^{II}) by hCTR1 as proposed previously,^[6] in spite of the release of ammine ligands upon binding to hCTR1_N. It has been suggested that the uptake of cDDP *in vivo* may only take several minutes.^[13] As the overall charge of [PtClX(NH₃)₂]⁺ is comparable to that of Cu^I, and its radius (2.4 Å)^[14] is smaller than the “narrowest” part of the hCTR1 pore (diameter ≈ 8 Å),^[15] it is reasonable to presume that *cis*-[PtCl(Met)(NH₃)₂]⁺ (**3**) is the major species that is transported by hCTR1. The anticancer drug may migrate by a methionine-based sulfur–sulfur exchange through the pore or channel formed by hCTR1. Indeed, a recent study on *E. coli* CusA did suggest a methionine-mediated transport mechanism for Cu^I efflux.^[16] It has also been demonstrated that the ¹⁵⁰MXXX¹⁵⁴M motif located at the second transmembrane domain of hCTR1 is important for both Cu and cDDP uptake,^[3,13,17] despite

contradictive data reported for cDDP uptake upon mutation of this pair of methionine residues. Once cDDP is taken up and activated by hCTR1, it would eventually reach downstream sulfur-containing proteins or small molecules in cytoplasm. These sulfur–platinum(II) complexes may serve as an intermediate prior to DNA platination.^[18] It has been recently demonstrated that square-planar Pt bound to the thioether group of methionine as well as the guanine-N7 unit on a DNA–histone adduct,^[19] and such phenomena may also exist *in vivo*.

In summary, the extracellular domain of hCTR1 (hCTR1_N) is likely to serve as a “pool” for the anticancer drug by binding cDDP to its methionine residues, thus increasing the likelihood of drug uptake. The drug could subsequently utilize the Cu transporter to enter the cell by methionine-based sulfur–sulfur exchange. Platinum thioether species may actually represent an active form of cDDP even though the platinated DNA is the thermodynamically favored final product.

Experimental Section

The WT and mutant proteins were overexpressed and purified as described in the Supporting Information. Methionine and histidine residues were modified using iodomethane and diethylpyrocarbonate respectively, as described previously.^[20] [¹⁵N]cDDP was synthesized from [¹⁵N]NH₄Cl and K₂PtCl₄ by following a previously reported procedure,^[21] and the purity of the final product (>95 %) was confirmed by 2D [¹H,¹⁵N] HSQC NMR spectroscopy.

NMR experiments: purified proteins in 20 mM phosphate buffer, 100 mM NaCl, pH 7.4 were mixed with [¹⁵N]cDDP at a 1:1 molar ratio with a final concentration of 1.2 mM. Both 1D ¹H and 2D [¹H,¹⁵N] SOFAST-HMQC spectra were recorded at 298 K on a Bruker Avance 600 spectrometer equipped with a cryogenic TCI probe. All NMR spectra were processed using Topspin 2.01 and analyzed using Sparky. The kinetic data obtained from NMR spectra were fitted using Scientist 3.0 (Micromath) to the proposed scheme adapted from a scheme of cDDP binding to DNA.^[22] Detailed information can be found in the Supporting Information.

MALDI-TOF MS studies: apo forms of WT or MutC (15 μM, in 0.2 mM phosphate buffer, 100 mM NaCl, pH 6.8) were incubated with 5 molar equivalents of [¹⁵N]cDDP for 9 or 24 h. Excess [¹⁵N]cDDP was removed and the proteins or cDDP–protein adducts were desalted by passing through a HiTrap column eluting with 10 mM ammonium bicarbonate buffer (pH 8.0), prior to lyophilization. The lyophilized samples were redissolved in a solution of CHCA (α-cyano-4-hydroxycinnamic acid) matrix and analyzed in a 4800 MALDI TOF/TOF Analyzer. Detailed information can be found in the Supporting Information.

Received: October 27, 2010

Revised: January 19, 2011

Published online: February 24, 2011

Keywords: amino acids · antitumor agents · cisplatin · isotopic labeling · platinum

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