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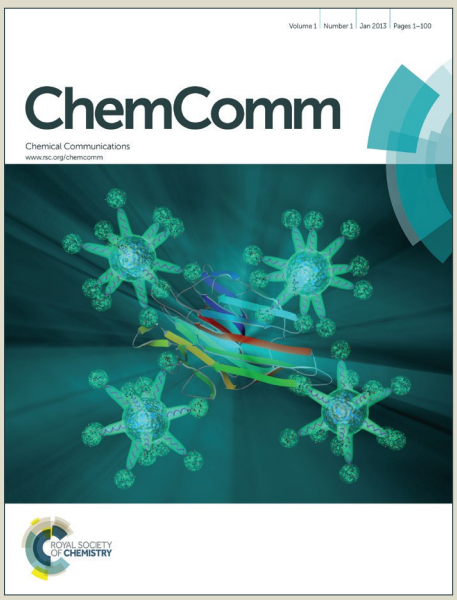

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Destructive Interactions of Dirhodium(II) Tetraacetate with β Metallothionein rh1a

D. L. Wong,[a] and M. J. Stillman[a]

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Metal-based therapeutics are vital tools in medicine. Metal-chelating proteins can dramatically decrease drug efficacy. Dirhodium(II) tetraacetate, a potential anticancer compound, binds *in vitro* to 8 cysteines of the human metallothionein 1a β-fragment. Electrospray ionization mass spectrometry shows that the final product is the Rh_2^{4+} core encapsulated by the β-MT protein.

Metallodrugs have a long and successful history in curing many different classes of disease; for example, cisplatin for testicular cancer, sodium aurothiomalate for rheumatoid arthritis, Li salts for psychological disorders, and Bi salts for gastrointestinal distress.[1-2] Challenges in the development of new metallopharmaceuticals include overcoming the cellular metal defences protecting the target. Metal-chelating gate-keeper proteins can result in dramatically decreased drug efficacy. In this paper, we report that one such gate-keeping protein, metallothionein, both binds dirhodium acetate, a potential anticancer metallodrug, and sequentially strips off the acetate ligands reducing the complex to just a thiolate-coordinated dirhodium core. This reaction may indicate a possible mechanism for a cellular metallodrug resistance pathway.

Metallothioneins (MTs) are sulfur rich proteins that bind a variety of metals including toxic metals and those of medicinal interest. Current metallodrug designs aim to overcome these defences, for example using non-platinum compounds, like the robust series of half-sandwich arene Ru(II) anti-cancer compounds.[3-5] While the over-expression of the cysteine-rich MT has been correlated to drug resistance,[6-7] there is very little direct, quantitative evidence of the mechanism that may be involved in the proposed interactions. In addition, there have been no reports of the subsequent fate of the metallodrug following its interaction with MT. Studies with cisplatin have provided some insight into the rates of metal ion isolation,[8-9] but much more detailed information on the mechanism of destruction of the metal complex by the MT through ligand exchange is needed.

Metal-metal bonded dirhodium carboxylate complexes have gained interest as an alternative to classical platinum anti-cancer compounds.[10] Dirhodium complexes are 18 electron systems when the two axial positions are capped by solvent, with octahedral coordination of the rhodium metals. Ground work studies have shown high *in vivo* antitumor activity of $Rh_2(O_2CR)_4$ (R = Me, Et, Pr) against L1210 tumors, Ehrlich ascites, and the sarcoma 180 and P388 tumor lines,[11] as well as the ability to bind DNA and inhibit protein synthesis in a manner akin to cisplatin.[12] Current research has extended the applications of rhodium compounds to medical imaging and protein labelling, but the significant antitumor characteristics of these rhodium complexes remain of great interest.[13-16] However, the dirhodium carboxylates are particularly sensitive to sulfur coordination from cysteines in biomolecular targets.[17-18] This is significant because MT may stand in the way as a formidable defence for cancerous cells due to the tendency of cysteine coordination of any incoming metal. Herein, we report preliminary studies involving the .remarkable and systematic deconstruction of dirhodium(II) tetraacetate ($Rh_2(OAc)_4$), a compound with anti-tumour activity, by the β-domain fragment of human metallothionein 1a.

Electrospray Ionization mass spectrometry (ESI-MS) is a powerful technique that allows for the quantitative visualization of a reaction as it progresses. In particular, mass spectrometry is especially effective in monitoring drug-protein binding reactions, as it is able to provide information regarding intermediate species as they develop in real time. [19-20] ESI mass spectral data can be used in the characterization of species formed in a reaction by providing kinetic

[a]*Department of Chemistry*
The University of Western Ontario
London, ON, N6A 5B7, Canada†
E-mail:stillman@uwo.ca
Web: www.stillmangroup.ca

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and stoichiometric details. When combined with UV-Visible absorption and circular dichroism spectroscopic information, binding sites and changes of protein structure as a result of metal binding can be determined. These techniques provide a simple, yet information-rich method for assessing the viability of a preliminary study. While *in vivo* studies may be suited for determining mobility and target binding site information in a cell, the high detail of the *in vitro* studies shown here provide the mechanistic insight required to understand the protective function that MT may perform in such drug resistant cancer forms. In this study, ESI-MS, UV-Visible absorption and circular dichroism spectroscopy, together with molecular dynamics calculations were employed to monitor the binding reaction of $Rh_2(OAc)_4$ with apo-β-MT.

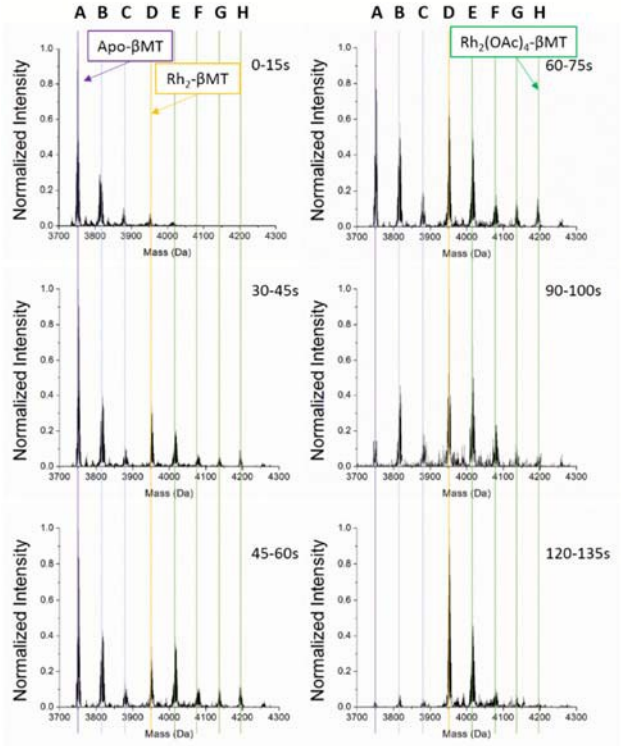

Figure 1. Deconvoluted time-dependent ESI-mass spectra recorded following the mixing of apo-β-MT with excess $Rh_2(OAc)_4$ at pH 7.3. The vertical axis shows the normalized intensity; the horizontal axis shows the mass in Da. Data were averaged for 15 seconds starting at 0, 30, 45, 60, 90 and 120 s from a data set collected over 60 minutes. The apo-β-MT is marked in track A, the final product with the Rh_2^{4+} bound is marked by track D. Track identifiers: A) Apo-β-MT; mass 3751. B) $Apo(OAc)_1$-β-MT; 3811. C) $Apo(OAc)_2$-β-MT; 3875. D) Rh_2-β-MT; 3951. E) $Rh_2(OAc)_1$-β-MT; 4013. F) $Rh_2(OAc)_2$-β-MT; 4075. G) $Rh_2(OAc)_3$-β-MT; 4136. H) $Rh_2(OAc)_4$-β-MT; 4192.

ESI mass spectra were recorded continuously over 60 min following the mixing of ($Rh_2(OAc)_4$) with apo-β-MT in water. Six representative mass spectra extracted at different times from the 60 minute collection are shown in Figure 1. The spectral data were averaged over 15 seconds meaning that the average data point time is 7.5 s from the start of the averaging in each spectrum resulting in data at 7.5, 37.5, 52.5, 67.5, 95.0 and 127.5 s.

Apo-β-MT is recognizable at a mass of ~3751 Da (track A), with the metallated species resulting in a series of higher mass species (tracks D-H). The metallated Rh_2-β-MT has a mass of ~3952 Da (track D), with each Rh replacing four of the cys thiol protons, which, we propose, results in an octahedral geometry for each Rh(II) in the dirhodium core, retaining its Rh-Rh bond. The remarkable feature of this binding reaction is that the data show, in sequence, the systematic removal of each of the four acetate groups, whose mass initially starts at ~4193 Da (track H) and with each acetate removal being noted at peaks M = ~4136 (track G), 4081 (track F), and 4016 Da (track E), respectively. We suggest that the released acetates in solution form surface adducts to positively charged amino acids (such as lysine) in the remaining MT, with these adducted species identified by a mass difference of 59 Da, and in proportional amount to the formation of Rh_2-β-MT. There was no other source of acetate in the solution. The time-dependent data indicate that loss of the acetates is faster than the initial interaction because the partially-ligated rhodium-MT complexes with 2, 3 or 4 acetates are in very low proportional concentration (tracks F-H) compared with the mono-acetate and Rh_2^{4+} core (track D). The overall binding rate can be seen from the decline in apo-β-MT abundance. As the Rh_2^{4+} core binds to the MT, the remaining apo-β-MT diminishes so that by 127.5 s almost none remains (tracks A-C).

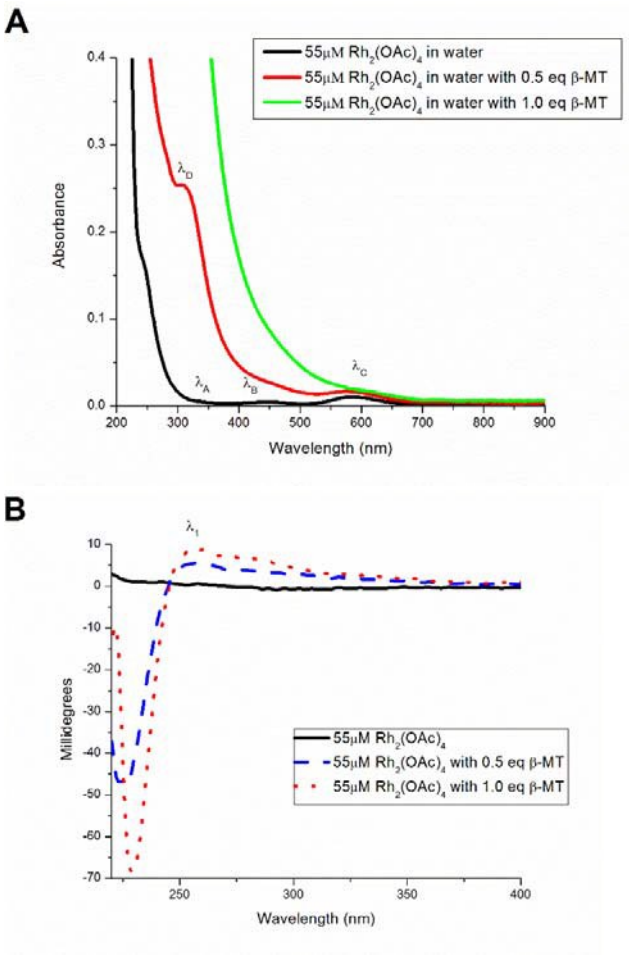

Figure 2. UV-Visible absorption spectra (A) and circular dichroism spectra (B) of a 55 μM $Rh_2(OAc)_4$ solution in water with 0.5 and 1.0 mol. eq. of apo-β-MT added. Figure 2A. Spectral assignments: λ_A = 350 nm; σ (H_2O) -> σ*(Rh_2). λ_B = 455 nm; π* (Rh_2) -> σ*(Rh-O). λ_C = 580 nm; π* (Rh_2) -> σ*(Rh_2). λ_D = 350 nm; σ (H_2O) -> σ*(Rh_2). Figure 2B. Spectral assignments: λ_1 = 255 nm, a new band appearing upon $Rh_2(OAc)_4$ binding.

The UV-visible absorption spectra recorded with 0.5 and 1 mol. eq. of $Rh_2(OAc)_4$ added to the apo-β-MT showed appearance of bands near 325 nm associated with a change in color from blue to yellow (Figure 2A). The Rh-Rh metal-metal bond was indicated by the band near 580 nm that only slightly blue shifted, as reported by others regarding rhodium axial coordination by solvents.[18]

The circular dichroism spectra show a new band forming near 255 nm that only appears as a result of the protein binding to the $Rh_2(OAc)_4$ (Figure 2B).

The ESI-mass spectral data together with the absorption spectral data, support our proposal that the $Rh_2(OAc)_4$ complex is bound as the Rh_2^{4+} core after each of the acetate bridging ligands is released and replaced by pairs of cysteinyl thiolates from the MT. In this mechanism, the thiolates displace the acetates stepwise, allowing the Rh-Rh bond to survive. However, the proposal does require a test of whether the Rh_2^{4+} core could exist within the peptide using a reasonable folding motif achievable from the 9 cysteines of the apo-β-MT fragment. To test this, we used an energy minimized structure of Cd_3β-MT1a to establish a typical host volume.[21] Using the $Rh_2(OAc)_4$ complex structure as a starting point, we deleted the 3 Cd^{2+} ions in the protein binding site and inserted the Rh_2^{4+} core aligned by the acetates. The alignment was locked as the MM3 system has no information about the electronic structure of the Rh_2^{4+} metal-metal bond requirement. The 8 coordination points for the 8 x/y aligned thiolate ligands are defined in the electronic structure as being eclipsed. There are two further z-axis coordination points frequently used by solvents.[18] The cysteinyl thiolate alignments following deletion of the three Cd^{2+} ions were used to guide bond formation to the Rh_2^{4+} core. The MD calculation trajectory showed that the peptide backbone realigned within 10 ps, indicating that the Rh_2^{4+} core required very little rearrangement to use the volume previously occupied by the 3 Cd^{2+} ions. Figure 3 shows the space filling representation and the ribbon alignment of the orientation at 200 ps. No significant change in the energy was observed for over 170 ps. The ribbon (Figure 3, right) shows that the peptide encapsulates the Rh_2^{4+} core. Emission data from Cu^+ binding studies to metallothioneins support the proposal that when the MT peptide binds using all the cysteines in a cluster that there is little access to the solvent, [22] so we have not included water in the binding region. The ribbon depiction of the peptide orientation in Cd_3-β-MT was virtually the same as shown in Figure 3, confirming that replacement of the three Cd^{2+} ions by the Rh_2^{4+} core did not require major reorientation of the cysteines. We do note, though, that the structure in Figure 3 is a hypothetical model designed to indicate the possible conformational changes necessary for the 38-amino acid, 9 cysteine apo-β-MT to bind the Rh_2^{4+} core. In later work we will use the ONIOM method to calculate the electronic structure of the Rh_2^{4+} core bound to 8 cysteinyl sulfurs within the relaxed protein environment. However, at this present time, the value of the minimized structure is in demonstrating that the protein backbone with its 9 cysteines can wrap around the Rh_2^{4+} core with little change from its normal conformation. Similarly, the experimental bonding parameters will be obtained in the future, but because crystallization of MT has proven to be almost impossible, we will need to use EXAFS methods. However, EXAFS methods require both high concentrations and stability, which will be the focus of a subsequent study.

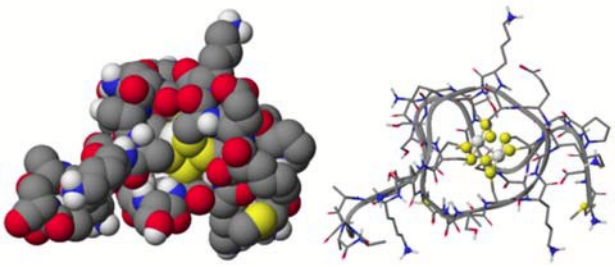

Figure 3. Results of a molecular dynamics calculation where the Rh_2^{4+} core was inserted into the apo-β-MT1a binding site. The calculation was carried out for 200 ps at an average temperature of 355 K with a dielectric constant set to the 78.5 of water. The yellow spheres are cysteinyl thiolates while the white spheres (right) are the two Rh^{2+} ions. We note that the intention of this calculation was to examine if the Rh_2^{4+} core could bind using 8 cys S without major change to the normal conformation.

Here, we report investigation of the simplest dirhodium carboxylate, dirhodium(II) tetraacetate ($Rh_2(OAc)_4$), and its deconstruction *in vitro* by the human MT1a β-domain fragment of the metal-defence protein, MT. Metallothioneins have a well-documented chemistry scavenging toxic metals that enter the cell and we and others have suggested that this property can contribute to greatly reduced efficacies for metal-based drugs. $Rh_2(OAc)_4$ is a complex with a dimetallic Rh(II) core, with four bridging acetates and the axial position occupied by the solvent, with each Rh(II) being octahedrally coordinated. Electrospray Ionization mass spectrometry was used to monitor the reaction between the rhodium complex and the protein in real time, with UV-Visible absorption and circular dichroism spectroscopies providing evidence of ligand exchange. The human MT1a β-domain fragment comprises a 38 amino acid peptide chain with 9 cysteine residues. We propose 8 of these 9 cysteinyl thiolates displace the four acetate ligands, leaving the Rh_2^{4+} core completely encapsulated by the protein. In the reaction, the four linker acetates are replaced stepwise by the more aggressive cysteinyl thiolates of the metallothionein, as the protein wraps around the rhodium complex, bringing the cysteinyl thiolates closer to the interior core.

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