Unusual Structural Features in the Lysozyme Derivative of the Tetrakis(acetato)chloridodiruthenium(II,III) Complex**

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Abstract: The reaction between the paddle-wheel tetrakis-(acetato)chloridodiruthenium(II,III) complex, [Ru$_2$(µ-O$_2$CCH$_3$)$_4$Cl] and hen egg-white lysozyme (HEWL) was investigated through ESI-MS and UV/Vis spectroscopy and the formation of a stable metal–protein adduct was unambiguously demonstrated. Remarkably, the diruthenium core is conserved in the adduct while two of the four acetate ligands are released. The crystal structure of this diruthenium–protein derivative was subsequently solved through X-ray diffraction analysis to 2.1 Å resolution. The structural data are in agreement with the solution results. It was found that HEWL binds two diruthenium moieties, at Asp101 and Asp119, respectively, with the concomitant release of two acetate ligands from each diruthenium center.

*T*he tetrakis(acetato)chloridodiruthenium(II,III) complex [Ru$_2$(µ-O$_2$CCH$_3$)$_4$Cl] (**1**) was first prepared by Stephenson and Wilkinson[1] and then structurally characterized by Cotton and co-workers.[2] Notably, this compound bears a dimetallic center in which two mixed-valence ruthenium ions are directly connected through a strong Ru−Ru bond, with a bond order of 2.5 and a short distance of 2.28 Å (Figure 1). In the crystal state, this compound exhibits a polymeric structure in which the diruthenium centers, coordinated to four bridging acetates, are linked by chloride axial ligands into an infinite zig-zag chain.[2] The unique structural,[2] electronic,[2–3] spectroscopic,[3–4] electrochemi-
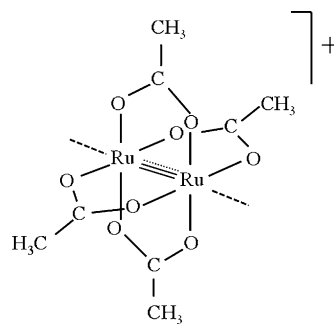

Figure 1. The typical paddle-wheel structure of the [Ru$_2$(µ-O$_2$CCH$_3$)$_4$]$^+$ unit.

cal,[5] and magnetic[6] properties of this peculiar dimetallic center were investigated and elucidated afterwards in a series of physicochemical studies.[2a,7] Remarkably, compound **1** is the precursor of a class of diruthenium complexes that have found widespread application in a variety of fields.

For instance, diruthenium tetracarboxylates can act as building blocks for cubic 3D network structured molecule-based magnets[8] and as catalysts.[9] Early studies reported their potential as catalysts for the hydrogenation of alkenes and alkynes[9a,b] and for the competitive cyclopropanation of alkenes.[9c] More recently, compound **1** and its derivatives have been reported to act as catalysts for the oxidative transformation of secondary amines into imines,[9d] the oxygenation of organic sulphides,[9e] the oxidation of alcohols,[9f] and the skeletal reorganization of enynes.[9g]

Furthermore, diruthenium tetracarboxylate compounds have been evaluated as prospective pharmaceutical agents.[10] Compound **1** has been used as a precursor to prepare a novel class of diruthenium(II,III) metallodrugs of the general formula [Ru$_2$(RCOO)$_4$Cl], where RCOO$^-$ is a carboxylate anion derived from a therapeutic drug.[10c] In particular, metallodrugs containing carboxylate ligands derived from the nonsteroidal anti-inflammatory drug ibuprofen[10d,e] or from γ-linolenic acid[10f,g] were found to be active against glioma tumor models and have been investigated for partial elucidation of their mechanism of action. Moreover, the diruthenium–ibuprofenate complex shows anti-inflammatory properties with reduced gastric ulceration in vivo compared to the ibuprofen parent drug.[10h]

As a result of the high degree of interest of this class of compounds for bioinorganic chemistry and biomedical applications, compound **1** was recently investigated for reactivity with some low-molecular-weight biomolecules such as amino

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acids and a few natural reducing agents (ascorbic acid and glutathione).[11]

However, no detailed studies on the interactions of compound **1** and its analogues with proteins have been reported. This prompted us to analyze in depth the reaction of tetrakis(acetato)chloridodiruthenium(II,III) with the model protein hen egg-white lysozyme (HEWL) through independent biophysical methods to describe the resulting derivatives. HEWL has already been used as a scaffold to characterize a variety of metal adducts and thus unravel the molecular basis for protein–metal complex recognition.[12]

At first, the behavior of compound **1** in a reference buffer at pH 6.8 was investigated by UV/Vis spectroscopy (Figure 2 a). The initial spectrum is characterized by a main band at 423 nm that is assigned to the $\pi(Ru-O)\rightarrow\pi^*(Ru_2)$ elec-
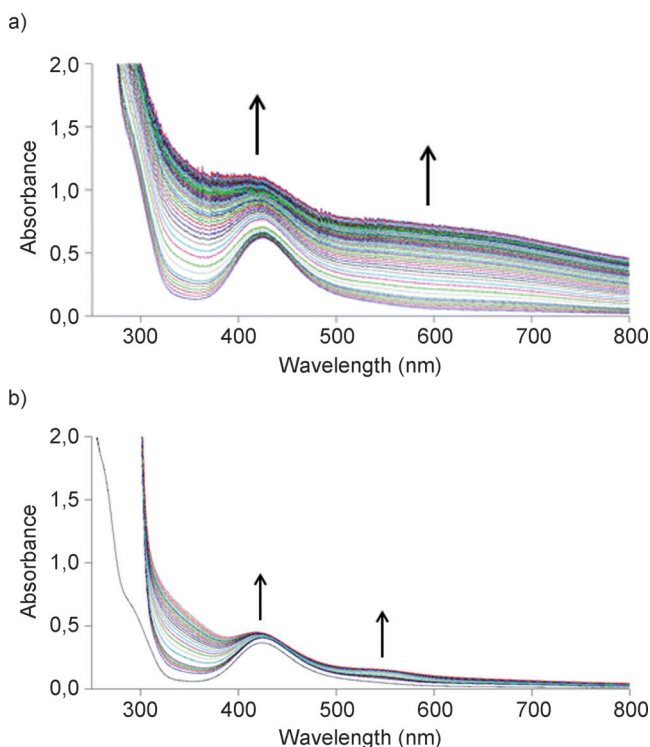
a)

b)

Figure 2. Time-dependent UV/Vis spectra (up to 24 h) of compound **1** 10^{-3} M dissolved in 20 mmol L^{-1} ammonium acetate buffer, pH 6.8 (a), and in the presence of HEWL (b). [compound **1**] $= 5\times10^{-4}$ mmol L^{-1}, complex/protein $= 1:1$.

tronic transition.[3] The maximum wavelength of this band indicates the predominance in solution of the neutral axially substituted $[Ru_2(\mu-O_2CCH_3)_4(H_2O)_2]^+$ species.[11a] Monitoring the electronic spectra over 24 h shows specific and progressive increases in the absorbance in the UV (300–370 nm) and visible (500–700 nm) regions. The new bands appearing in these regions might be related to replacement of the axial ligands as previously reported for the reactions of $[Ru_2(\mu-O_2CCH_3)_4(H_2O)_2]^+$ with amino acids.[11a] A generalized increase in absorbance is also detected over the whole 300–800 nm spectral interval, a result that might be attributed to

the occurrence of aggregation phenomena and the formation of oligomeric species.

Later, the same process was monitored by UV/Vis spectroscopy in the presence of an equimolar amount of HEWL (Figure 2 b). In this case, a marked increase in absorbance is detected in the 300–400 nm region and a less pronounced increase is detected between 500 and 600 nm, while the band at 423 nm is basically conserved. By contrast, the generalized increase in absorbance over the whole spectral window is no longer observed. At the end of the process, ultrafiltration was carried out with a cut-off of 3 kDa, and the two resulting fractions were analyzed by UV/Vis spectroscopy. Notably, the band at 423 nm is associated with the low-MW fraction while the band at 330 nm is mainly associated with the protein fraction (see the Supporting Information). These results imply that the presence of the protein greatly hinders the aggregation/oligomerization processes; ruthenium binding to the protein results in the appearance of a characteristic broad band centered at 330 nm.

Electrospray ionization mass spectrometry (ESI-MS) measurements were then carried out to better characterize adduct formation between compound **1** and HEWL. ESI-MS is a potent investigation tool that can be used to identify the exact nature of protein-bound metal fragments and precisely assess the metal/protein stoichiometry in this kind of adduct.[13] The resulting ESI-MS spectra are shown in Figure 3. Remarkably, a rather intense new peak is detected at 14656.6 Da, which corresponds to a complex formed by HEWL with a fragment of the type $[Ru_2(\mu-O_2CCH_3)_2-(H_2O)_2]^{3+}$. The intensity of this peak progressively increases with time over 72 h.
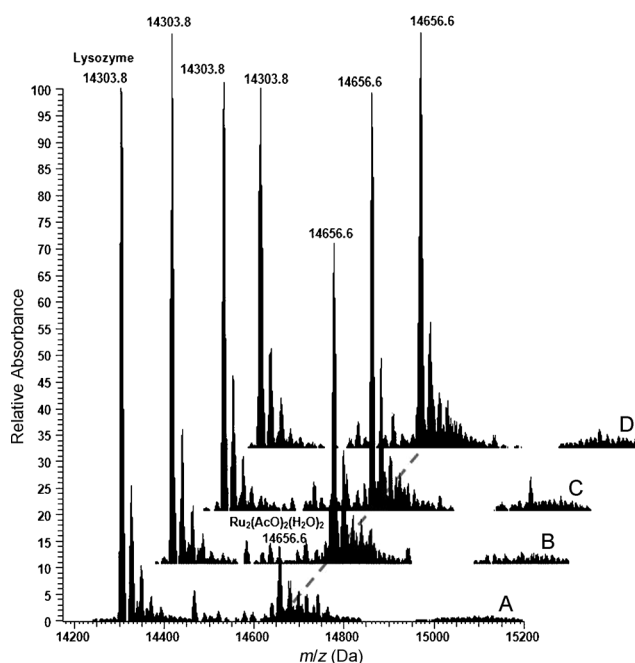

Figure 3. Deconvoluted ESI-MS of HEWL treated with 10^{-3} mol L^{-1} $[Ru_2(\mu-O_2CCH_3)_4Cl]$ (complex/protein $= 10:1$) in 20 mmol L^{-1} ammonium acetate buffer, pH 6.8 recorded after 6 h (A), 24 h (B), 48 h (C), and 72 h (D) of incubation at RT.

The observation of this new peak implies that the dimetallic center is retained upon protein binding, while two of the four acetate ligands are lost owing to ligand-replacement reactions.

To further elucidate the structure of the metal–protein adduct, we tried to crystallize the protein in the presence of compound **1**. Crystals suitable for X-ray diffraction studies were grown (see the Supporting Information). The structure was refined at 2.15 Å resolution to an R-factor of 18.9 (R-free 25.4) and deposited in the Protein Data Bank (PDB ID 4OOO, see the Supporting Information). The overall protein conformation is not significantly affected by the binding of compound **1** (Figure 4): in fact, the HEWL structure in the complex is almost identical to that of the native protein (PDB code 193L), with root mean square deviation in the positions of the backbone α-carbon atoms as low as 0.26 Å.
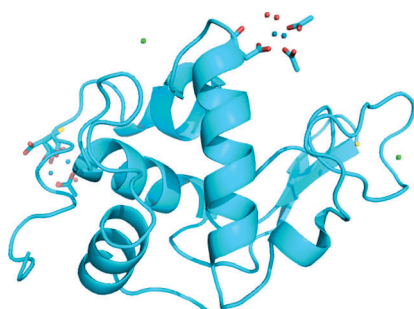

Figure 4. Overall structure of HEWL–compound **1**. The diruthenium centers are covalently attached to the Asp101 or Asp119 side chains.

Inspection of F_0-F_c electron density maps led to the identification of two distinct binding sites for the bis-(acetato)diruthenium fragments on the HEWL surface (Figure 5).

The side-chain oxygen atoms of Asp101 or Asp119 are found to coordinate to the diruthenium center in a fashion leading to a distorted lantern-like structure. In both sites, the Ru atoms have occupancy equal to 0.5. Selected bond lengths and angles for the diruthenium center in the adduct are given in Table S1 in the Supporting Information.

The structural results provide valuable hints for the likely mechanism of protein metalation. It may be postulated that the aspartate group is able to displace one of the acetate ligands coordinated to the diruthenium core in compound **1** and to replace it as a bidentate ligand; a second acetate is then removed, possibly owing to steric hindrance with the protein, and is replaced by two water molecules. Despite the evident similarity of the metal–protein interactions at the two distinct sites, it must be noted that in one case, the two remaining acetate ligands are *trans* to each other, while in the other case, they have a *cis* configuration.

As noted above, the degree of occupancy of the two aspartate binding sites by the diruthenium fragment is similar, thus comparable affinities may be inferred. The resulting adduct is apparently very stable as indicated by the time-course absorption spectra.
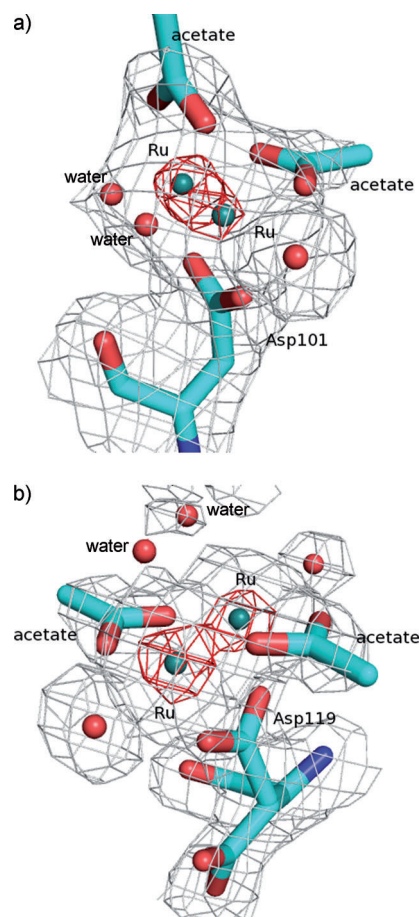

Figure 5. Details of the binding sites of the compound **1** moieties in HEWL. The adduct contains diruthenium diacetate fragments coordinated to Asp101 (a) or Asp119 (b) side chains. In both sites, the binding of compound **1** to HEWL is accompanied by the loss of two acetate groups. The $2F_o-F_c$ electron density maps are contoured at the 3σ (red) and 1σ (grey) level.

In conclusion, we have shown that the $[Ru_2(\mu\text{-}O_2CCH_3)_4]^+$ paddle-wheel complex is able to interact with the model protein HEWL to form a stable adduct. The adduct was characterized in detail based on largely consistent ESI-MS and X-ray diffraction results. In particular, the peculiar structural features of the adduct were elucidated through high-resolution crystallographic data. Interestingly, the HEWL derivative of compound **1** bears two diruthenium centers tightly anchored to two distinct aspartate groups in positions that are accessible to the solvent. In both cases, association of the diruthenium moieties with the protein is achieved through the formation of two coordination bonds between the two oxygen atoms of the side-chain carboxylate group of Asp and the two ruthenium atoms of the diruthenium motif. To our knowledge, this peculiar mode of association between the diruthenium motif and the protein is unprecedented. However, it is worth noting that quite similar behavior has emerged for dirhodium tetracarboxylate complexes in their interactions with a variety of peptides as described by Ball and co-workers.[14] Remarkably, association of the dirhodium motif to two aspartate groups of the ZF

peptide after the release of two acetate ligands, in a fashion that closely resembles the behavior of the diruthenium complex with lysozyme, has been reported.[14a]

Upon consideration of the known and established catalytic properties of various diruthenium centers, it is conceivable that this novel diruthenium–HEWL derivative may gain new catalytic properties compared to the native protein and may thus behave as an artificial metalloenzyme. The likely occurrence of innovative and peculiar catalytic properties for this HEWL derivative is currently being explored.

[1] T. A. Stephenson, G. Wilkinson, *J. Inorg. Nucl. Chem.* **1966**, *28*, 2285–2291.

[2] a) *Multiple Bonds between metal atoms*, 3rd ed. (Eds.: F. A. Cotton, C. A. Murillo, R. A. Walton), Springer, Heidelberg, **2005**; b) M. J. Bennett, K. G. Caulton, F. A. Cotton, *Inorg. Chem.* **1969**, *8*, 1–6; c) A. Bino, F. A. Cotton, T. R. Felthouse, *Inorg. Chem.* **1979**, *18*, 2599–2604.

[3] a) J. G. Norman, Jr., H. J. Kolari, *J. Am. Chem. Soc.* **1978**, *100*, 791–799; b) J. G. Norman, Jr., G. E. Renzoni, D. A. Case, *J. Am. Chem. Soc.* **1979**, *101*, 5256–5267.

[4] a) C. R. Wilson, H. Taube, *Inorg. Chem.* **1975**, *14*, 2276–2279; b) S. M. Tetrick, V. T. Coombe, G. A. Heath, T. A. Stephenson, R. A. Walton, *Inorg. Chem.* **1984**, *23*, 4567–4570.

[5] a) M. Mukaida, T. Nomura, T. Ishimori, *Bull. Chem. Soc. Jpn.* **1972**, *45*, 2143–2147; b) M. McCann, A. Carvill, C. Cardin, M. Convery, *Polyhedron* **1993**, *12*, 1163–1169; c) M. W. Cooke, C. A. Murphy, T. S. Cameron, J. C. Swarts, M. A. S. Aquino, *Inorg. Chem. Commun.* **2000**, *3*, 721–725; d) M. W. Cooke, T. S. Cameron, K. N. Robertson, J. C. Swarts, M. A. S. Aquino, *Organometallics* **2002**, *21*, 5962–5971.

[6] F. A. Cotton, Y. Kim, T. Ren, *Polyhedron* **1993**, *12*, 607–611; R. Jiménez-Aparicio, F. A. Urbanos, J. M. Arrieta, *Inorg. Chem.* **2001**, *40*, 613–619; Y. Sayama, M. Handa, M. Mikuriya, I. Hiromitsu, K. Kasuga, *Bull. Chem. Soc. Jpn.* **2003**, *76*, 769–779.

[7] a) M. A. S. Aquino, *Coord. Chem. Rev.* **1998**, *170*, 141–202; b) M. A. S. Aquino, *Coord. Chem. Rev.* **2004**, *248*, 1025–1045.

[8] T. E. Vos, Y. Liao, W. W. Shum, J. H. Her, P. W. Stephens, W. M. Reiff, J. S. Miller, *J. Am. Chem. Soc.* **2004**, *126*, 11630–11639; B. S. Kennon, J. S. Miller, *Inorg. Chem.* **2010**, *49*, 5542–5545.

[9] a) P. Legzdins, R. W. Mitchell, G. L. Rempel, J. D. Ruddick, G. Wilkinson, *J. Chem. Soc. A* **1970**, 3322–3326; b) A. J. Lindsay, G. McDermott, G. Wilkinson, *Polyhedron* **1988**, *7*, 1239–1242; c) A. F. Noels, A. Demonceau, E. Carlier, A. J. Hubert, R.-L. Márquez-Silva, R. A. Sánchez-Delgado, *J. Chem. Soc. Chem. Commun.* **1988**, 783–784; d) S. I. Murahashi, Y. Okano, H. Sato, T. Nakae, N. Komiya, *Synlett* **2007**, 1675–1678; e) L. Villalobos, J. E. B. Paredes, Z. Cao, T. Ren, *Inorg. Chem.* **2013**, *52*, 12545–12552; f) C. N. Kato, M. Ono, T. Hino, T. Ohmura, W. Mori, *Catal. Commun.* **2006**, *7*, 673–677; g) T. Nakae, T. Yasunaga, M. Kamiya, Y. Fukumoto, N. Chatani, *Chem. Lett.* **2013**, *42*, 1565–1567.

[10] a) E. J. van Rensburg, E. Kreft, J. C. Swarts, S. R. Dalrymple, D. M. MacDonald, M. W. Cooke, M. A. S. Aquino, *Anticancer Res.* **2000**, *22*, 889–892; b) B. K. Keppler, M. Henn, U. M. Juhl, M. R. Berger, R. Niebl, F. E. Wagner, *Prog. Clin. Biochem. Med.* **1989**, *10*, 41–69; c) D. de Oliveira Silva, *Anti-Cancer Agents Med. Chem.* **2010**, *10*, 312–323; d) G. Ribeiro, M. Benadiba, A. Colquhoun, D. de Oliveira Silva, *Polyhedron* **2008**, *27*, 1131–1137; e) M. Benadiba, R. R. P. Santos, D. de Oliveira Silva, A. Colquhoun, *J. Inorg. Biochem.* **2010**, *104*, 928–935; f) G. Ribeiro, M. Benadiba, D. de Oliveira Silva, A. Colquhoun, *Cell Biochem. Funct.* **2010**, *28*, 15–23; g) J. A. Miyake, M. Benadiba, G. Ribeiro, D. de Oliveira Silva, A. Colquhoun, *Anticancer Res.* **2014**, *34*, 1901–1911; h) A. Andrade, S. F. Namora, R. G. Woisky, G. Wiezel, R. Najjar, J. A. A. Sertie, D. de Oliveira Silva, *J. Inorg. Biochem.* **2000**, *81*, 23–27; i) R. L. S. R. Santos, A. Bergamo, G. Sava, D. de Oliveira Silva, *Polyhedron* **2012**, *42*, 175–181.

[11] a) R. L. S. R. Santos, R. van Eldik, D. de Oliveira Silva, *Inorg. Chem.* **2012**, *51*, 6615–6625; b) R. L. S. R. Santos, R. van Eldik, D. de Oliveira Silva, *Dalton Trans.* **2013**, *42*, 16796–16805.

[12] a) A. Vergara, G. D'Errico, D. Montesarchio, G. Mangiapia, L. Paduano, A. Merlino, *Inorg. Chem.* **2013**, *52*, 4157–4159; b) L. Messori, F. Scaletti, L. Massai, M. A. Cinellu, C. Gabbiani, A. Vergara, A. Merlino, *Chem. Commun.* **2013**, *49*, 10100–10102; c) L. Messori, T. Marzo, C. Gabbiani, A. A. Valdes, A. G. Quiroga, A. Merlino, *Inorg. Chem.* **2013**, *52*, 13827–13829; d) J. R. Helliwell, S. W. Tanley, *Acta Crystallogr. Sect. D* **2013**, *69*, 121–125; e) L. Messori, A. Merlino, *Dalton Trans.* **2014**, *43*, 6128–6131.

[13] a) A. Casini, G. Mastrobuoni, W. H. Ang, C. Gabbiani, G. Pieraccini, G. Moneti, P. J. Dyson, L. Messori, *ChemMedChem* **2007**, *2*, 631–635; b) L. Messori, F. Scaletti, L. Massai, M. A. Cinellu, I. Russo Krauss, G. di Martino, A. Vergara, L. Paduano, A. Merlino, *Metallomics* **2014**, *6*, 233–236.

[14] a) A. N. Zaykov, K. R. Mackenzie, Z. T. Ball, *Chem. Eur. J.* **2009**, *15*, 8961–8965; b) Z. Chen, F. Vohidov, J. M. Coughlin, L. J. Stagg, S. T. Arold, J. E. Ladbury, Z. T. Ball, *J. Am. Chem. Soc.* **2012**, *134*, 10138–10145; c) R. Sambasivan, Z. T. Ball, *J. Am. Chem. Soc.* **2010**, *132*, 9289–9291; d) A. N. Zaykov, B. V. Popp, Z. T. Ball, *Chem. Eur. J.* **2010**, *16*, 6651–6659.