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Aerobic Reactions of Antitumor Active Dirhodium(II) Tetraacetate $\text{Rh}_2(\text{CH}_3\text{COO})_4$ with Glutathione

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Abstract

The aerobic reaction between glutathione (H_3A) and dirhodium(II) tetraacetate, $\text{Rh}_2(\text{AcO})_4$ ($\text{AcO}^- = \text{CH}_3\text{COO}^-$), in aqueous solution ($\text{pH} = 7.4$) breaks up the direct $\text{Rh}^{\text{II}}\text{-Rh}^{\text{II}}$ bond and its carboxylate framework, as evidenced by UV-vis spectroscopy. After purifying the reaction product using size exclusion chromatography, electrospray ionization mass spectrometry (ESI-MS) of the solution showed binuclear $[\text{Rh}^{\text{III}}_2(\text{HA})_4]^{2-}$ and $[\text{Rh}^{\text{III}}_2(\text{HA})_5]^{4-}$ ions. Evaporation yielded a solid compound, $\{\text{Na}_2[\text{Rh}^{\text{III}}_2(\text{HA})_4] \cdot 7\text{H}_2\text{O}\}_n$, for which Rh K-edge extended X-ray absorption fine structure (EXAFS) spectroscopy revealed ~ 2 Rh-O (2.08 ± 0.02 Å) and ~ 4 Rh-S (2.33 ± 0.02 Å) bond distances around each Rh^{III} center, and the $\text{Rh}^{\text{III}}\text{--Rh}^{\text{III}}$ distance 3.11 ± 0.02 Å, close to that in dirhodium(III) complexes with three bridging thiolates connecting Rh^{III}_2 units. The ^{13}C CPMAS NMR spectrum of the Rh^{III} -glutathione complex showed a change $\delta_{\text{C}} > 6$ ppm in the chemical shift of the COO^- signal, indicating some carboxylate coordination to the Rh^{III} ions. This study shows that under aerobic conditions glutathione enables oxidation of $\text{Rh}_2(\text{AcO})_4$ and thus reduces its antitumor efficiency.

Graphical abstract

The reaction of $\text{Rh}_2(\text{AcO})_4$ with glutathione was investigated by ESI-MS, UV-vis, ^{13}C NMR and X-ray absorption spectroscopy, revealing that glutathione breaks down the carboxylate framework enabling oxidation of the Rh_2^{4+} core to Rh^{III} dimeric units, bridged by three thiolates.

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Electronic Supplementary Material

ESI-mass spectra of Rh^{III} -GSH solid (2), and the $\text{Rh}_2(\text{AcO})_4$ -glutathione solution mixture at 0 and 80 V measured in (–) and (+) ion modes; separate contributions of different scattering paths in the EXAFS spectra of compound 2; S K-edge XANES spectrum of Rh^{III} -GSH solid (2) and the Rh^{III} *N*-acetylcysteine compound (3). For the $\text{Rh}_2(\text{AcO})_4$ -glutathione reaction at the pH of mixing (acidic) see Appendix 1, with UV-vis and ESI-mass spectra for the $\text{Rh}_2(\text{AcO})_4$ -glutathione solution.

Keywords

Glutathione; Dirhodium(II) tetraacetate; O₂ reduction; Structure; Spectroscopy; ESI-MS; EXAFS; NMR

1. Introduction

Antitumor activities of metal-based drugs are primarily based on their ability to: (a) target DNA, inhibiting transcription and DNA replication, (b) act as antimetastatic agents, or (c) affect mitochondrion and vital intracellular signalling pathways, resulting in cell damage or cell death.[1] The introduction of a class of platinum compounds including cisplatin, *cis*-[Pt(NH₃)₂Cl₂], for the treatment of certain tumors by Rosenberg *et al.* in 1969,[2] was followed by numerous efforts to design more efficient antitumor agents than the cisplatin family. Although not in clinical use, dirhodium(II) carboxylates have attracted considerable attention since the 1970s due to their marked antitumor activity.[3, 4] They can target DNA and form interstrand cross-links, which ultimately leads to cell death,[5, 6] but also bind to proteins and enzymes. An early *in vitro* study by Bear and co-workers concluded that dirhodium(II) carboxylates merely inhibit enzymes that have thiol groups at or near their active sites. This observation was linked to the ability of *L*-cysteine to break down the carboxylate framework around the dirhodium(II) core.[7] Later it was found that some dirhodium(II) complexes, retaining at least two of the bridging carboxylates, could inhibit transcription through redox reactions with cysteine residues of T7-RNA polymerase.[8] A recent *in vitro* ESI-MS study on the interaction between Rh₂(AcO)₄ (**1**, AcO[−] = CH₃COO[−]) and the β-domain fragment of human metallothionein 1a showed that the Rh₂⁴⁺ core binds to 8 cysteine residues.[9]

Glutathione (GSH, γ-*L*-glutamyl-*L*-cysteinyl-glycine; here denoted H₃A in its triprotonated form; see Scheme 1) is the most abundant thiol-containing peptide found in cells (0.5 – 10 mM),[10] with many important functions including protection by coordinating heavy metal ions *via* the thiol group. The reported microscopic acid dissociation constants obtained by means of ¹H NMR spectroscopy are: pK_{a1} = 2.19 (glutamyl –COOH), pK_{a2} = 3.22 (glycyl –COOH), pK_{a3} = 8.97 (cysteinyl –SH), and pK_{a4} = 9.17 (–NH₃⁺).[11]

The interactions between GSH and some antitumor active complexes including cisplatin and ruthenium-based compounds have been extensively studied, showing that GSH sometimes can reduce their efficiency.[12–14] For the antitumor activity of dirhodium(II) carboxylates it appears essential to retain the Rh^{II}–Rh^{II} bond.[4] Therefore, studies of their interactions with glutathione are needed, also to investigate potential metabolic routes of the reaction products in the cell. The reaction of a dirhodium(II) bipyridine (bpy) compound, [Rh₂(AcO)₂(bpy)₂(CH₃CN)₂](BF₄)₂, with glutathione resulted in a blue precipitate, which was proposed to contain thiolate ligands bound to a dirhodium(II) core.[15] Another study based on UV-vis spectroscopy reported reduction by GSH of dirhodium(II) carboxylates containing bpy or phen (1,10-phenantroline) to polynuclear Rh(I) species, while proposing [Rh₂(AcO)₄(H₂O)₂] to form a stable adduct [Rh₂(AcO)₄(GSH)₂].[16] Later, live cell cytotoxicity studies using a class of dirhodium(II) complexes with general formula [Rh₂(μ-

$\text{AcO})_2(\eta^1\text{-AcO})(\text{L})(\text{CH}_3\text{OH})]^+$ (e.g. with L = bpy, phen) showed that changes in the glutathione level in the cells had minimal effect on the cytotoxicity of this specific family of dirhodium(II) complexes.[5] Another recent spectroscopic, ESI-MS and computational study suggested that $\text{Rh}_2(\text{AcO})_4(\text{GSH})_{1-2}$ species form under anaerobic conditions in aqueous solution.[17]

Only one crystal structure in the Crystal Structure Database (CSD) shows a thiol group (i.e. benzyl thiol) axially coordinated to dirhodium(II) tetraacetate ($\text{Rh}^{\text{II}}\text{-Rh}^{\text{II}}\text{-SH}$), leaving the metal-metal bond intact.[18] The only Rh K-edge EXAFS spectroscopy study on a reaction of $\text{Rh}_2(\text{AcO})_4$ with biomolecules was on adenosine coordinating to its axial positions.[19]

To date, there is no structural information about the reaction product(s) between glutathione and dirhodium(II) carboxylate complexes. Here, we report the results of $\text{Rh}_2(\text{AcO})_4$ reacting with GSH in aqueous solution (mole ratios 1:4 and 1:10; pH 7.4) under aerobic conditions using ESI-MS, UV-vis, ^{13}C NMR and Rh K-edge EXAFS spectroscopic techniques. Even though our chosen final experimental pH 7.4 is close to the physiological pH values in normal cells, many tumors show oxygen deficiency (*hypoxia*) [20], which may induce slightly different (mildly acidic) intracellular environments [21].

2. Material and methods

L-Glutathione (reduced) and sodium hydroxide were purchased from Sigma-Aldrich and used without further purification. Anhydrous dirhodium(II) tetraacetate (**1**) was used as supplied by Pressure Company Co. Deoxygenated water was prepared by bubbling argon gas through boiled distilled water. For pH measurements, a calibrated Thermo Scientific Orion Star semi-micro electrode was used.

2.1. Aerobic reaction of $\text{Rh}_2(\text{AcO})_4$ with glutathione

To a solution of glutathione (0.228 mmol) in 18 mL degassed boiled water, a suspension of emerald green $\text{Rh}_2(\text{AcO})_4$ (0.057 mmol) in 6 mL O_2 -free water was added dropwise under a stream of argon gas to avoid oxidation of glutathione to GSSG. A clear emerald green solution (pH 3.2) was obtained and its pH was then adjusted to 7.4 by dropwise addition of 1.0 M NaOH prepared in O_2 -free water. The resulting dark red solution was stirred for 2 hours under argon flow at room temperature, and then exposed to air for 46 hours, while keeping the pH at 7.4 ($C_1 = 2.3$ mM; total volume = 24.5 mL). Aliquots of the mixture were taken for the ESI-MS time study at 0.5, 2, 24 and 48 hours after the initial pH adjustment; see Figure S1-a. After 48 hours, the solution was concentrated and passed through a Sephadex G-15 size exclusion chromatography column, using Millipore water as eluent. Different parts of the orange band on the column were sampled for ESI-MS, all showing similar mass peaks. One part of the solution was concentrated and used for EXAFS measurement, while another part was evaporated to dryness under vacuum at room temperature. Elemental analysis and thermogravimetry (TG, using a Netzsch STA 409 instrument) showed for this dark red solid sample $\text{Rh}^{\text{III}}\text{-GSH}$ (**2**): Calcd. for $\{\text{Na}_2[\text{Rh}_2^{\text{III}}(\text{HA})_4]\cdot 7\text{H}_2\text{O}\}_n$ ($\text{Rh}_2\text{C}_{40}\text{H}_{74}\text{N}_{12}\text{O}_{31}\text{S}_4\text{Na}_2$): %C 30.04, %H 4.66, %N 10.51 (7.88% H_2O); Found %C 30.31, %H 4.76, %N 9.99 (TG: 7.30% H_2O); yield = 78.1%.

Magnetic susceptibility measurement with a Johnson Matthey magnetic susceptibility balance (MSB) confirmed that the solid compound was diamagnetic.

For UV-vis measurement a dilute solution was prepared using 0.096 mmol glutathione and 0.024 mmol $\text{Rh}_2(\text{AcO})_4$ following the above procedure (total volume = 24.3 mL; $C_1 = 1.0$ mM). Aliquots of the reaction mixture were taken right after addition of $\text{Rh}_2(\text{AcO})_4$ (pH = 3.2), and then at 5 minutes, 6 hours and 48 hours after adjusting the pH to 7.4.

A similar solution with $\text{Rh}_2(\text{AcO})_4$: GSH in mole ratio 1:10 ($C_1 = 1.9$ mM) was prepared for ESI-MS study following the above procedure.

2.2. Electrospray ionization mass spectrometry

ESI-mass spectra were collected both in positive (+) and negative (–) ion modes on an Agilent 6520 Q-ToF instrument by direct infusion of the Rh-glutathione reaction mixtures, as well as different parts of the orange band formed on the Sephadex G-15 column; water was used as the mobile phase. For technical details, see *Ref.* [22]. The assignments of the mass peaks were verified with the Isotope Distribution Calculator from Agilent and with a high resolution calculation method from Scientific Instrument Services. [23]

2.3. Electronic spectroscopy

A Cary 300 UV-vis double-beam spectrophotometer was used to measure the UV-vis spectra of $\text{Rh}_2(\text{AcO})_4$ and glutathione solution mixtures (for sample preparation, see Section 2.1). Quartz cells with a path length of 1 mm were used to hold the samples, using water as blank.

2.4. ^{13}C NMR spectroscopy

Carbon-13 cross-polarization magic angle spinning (CPMAS) NMR spectra were measured at room temperature using a Bruker Avance 500 MHz spectrometer with high power proton decoupling, setting α -glycine carbonyl at 176.50 ppm as reference.[24] The Rh^{III} -GSH solid (**2**) and glutathione were packed separately into a 4-mm zirconia rotor, spinning at 12 kHz MAS rate, collecting ~100 scans with 10 s recycle delay for each sample.

2.5. X-ray absorption spectroscopy: Data collection

Rh K-edge EXAFS spectra were measured at room temperature at BL 7–3 (500 mA) at the Stanford Synchrotron Radiation Lightsource (SSRL) operating under 3 GeV. Higher-order harmonics were rejected by detuning a Si(220) ($\phi = 0^\circ$) double-crystal monochromator to 50% of maximum intensity at the end of the Rh K-edge scan range. The X-ray energy was internally calibrated by assigning the first inflection point of the absorption edge of a Rh foil placed between the ion chambers I_1 and I_2 to 23219.80 eV. Three ion chambers (I_0 , I_1 and I_2) were filled with nitrogen (N_2) and the Lytle detector with argon (Ar). The concentrated solution of Rh^{III} -GSH (**2**) was held between 5 μm polypropylene windows of a 5 mm Teflon frame, placed between ion chambers I_0 (detecting the incident beam) and I_1 . Solid samples of **1** and **2** were mixed with boron nitride (sample: BN = 70:30 w/w), finely ground and pressed in a 1 mm thick Al frame with Mylar tape as window material. Five scans were collected for **1** and **2** in fluorescence and transmission modes, respectively. All individual

scans were compared prior to averaging to ensure that no radiation damage occurred during measurement.

2.6. X-ray absorption spectroscopy: Data analysis

EXAFS oscillations were extracted using the WinXAS 3.1 program,[25] by subtracting the background in the pre-edge region using a first-order polynomial, followed by normalization of the edge step. The threshold energy (E_0) was used for converting the energy unit to k ($\text{\AA}^{-1}$), where $k = [(8\pi^2 m_e / h^2)(E - E_0)]^{1/2}$; E_0 values were 23226.0 eV for **1** and 23226.3 eV for **2**. The EXAFS oscillations overlapped closely for the Rh^{III} -GSH product **2** as solid and in concentrated solution.

For modelling theoretical EXAFS oscillations and to generate the input file for the ATOMS program, which creates the input file for the FEFF 7.0 program,[26, 27] the coordinates of the following crystal structures were used: $[\text{Rh}_2(\text{AcO})_4(\text{H}_2\text{O})_2]$,[28] and Λ - $[\text{Rh}\{\text{Ir}(\text{aet})_3\}_2]\text{Br}_3$ (CSD: AQUVOX; Haet = 2-aminoethanethiol).[29] In the latter structure, due to the similarity in ionic radii of Rh^{3+} and Ir^{3+} ions (0.665 $\text{\AA}$ and 0.68 $\text{\AA}$, respectively), [30] the terminal Ir atoms were replaced by Rh in the ATOMS input file.

The least-squares curve-fitting procedure was performed over the k -range 2.8 ~ 18 $\text{\AA}^{-1}$. For each backscattering path, the structural parameters that were refined included the bond distance (R), the Debye–Waller factor parameter (σ^2) and in some cases also the coordination number (CN). The amplitude reduction factor (S_0^2) was refined to 0.92 with fixed coordination numbers for solid $\text{Rh}_2(\text{AcO})_4$ (**1**) and was then used for sample **2**. The

E_0 parameter was refined as a common value for all scattering paths of each sample. The accuracy of the bond distances R , σ^2 and refined CN values is within ± 0.02 $\text{\AA}$, ± 0.001 $\text{\AA}^2$ and ± 10 –15%, respectively.

3. Results and Discussion

3.1. Electronic Absorption Spectroscopy

The aerobic reaction of $\text{Rh}_2(\text{AcO})_4$ with glutathione was investigated at the pH of mixing (acidic) and at physiological pH. An aqueous solution of $\text{Rh}_2(\text{AcO})_4$ is quite stable in air. The UV-vis spectrum of the emerald green aqueous solution of $\text{Rh}_2(\text{AcO})_4$ and GSH (mole ratio 1:4) showed at the pH of mixing (3.2) the characteristic broad band of $[\text{Rh}_2(\text{AcO})_4(\text{H}_2\text{O})_2]$ with $\lambda_{\text{max}} = 584$ nm (Figure 1), which has been attributed to the $\pi^*(\text{Rh}_2^{4+}) \rightarrow \sigma^*(\text{Rh}_2^{4+})$ transition (band I) and is characteristic of its lantern structure.[31–33]

The acidic solution for which the reaction is slow shows a gradual color change to orange-brown, with an emerging UV-vis absorption peak at $\lambda_{\text{max}} = 347$ nm, attributed by ESI-MS to formation of a deprotonated $\text{Rh}^{\text{III}}_2(\text{AcO})_4(\text{GSH})_2$ complex ($-m/z = 526.00, 1053.01$), with $\text{S}^- \rightarrow \text{Rh}^{3+}$ ligand-to-metal charge transfer (LMCT); see Figure A1 in Appendix 1, Electronic Supplementary Material (ESM). Oxidation of Rh_2^{4+} to Rh_2^{6+} may occur by reducing oxygen to hydrogen peroxide,[15] the presence of which in the solution was confirmed using an iodine test (see Ref. [22]).[34] Recently a similar two-electron O_2 reduction in the presence of a proton source was reported for a bis(μ -thiolato) $\text{Mn}(\text{II})$ dimer

with a coordinated thiol ligand, $[\text{Mn}_2^{\text{II}}(\text{LS})(\text{LSH})]\text{ClO}_4$, which oxidizes to a dinuclear Mn(III) complex, $[\text{Mn}_2^{\text{III}}(\text{LS})_2]^{2+}$, with two bridging and two terminal thiolate groups, assuming $[\text{Mn}_2^{\text{III}}(\text{LS})_2(\text{OOH})]^+$ as the intermediate.[35] It is well established that the high covalency of metal-(*S*-cysteinate) bonds, and the charge donation by thiolate ligands, significantly will lower the reduction potential of the metal centre and catalyze the reduction of dioxygen.[35, 36, 37] Also in the present study, it appears that the *two* deprotonated *S*-bound GSH ligands decrease the redox potential of $\text{Rh}_2(\text{AcO})_4$ and promote its oxidation. The reactions (1) and (2) below resemble those observed for $\text{Rh}_2(\text{AcO})_4$ and *N*-acetylcysteine (see Figure A2).[22]

However, it seems likely that in $[\text{Rh}_2^{\text{II}}(\text{AcO})_4(\text{H}_2\text{A})]^-$ the glutathione glycyl –COOH group of the H_2A^- ligand is deprotonated at the acidic pH of mixing $\text{Rh}_2(\text{AcO})_4$ and GSH, considering its low pK_a value (3.22), rather than the coordinated cysteine thiol group (as assumed by Wong et al.)[17]; see Scheme 2.

Recently Wong et al. detected a shoulder at ~353 nm for a golden-yellow aqueous solution of $\text{Rh}_2(\text{AcO})_4$ and GSH (pH = 3.0), measured under anaerobic conditions. This band was attributed to LMCT from *S*-glutathionate (GS^-) dominated molecular orbitals to $\sigma^*(\text{Rh}_2^{4+})$ in $[\text{Rh}_2(\text{AcO})_4(\text{GS})(\text{H}_2\text{O})]^-$ species, with axially coordinated thiolate to $\text{Rh}_2(\text{AcO})_4$, retaining its paddlewheel structure.[17]

In the current article we focus on the results from the aerobic reaction of $\text{Rh}_2(\text{AcO})_4$ with glutathione at physiological pH. When adjusting the pH to 7.4 under aerobic conditions, the solution turned dark red with gradual loss of intensity for the band at 584 nm, which virtually disappeared after 48 hours (Figure 1), suggesting cleavage of the $\text{Rh}^{\text{II}}\text{-Rh}^{\text{II}}$ bond upon oxidation of Rh(II) to Rh(III).

3.2. Electrospray ionization mass spectrometry

ESI-mass spectra were measured in both positive (+) and negative (–) ion modes for the solutions containing $\text{Rh}_2(\text{AcO})_4$ and glutathione (mole ratios 1:4 and 1:10), and for the Rh^{III} -GSH solid **2** (dissolved in water). Spectra of the reaction mixture were obtained at 0.5, 2, 24 and 48 hours after the initial pH adjustment at 7.4 (see Figure S1-a). The ESI-mass spectra measured in (+) ion mode (Figure S2a, b) showed that some $\text{Rh}_2(\text{AcO})_4$ remained after 48 hours in the solution mixture with mole ratio 1:4. However, with excess glutathione ($C_{\text{GSH}} = 19 \text{ mM}$), which was used to simulate more biologically relevant conditions closer to high cellular GSH levels (~10 mM)[10], and low $\text{Rh}_2(\text{AcO})_4$ concentration ($C_1 = 1.9 \text{ mM}$), all $\text{Rh}_2(\text{AcO})_4$ had reacted. In both cases similar Rh-glutathione mass ions were detected in (–) ion mode, despite different ligand concentrations. Also the ESI-mass

spectrum of the solid **2** in (+) ion mode did not show any peak related to $\text{Rh}_2(\text{AcO})_4$ or any other Rh-containing mass ion (Figure S2c).

Figure 2 displays the ESI-mass spectra of the $\text{Rh}_2(\text{AcO})_4$ -glutathione solution mixture after 48 hours and of **2** in (–) ion mode (fragmentor voltage 80 V); all Rh-glutathione mass ions detected in these spectra contained Rh(III) ions (see Table 1). The assignment of the Rh-glutathione peaks is facilitated by the isotopic pattern for the stable sulfur isotopes ^{32}S , ^{33}S , ^{34}S and ^{36}S with natural abundance 95.02, 0.75, 4.21 and 0.02 atom%, respectively.[38]

In these spectra, mass ion peaks associated with dirhodium(III) species bound to three ($-m/z = 372.66, 559.50$), four ($-m/z = 475.03, 713.04$), and five GSH ($-m/z = 432.79, 866.58$) appear. No peak could be observed for $\text{Rh}^{\text{III}}_2(\text{GSH})_{1-2}$ in either (+) or (–) ion modes. When repeating the ESI-MS measurement for the Rh-glutathione solution (mole ratio 1:4, pH = 7.4) at fragmentor voltage 0 V, the peak at $-m/z = 372.66$ disappeared and the intensity of the peak at $-m/z = 559.50$ decreased considerably (Figure S1-b), indicating that the $\text{Rh}^{\text{III}}_2(\text{GSH})_3$ mass ions produced in the instrument at 80 V were fragments of larger species.

The presence of mass ions associated with $\text{Rh}^{\text{III}}_2(\text{GSH})_{4-5}$ species in the ESI-mass spectrum of the diamagnetic solid Rh^{III} -GSH (**2**) dissolved in water is consistent with an oligomeric structure for the composition $\{\text{Na}_2[\text{Rh}^{\text{III}}_2(\text{HA})_4] \cdot 7\text{H}_2\text{O}\}_n$ based on the elemental analysis (see *Experimental Section*). No mononuclear Rh(III) mass ion was detected in either (+) or (–) ion mode (Figures 2 *right* and S2c).

The hydrogen peroxide generated at the oxidation of Rh_2^{4+} to Rh_2^{6+} , [15, 22] then partially oxidizes the Rh(III) bound thiolato groups to form sulfenato complexes, as evidenced by the ($-m/z + 8$) mass ions with low intensity in the ESI-mass spectra, such as the $[\text{Rh}^{\text{III}}_2(\text{HA})_4 + \text{O}]^{2-}$ complex with 721.04 amu ($= 713.04 + 8$); see Figure 2 (*left*) and Table 1. However, the lack of a peak at ~2476 eV in the S K-edge X-ray absorption near-edge structure (XANES) spectrum of the solid Rh^{III} -GSH product (**2**) indicates that this formation of sulfenato ($\text{S}=\text{O}$) groups is only minor (Figure S3-a), [39] in contrast to the distinct feature that appears at 2475.6 eV for the corresponding penicillamine compound, $\text{Na}_2[\text{Rh}_2(\text{Pen})_2(\text{Pen}(\text{SO}))_2] \cdot 4.5\text{H}_2\text{O}$. [22] Similar oxidation has been reported for thiolato Co(III) and thiolato Ru(II) polypyridyl complexes, as well as for a GSH complex with an antitumor active Ru(II) arene compound. [14, 40, 41] Oxygenation of thiolato groups bound to Fe(II) or Ni(II) ions by O_2 has also been reported. [42, 43]

ESI-mass peaks associated with oxidized glutathione (GSSG) were observed for both the $\text{Rh}_2(\text{AcO})_4 + 4$ GSH solution mixture and for the solid **2** (Figure 2). Oxidation of GSH to GSSG can occur by the peroxide that is generated in the solution, or by oxygen during stirring the solution 46 h under aerobic conditions. A low intensity peak at $-m/z = 611.14$ amu for $[\text{GSSG} - \text{H}^+]^-$ was also observed for different parts of the orange band eluted from the Sephadex G-15 size-exclusion column. Although the solid **2** formed at evaporation then could contain some minor GSSG contamination, it would not affect the EXAFS results and our conclusion about the structure of **2**. No mass peak associated with a deprotonated GSSG

coordinated (via its COO^- groups) to a Rh(III) ion, e.g. in $[\text{Rh}^{\text{III}}_2(\text{GSH})_3(\text{GSSG}) - 8\text{H}^+]^{2-}$ (calculated $-m/z = 865.57$), was detected.

3.3. Rh K-edge EXAFS Spectroscopy

The comparison of the k^3 -weighted Rh K-edge EXAFS spectra and the corresponding Fourier transforms of solid $\text{Rh}_2(\text{AcO})_4$ (**1**) with the concentrated aqueous solution of the Rh^{III} -GSH product (**2**) in Figure 3 shows a clear difference in the local structure around the Rh ions. Distinct Rh-Rh scattering is absent in the EXAFS oscillation of the Rh^{III} -GSH product (**2**), which lacks high EXAFS amplitude at high k ($> 10 \text{ \AA}^{-1}$) when compared to that of solid $\text{Rh}_2(\text{AcO})_4$ (**1**); see Figure 3 *left*. Least-squares curve-fitting of simulated EXAFS oscillations for a model of the local environment around the Rh ions in **1** results in mean distances very close to the crystallographic values for $[\text{Rh}_2(\text{AcO})_4(\text{H}_2\text{O})_2]$: (Rh-O_{eq} 2.047 Å, Rh-Rh 2.385 Å, Rh-C 2.886 Å and Rh-O'_{eq} 3.079 Å); see Table 2.[28]

For the concentrated Rh^{III} -GSH (**2**) solution the Fourier-transform of the EXAFS oscillation shows a small peak at $\sim 2.8 \text{ \AA}$ (not corrected for phase shift); see Figure 3 *right (b)*. This FT peak was well explained by including an Rh...Rh interaction in the EXAFS fitting model. Using an EXAFS model consisting of Rh-O, Rh-S and Rh...Rh scattering paths for the least squares curve-fitting procedure, led to 2.5 Rh-O ($2.08 \pm 0.02 \text{ \AA}$), 4 Rh-S ($2.33 \pm 0.02 \text{ \AA}$) and 0.85 Rh...Rh ($3.11 \pm 0.02 \text{ \AA}$) interactions; see Table 2. Figure S4 displays the contribution of each scattering path in the fitted EXAFS model.

The refined Rh...Rh distance ($3.11 \pm 0.02 \text{ \AA}$), which is similar to that of the *N*-acetylcysteine compound $\{\text{Na}_2[\text{Rh}_2(\text{NAC})_4] \cdot 4.5\text{H}_2\text{O}\}_n$ (**3**) (Rh...Rh $3.10 \pm 0.02 \text{ \AA}$),[22] is longer than the normal range ($\sim 2.5 - 2.7 \text{ \AA}$) for an $\text{Rh}^{\text{II}}\text{-Rh}^{\text{II}}$ bond,[44] and shorter than the $\text{Rh}^{\text{III}}\text{...Rh}^{\text{III}}$ distance ($\sim 3.51 - 3.57 \text{ \AA}$) in dithiolato-bridged dirhodium(III) complexes; see the CSD survey in *Ref.* [22]. This survey also shows dirhodium(III) complexes with three bridging thiolato groups, all of which with the general formula $\text{Cp}^*\text{Rh}(\mu\text{-S})_3\text{RhCp}^*$ (with axial $\text{Cp}^* = \eta^5\text{-C}_5\text{Me}_5$ pentamethylcyclopentadienyl ligands) and with $\text{Rh}^{\text{III}}\text{...Rh}^{\text{III}}$ distances within the range $3.21 - 3.36 \text{ \AA}$,[22] and a mean bridging Rh-S distance of $2.40 \text{ \AA}$. Based on this similarity, we propose three bridging thiolato groups between the Rh(III) ions in **2**.

3.4. ^{13}C NMR Spectroscopy

Figure 4 compares ^{13}C CPMAS NMR spectra of the solids Rh^{III} -GSH (**2**), $\text{Rh}_2(\text{AcO})_4$ and glutathione. The broadness of the peaks for **2** can be attributed to Rh(III) sites with similar but not identical morphology, resulting in slightly different local environments around the C atoms in glutathione. Resonances for the carbonyl groups in glutathione were found at 178.8 (C4), 174.8 (C5), 173.6 (C10) and 171.2 ppm (C8), slightly shifted relative to the corresponding reported values: 178.6, 174.3, 173.1 and 170.7 ppm,[45] due to the different reference value used for the glycine C=O chemical shift (176.03 ppm). For **2** there are two broad peaks in the carbonyl region at 175.0 and 185.1 ppm, with the latter considerably shifted downfield ($> 6 \text{ ppm}$) relative to the C=O peaks for pure glutathione. A similar downfield shift observed in the carboxylate region of ^{13}C CPMAS NMR spectra for the reaction products of $\text{Rh}_2(\text{AcO})_4$ with penicillamine or cysteine in mole ratio 1:4 was

attributed to the Rh(III)-coordinated COO^- group.[22] It is unlikely that the peak at $\delta_{\text{C}} = 185.1$ ppm in the spectrum of **2** is due to coordination of an amide $-\text{NH}-\text{C}=\text{O}$ group to Rh(III) ($\delta(\text{C4}) = 178.8$ ppm in pure GSH). The reason is that neither the amide $\text{C}=\text{O}$ nor the COO^- group of an *N*-acetylcysteine ligand is involved in Rh(III) coordination in $\{\text{Na}_2[\text{Rh}_2(\text{NAC})_4].4.5\text{H}_2\text{O}\}_n$ (**3**), as evidenced by the downfield shift of only ~ 2 ppm relative to the corresponding ^{13}C NMR signals of pure solid *N*-acetylcysteine.[22] Therefore, we conclude that for some of the glutathione ligands in **2**, *tailing* COO^- groups in a $\text{Rh}^{\text{III}}_2\text{S}_3$ core are bound to Rh(III) ions of the neighbouring $\text{Rh}^{\text{III}}_2\text{S}_3$ units. Similar COO^- coordination has been observed in a silver(I) *N*-acetylmethioninate complex.[46]

Scheme 3 displays our proposed structure for the oligomeric compound $\{\text{Na}_2[\text{Rh}_2(\text{HA})_4].7\text{H}_2\text{O}\}_n$ (**2**) with the non-terminal Rh(III) ions generally surrounded by four S and two O atoms, either from water or from a COO^- group from a neighbouring glutathione. A minor number of the terminal thiolates could be oxidized to sulfenato ($\text{S}=\text{O}$) groups by peroxide, as evidenced by ESI-MS. High stability of the repeating triply bridged $\text{Rh}^{\text{III}}_2\text{S}_3$ core units seems to be the reason for the absence of any mass peak (+ or –) related to $\text{Rh}^{\text{III}}_2(\text{GSH})_{1-2}$ fragments in ESI-MS (see Section 3.2).

There are two minor differences between the proposed structures for the glutathione compound $\{\text{Na}_2[\text{Rh}_2(\text{HA})_4].7\text{H}_2\text{O}\}_n$ (**2**) and the *N*-acetylcysteine compound $\{\text{Na}_2[\text{Rh}_2(\text{NAC})_4].4.5\text{H}_2\text{O}\}_n$ (**3**),[22] which according to EXAFS spectroscopy have similar local structure around the rhodium ions: a) For the oligomeric compound **3** we proposed $\text{Rh}^{\text{III}}_2\text{S}_3$ units connected by one or two bridging thiolate groups,[22] rather than with just one bridging thiolate in **2** because of the more bulky glutathione ligand. That difference is consistent with the slightly higher FT magnitude and refined coordination number for the Rh-S path in **3** ($\text{CN} = 4.6$) than in **2** ($\text{CN} = 4.1$); see Table 2 and the Supporting Information in *Ref.* [22]. b) In the *N*-acetylcysteine compound **3**, the COO^- group is not bound to the Rh(III) ions.[22]

The structure in Scheme 3 is to some extent similar to the $[\text{Rh}^{\text{II}}(\eta^1\text{-C}_6\text{H}_5\text{S})(\mu\text{-C}_6\text{H}_5\text{S})(\text{bpy})]_2$ complex proposed as structural model for an “oxidatively unstable intermediate” in the reaction between dirhodium(II) bis-polybipyridines $[\text{Rh}_2(\text{AcO})_2(\text{bpy})_2(\text{CH}_3\text{CN})_2](\text{BF}_4)_2$ and thiol-containing molecules such as glutathione.[15] This intermediate has two bridging thiolato ligands in a butterfly arrangement and two thiolato groups in the axial positions of the $\text{Rh}^{\text{II}}_2\text{S}_2$ core in line with the Rh-Rh bond. In our proposed structure for the aerobic reaction product **2** of $\text{Rh}_2(\text{AcO})_4$ and GSH, $\{\text{Na}_2[\text{Rh}_2(\text{HA})_4].7\text{H}_2\text{O}\}_n$ (Scheme 2), an *S*-bridging glutathione and two O-donor ligands fill the space occupied by the two bipyridine ligands in that “intermediate” model compound.

4. Conclusion

The current study shows that under aerobic conditions in aqueous solution at physiological pH glutathione ($\text{GSH} = \text{H}_3\text{A}$) forms an oligomeric $\{\text{Na}_2[\text{Rh}^{\text{III}}_2(\text{HA})_4].7\text{H}_2\text{O}\}_n$ compound with triple thiolate bridges between Rh(III) ions ($\text{Rh}^{\text{III}} \dots \text{Rh}^{\text{III}} \sim 3.11 \pm 0.02 \text{ \AA}$) within dimeric $\text{Rh}^{\text{III}}_2(\text{HA})_3$ units, linked together with a single thiolate bridge. This behaviour resembles that of *N*-acetylcysteine with $-\text{SH}$ and COO^- functional groups, which forms

similar oligomers in the $\{\text{Na}_2[\text{Rh}^{\text{III}}_2(\text{NAC})_4] \cdot 4.5\text{H}_2\text{O}\}_n$ compound, with the difference that with glutathione also the COO^- groups to some extent are involved in binding to the Rh(III) ions.

Considering that preservation of the Rh_2^{4+} core in dirhodium(II) carboxylates seems to be essential for its cytotoxic effect via binding to DNA at physiological conditions, breakdown of that core structure in the presence of glutathione would reduce that activity. Our study supports an earlier investigation by Erck and co-workers, who soon after injecting ^{14}C -labeled $\text{Rh}_2(\text{AcO})_4$ in tumor-bearing Swiss mice observed exhalation of $^{14}\text{CO}_2$, suggesting breakdown in tumor cells of the $\text{Rh}_2(\text{AcO})_4$ structure to acetate/ CO_2 and Rh ions, as a result of its interaction with thiol-containing molecules.[47] The present study is the first to highlight the structural aspects of the reaction of dirhodium(II) tetraacetate with glutathione at physiological pH under aerobic conditions. Knowledge about the nature of the resulting products is important to predict how they would react within our body and how they can be mobilized to facilitate excretion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

EXAFS	X-ray absorption fine structure
ESI-MS	Electrospray ionization mass spectrometry
CPMAS	Cross-polarization magic angle spinning
XANES	X-ray absorption near-edge structure

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Figure 1.

UV-vis spectra of an aqueous solution of $Rh_2(AcO)_4$ ($C_1 = 3.0$ mM) compared with an aqueous solution containing $C_1 = 1.0$ mM and glutathione (mole ratio 1:4) at the pH of mixing (3.2) and at pH = 7.4 after 5 min, 6 h and 48 h from the initial pH adjustment; peak positions are obtained from second derivatives.

Figure 2. ESI-Mass spectra (– ion mode) of: (*left*) Rh₂(AcO)₄ + GSH solution mixture (mole ratio 1:4; C₁ = 2.3 mM; pH = 7.4) after 48 hours; (*right*) Rh^{III}-GSH solid **2** (dissolved in water); fragmentor voltage = 80 V.

Figure 3. (Left) The k^3 -weighted Rh K-edge EXAFS spectra, and (right) the corresponding Fourier transforms of: a) solid $\text{Rh}_2(\text{AcO})_4$ (1), b) concentrated aqueous solution of Rh^{III} -GSH (2). The curve-fitting results are presented in Table 2.

Figure 4.

Solid state ^{13}C CPMAS NMR spectra obtained for glutathione, $\text{Rh}_2(\text{AcO})_4$ (**1**), and the Rh^{III} -GSH reaction product (**2**).

Scheme 1.
Molecular structure of glutathione (GSH = H₃A), and two of its deprotonated forms

Scheme 2.

Proposed pathway for the aerobic reaction of $\text{Rh}_2(\text{AcO})_4$ with glutathione at pH of mixing (acidic). Both species were detected by ESI-MS; see Appendix 1 in ESM.

Scheme 3.

Proposed oligomeric structure for **2**, $\{\text{Na}_2[\text{Rh}_2(\text{HA})_4] \cdot 7\text{H}_2\text{O}\}_n$ ($\text{GSH} = \text{H}_3\text{A}$), with single thiolate bridges between triply bridged dimeric units. The terminal thiolate could be partially oxidized to sulfenato ($\text{S}=\text{O}$) groups. Sodium ions are not shown.

Table 1

Assignment of (–) Mass Ions in the ESI-Mass Spectra Shown in Figure 2 Compared to Calculated Mass Ions.^{a, b}

<i>m/z</i>	Assignment	Calcd.	<i>m/z</i>	Assignment	Calcd.
306.0747	[GSH – H ⁺] [–]	306.0760	721.0395	[2Rh ^{III} + 4GSH – 8H ⁺ + O] ^{2–}	721.0393
372.6648	[2Rh ^{III} + 3GSH – 9H ⁺] ^{3–}	372.6640	724.0323	[2Rh ^{III} + 4GSH – 9H ⁺ + Na ⁺] ^{2–}	724.0329
432.7894	[2Rh ^{III} + 5GSH – 10H ⁺] ^{4–}	432.7880	732.0302	[2Rh ^{III} + 4GSH – 9H ⁺ + Na ⁺ + O] ^{2–}	732.0303
475.0262	[2Rh ^{III} + 4GSH – 9H ⁺] ^{3–}	745.0253	735.0228	[2Rh ^{III} + 4GSH – 10H ⁺ + 2Na ⁺] ^{2–}	735.0238
559.4996	[2Rh ^{III} + 3GSH – 8H ⁺] ^{2–}	559.5000	746.0149	[2Rh ^{III} + 4GSH – 11H ⁺ + 3Na ⁺] ^{2–}	746.0148
577.3871	[2Rh ^{III} + 5GSH – 9H ⁺] ^{3–}	577.3866	866.5828	[2Rh ^{III} + 5GSH – 8H ⁺] ^{2–}	866.5838
584.7144	[2Rh ^{III} + 5GSH – 10H ⁺ + Na ⁺] ^{3–}	584.7139	877.5740	[2Rh ^{III} + 5GSH – 9H ⁺ + Na ⁺] ^{2–}	877.5748
592.0414	[2Rh ^{III} + 5GSH – 11H ⁺ + 2Na ⁺] ^{3–}	592.0412	888.5649	[2Rh ^{III} + 5GSH – 10H ⁺ + 2Na ⁺] ^{2–}	888.5657
599.3687	[2Rh ^{III} + 5GSH – 12H ⁺ + 3Na ⁺] ^{3–}	599.3685	896.5642	[2Rh ^{III} + 5GSH – 10H ⁺ + 2Na ⁺ + O] ^{2–}	896.5632
611.1423	[GSSG – H ⁺] [–]	611.1442	899.5556	[2Rh ^{III} + 5GSH – 11H ⁺ + 3Na ⁺] ^{2–}	899.5567
633.1233	[GSSG – 2H ⁺ + Na ⁺] [–]	633.1261	907.5521	[2Rh ^{III} + 5GSH – 11H ⁺ + 3Na ⁺ + O] ^{2–}	907.5542
655.1060	[GSSG – 3H ⁺ + 2Na ⁺] [–]	655.1081	910.5467	[2Rh ^{III} + 5GSH – 12H ⁺ + 4Na ⁺] ^{2–}	910.5477
713.0418	[2Rh ^{III} + 4GSH – 8H ⁺] ^{2–}	713.0419	921.5375	[2Rh ^{III} + 5GSH – 13H ⁺ + 5Na ⁺] ^{2–}	921.5387

^a Glutathione, GSH = C₁₀H₁₇N₃O₆S; oxidized glutathione, GSSG = C₂₀H₃₂N₆O₁₂S₂;

^b Calculations are based on the isotopic calculator from Scientific Instrument Services.[23]

Structural Parameters Derived from Least Squares Curve-Fitting of Simulated EXAFS Oscillations for Models of the Local Rh-ion Environment to the Experimental EXAFS spectra of **1** and **2** shown in Figure 3.^a

Table 2

Sample	Rh-O			Rh-S			Rh-Rh / Rh...Rh		
	CN	R (Å)	σ^2 (Å ²)	CN	R (Å)	σ^2 (Å ²)	CN	R (Å)	σ^2 (Å ²)
1 ^b	4 f	2.03	0.0029				1 f	2.38	0.0012
2	2.5	2.08	0.0034	4.1	2.33	0.0050	0.85	3.11	0.0046

^a Amplitude reduction factor (S_0^2) was refined to 0.92 for **1** and fixed at this value for fitting the spectrum of **2**; f = fixed value; estimated errors: $R \pm 0.02$ Å; $\sigma^2 \pm 0.001$ Å²; $CN \pm 10$ –15%;

^b Other refined paths: 4Rh-C ($R = 2.88$ Å, $\sigma^2 = 0.0034$ Å²), 4Rh...O' eq ($R = 3.09$ Å, $\sigma^2 = 0.0057$ Å²).