

CORM-3 Reactivity toward Proteins: The Crystal Structure of a Ru(II) Dicarbonyl–Lysozyme Complex

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S Supporting Information

ABSTRACT: CORM-3, [*fac*-Ru(CO)₃Cl(κ^2 -H₂NCH₂-CO₂)], is a well-known carbon monoxide releasing molecule (CORM) capable of delivering CO *in vivo*. Herein we show for the first time that the interactions of CORM-3 with proteins result in the loss of a chloride ion, glycinate, and one CO ligand. The rapid formation of stable adducts between the protein and the remaining *cis*-Ru^{II}(CO)₂ fragments was confirmed by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES), Liquid-Chromatography Mass Spectrometry (LC-MS), Infrared Spectroscopy (IR), and X-ray crystallography. Three Ru coordination sites are observed in the structure of hen egg white lysozyme crystals soaked with CORM-3. The site with highest Ru occupancy (80%) shows a *fac*-[(His15)Ru(CO)₂(H₂O)₃] structure.

The pleiotropic role of carbon monoxide (CO) in Biology and Medicine is now well recognized.^{1–4} As a result of its signaling functions, CO is involved in multiple defense mechanisms in physiologic and pathologic situations. For example, CO acts as a strong anti-inflammatory and antiapoptotic agent, prevents endothelial damage due to various oxidative stresses, and actively promotes endothelial healing. Administration of CO gas has produced significant therapeutic effects in many animal models of disease and is being tested in a clinical trial of kidney transplantation.⁵ However, in spite of its therapeutic potential, the safety and practicality of its application as an inhaled gas remains questionable due to its toxicity at high concentrations. To circumvent this problem, Motterlini, Mann and colleagues proposed the use of CO-releasing molecules (CORMs) as pharmaceutical agents.⁶ CORM-3 [*fac*-Ru(CO)₃Cl(κ^2 -H₂NCH₂-CO₂)], the first known air-stable and water-soluble CORM,^{7,8} has attracted great interest because of its activity in animal models of diseases with major clinical indications, such as transplantation,⁹ myocardial infarction,¹⁰ and rheumatoid arthritis.¹¹

The release of CO from CORM-3 to biological targets is exemplified by the deoxy-myoglobin carbonylation assay.⁷ Fresh solutions of CORM-3 (~50 μ M) rapidly generate (5–10 min) a maximal amount of carboxy-myoglobin, which corresponds to the transfer of 1 mol of CO per mol of CORM-3. It is often assumed that CO dissociates from CORM-3 into solution and is

then scavenged by deoxy-Mb to form CO-Mb. However, we found that no CO can be detected by Gas Chromatography-Thermal Conductivity Detection (GC-TCD) in the headspace of a closed vial containing a 10 mM solution of CORM-3 in different aqueous media including distilled H₂O, PBS pH 7.4, and Fetal Bovine Serum. Instead, CO₂ was detected in all cases except in H₂O at pH 1.9. CO₂ results from the already identified pH dependent water–gas shift reaction of CORM-3 with H₂O^{7,8} that ultimately forms CO₂ and H₂, as we confirmed by GC (see Supporting Information (SI) for details). These observations suggested to us that myoglobin did not simply act as a scavenger of free CO, and we hypothesized that CO may be mobilized from the Ru(II) complex by chemical interactions with the protein. Moreover, it has been shown that CORM-3 has a much shorter half-life in plasma (3.6 min) than in H₂O (98 h) or PBS (20.4 min) which means that after 3.6 min in plasma half of the initial amount of CORM-3 has lost the capacity to release CO to Mb.¹² Understanding such interactions is crucial for the design of pharmaceutical CORMs, which will ultimately circulate in the protein-rich plasma of humans. These interactions may be quite extensive and unexpected, as recently evidenced in the case of the antimetastatic Ru(III) drug NAMI-A,¹³ and have never been demonstrated for any therapeutically active CORM. After all how does CORM-3 achieve its pharmacological activity *in vivo* if it is so rapidly inactivated in the plasma without forming free CO?

Herein, we present our findings on the interaction of CORM-3 with several plasma proteins that may play a central role in its transport and activation *in vivo*. The interaction with lysozyme was also studied since it has been shown before that its scaffold is particularly suited to probe the fundamental interactions of proteins with metal substrates.^{14,15}

Horse heart myoglobin, human hemoglobin, human albumin, human transferrin, and hen egg white lysozyme (HEWL) were incubated at room temperature with an excess of CORM-3 for 1 h. After removal of any unbound compound by dialysis, analysis by ICP demonstrated the presence of Ru in all protein solutions, indicating that CORM-3 and/or derived Ru species were bound to proteins. To facilitate the structural characterization of the metalated protein adducts, crystallization of the CORM-3 reacted proteins was attempted under a wide variety of conditions, but no diffracting crystals could be obtained. Soaking of protein

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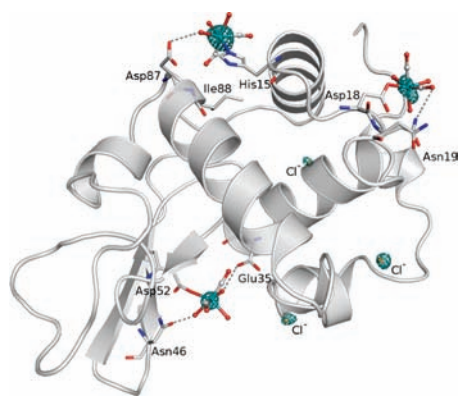


Figure 1. Overall structure of HEWL bound to three CORM-3 moieties. The three chloride ions found in the structure are also depicted. Anomalous electron density maps are contoured at 3.0σ (teal). Hydrogen bonds between CORM-3 and the protein are depicted in dashed lines. Pictures have been prepared using Pymol software.¹⁶

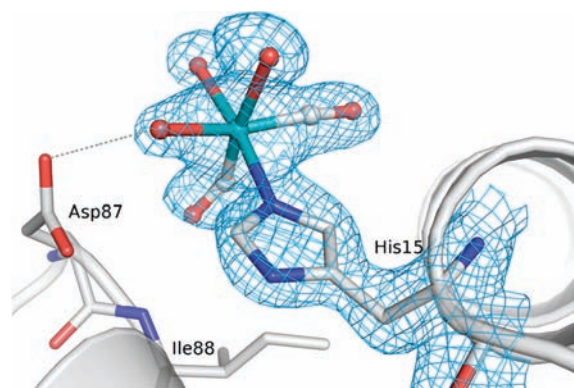


Figure 2. CORM-3 covalently bound to His15 of lysozyme adopting an octahedral geometry. $2F_o - F_c$ electron density map is contoured at 1.0σ .

crystals with CORM-3 solutions was unsuccessful in all cases, except the HEWL model protein. Indeed, crystals of lysozyme soaked with CORM-3 were measured using synchrotron radiation, and high-resolution data (1.67 \AA) were collected.

The crystals belong to a tetragonal space group (PDB accession code 2XJW), and the structure was solved using an HEWL model¹⁷ belonging to the same space group (PDB code 193L). The resulting electron density map shows that CORM-3 is bound to the protein at three different exposed sites: His15, Asp18, and Asp52 (Figure 1). The binding of different transition metals to these same sites has been observed in other deposited lysozyme structures.^{14,15,18–20}

At His15, with a 0.8 Ru occupancy, Ru adopts an octahedral geometry with the $N\epsilon 2$ of histidine side chain, two CO molecules, and three water molecules (Figure 2). The data unequivocally show that the chloride ion present in CORM-3 is absent in this protein–Ru adduct. Its absence was ascertained by (1) B-factor analysis (the B-factor variance is homogeneous among all atoms surrounding the metal) and (2) by inspection of anomalous electron density maps. Anomalous peaks are found at the Ru positions and for the chloride ions present at the protein surface (Figure 1) (also present in other deposited structures) but not in the first coordination sphere of Ru.

The electron density is not conclusive at the two other Ru sites, due to lower Ru occupancies: 0.5 bound to Asp18 and 0.4

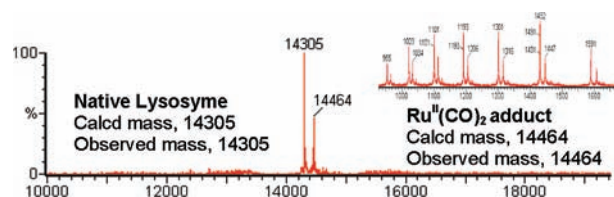


Figure 3. ESI-MS spectrum of HEWL (2.0 mg/mL) incubated with CORM-3 (10 equiv) in H_2O ($pH \approx 6$) for 10 min at room temperature.

bound to Asp52. Therefore, these two protein–CORM-3 adducts cannot be modeled as accurately as the His15 adduct, which is reflected in the larger B factors of the metal ligands. For the three sites, carbon monoxide ligands are found at equatorial positions in a linear and bent mode. The values for the bond distances and angles within the $Ru^{II}(CO)_2$ adduct ($Ru-CO$ distances of 1.8 to 2.0 \AA and $Ru-C-O$ angle of 139° to 178°) are within the range that has been described for small molecule structures reported in the Cambridge Structural Database (CSD)²¹ (GIRJIA,²² FOJVAB²³). Water molecules are occupying the remaining coordination positions at 2.1 to 2.4 \AA from the metal. These distances are also in the range of reported values for Ru complexes. Comparing the obtained model with other structures where similar carbonyl-metal centers are bound to HEWL (3KAM¹⁴ and 2Q0M¹⁵), the overall structure of the protein is maintained apart from Arg14 and Asn46. The side chain of Arg14, which is close to the His15–Ru site, has moved from its original position in the native protein allowing CORM-3 to bind to His15. The side chain of Asn46 is hydrogen bonded to one of the water ligands of the Asp52–Ru complex and has moved toward the surface of the protein. These results reveal that the reaction of CORM-3 with HEWL leads to protein– $Ru^{II}(CO)_2$ adducts.

To gain insight into the dynamics of the reaction of CORM-3 with HEWL, this interaction was studied in solution by LC-MS. Reaction mixtures with different ratios of CORM-3 to protein were analyzed. When lysozyme (2.0 mg/mL) was reacted with CORM-3 (1 or 10 equiv) in water at room temperature for 10 min, a single metal–protein adduct corresponding to the addition of one unit of $Ru^{II}(CO)_2$ (157 m/z) to lysozyme was observed (Figure 3). The fast formation of this single protein– $Ru^{II}(CO)_2$ complex at various reaction stoichiometries suggests that this complex is the kinetically and thermodynamically favored product. LC-MS analysis of dissolved crystals of lysozyme soaked with CORM-3 also confirmed the presence of the $Ru^{II}(CO)_2$ fragment in excess of the protein mass (see SI for details). These adducts contrast with those formed between the human- β -amyloid peptide 1–28, A β 28, and the complex $[Ru(CO)_3-Cl_2(N-1,3-thiazole)]$ which retains the $Ru^{II}(CO)_3$ fragment.²⁴

The formation of HEWL– $Ru^{II}(CO)_2$ adducts was further confirmed by the IR of crystals soaked with CORM-3. The IR spectrum of the crystals (ground and deposited in KBr pellets) shows two bands at 2060 and 1986 cm^{-1} which are indicative of a *cis*- $Ru^{II}(CO)_2$ fragment and are different from the CO bands of CORM-3, tricarbonylrhenium-lysozyme,¹⁴ and tricarbonylmanganese-lysozyme¹⁵ adducts (Figure 4). Further, iCORM-3 solutions contain unidentified species with *cis*- $Ru^{II}(CO)_2$ fragments that produce IR spectra rather similar to that of the HEWL– $Ru^{II}(CO)_2$ adducts in Figure 4 (see SI for details). Interestingly, the IR spectra of samples of HEWL incubated with CORM-3 for 1 h (in water, then dialyzed and lyophilized) present two pairs of CO stretching vibrations assignable to *cis*- $Ru^{II}(CO)_2$

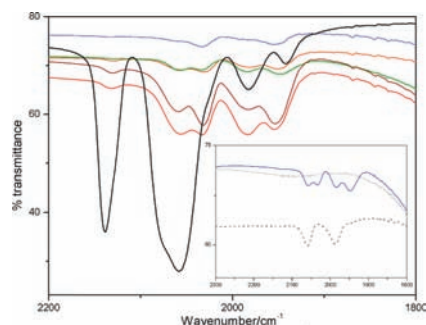


Figure 4. Infrared spectra (KBr pellets; cm^{-1}) of protein samples after incubation with CORM-3 (dialyzed and lyophilized): horse heart myoglobin in brown; human hemoglobin in red; human albumin in orange; human transferrin in blue; and HEWL in green. In black is the spectrum of CORM-3. In the insert are the IR spectra of crystals of HEWL soaked with CORM-3 (dashed line), HEWL samples after incubation with CORM-3 (blue), and native HEWL (dotted line).

fragments: one with bands at 2056 and 1981 cm^{-1} and another with bands at 2038 and 1950 cm^{-1} (inset in Figure 4). The position of these bands is the same for IR spectra taken on KBr pellets or on oil mulls.

The pair of vibrations at higher energies is the same as the one seen in the IR spectrum of the HEWL–CORM-3 crystals and is assigned to the cationic complex $\text{cis-}[\text{Ru}^{\text{II}}(\text{CO})_2(\text{H}_2\text{O})_3(\text{His15})]^{2+}$ identified in Figure 2. The origin of the second, lower energy pair of vibrations is not obvious, but we propose that it arises from a negatively charged protein-bound $[\text{cis-Ru}^{\text{II}}(\text{CO})_2\text{X}_4]^{z-}$ complex, such as the complexes linked to the carboxyl groups of Asp18 and Asp52 that were observed in the crystal structure (Figure 1). This proposal is supported by the position of the corresponding IR bands of the negatively charged complex, $[\text{Ru}(\text{CO})_2\text{Cl}_4]^{2-}$, which are at 2036 and 1935 cm^{-1} .²⁵ When we reacted horse heart myoglobin, human hemoglobin, human albumin, and human transferrin with CORM-3 (water; 1 h; dialysis as stated above), the IR spectra collected from the lyophilized samples all showed the same pattern of $\text{cis-Ru}^{\text{II}}(\text{CO})_2$ vibrations as described above for HEWL (see Figure 4): one pair at ca. 2060 and 1980 cm^{-1} and another pair at ca. 2030 and 1950 cm^{-1} (see SI for details).

In order to identify the fate of the missing CO ligand, CORM-3 was incubated with bovine serum albumin. Head space analysis by GC/TCD revealed CO_2 but not CO. Similarly, sheep plasma led to even more extensive liberation of CO_2 , but no CO. In modeling the experiments with lysozyme, the addition of excess 1-methylimidazole to CORM-3 at pH 4.0 also failed to release CO gas (see SI for details). In contrast to what is known for deoxy-Mb, incubation of CORM-3 with myoglobin (5:1 molar ratio; see SI for details) under air failed to produce COMb but formed a Mb– $\text{Ru}^{\text{II}}(\text{CO})_2$ adduct (Figure 4).

In summary, our studies of the reaction of the CO-releasing molecule CORM-3 with various proteins identified protein– $\text{Ru}^{\text{II}}(\text{CO})_2$ adducts as the reaction products. The reactions were fast, eliminating all non-CO ligands from CORM-3 in addition to one CO ligand, which is released in the form of CO_2 . This rapid formation of protein– $\text{Ru}^{\text{II}}(\text{CO})_2$ adducts led us to hypothesize that such protein adducts generated *in vivo* are the source of the reported COHb levels that were sustained over 24 h after a single dose of CORM-3.¹⁰ These findings may be of significance for the design of CORMs for therapeutic uses.

■ ASSOCIATED CONTENT

S Supporting Information. Full experimental details and characterization. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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