



Cite this: *Dalton Trans.*, 2026, **55**, 4051

Received 26th January 2026,  
 Accepted 18th February 2026

DOI: 10.1039/d6dt00205f

rsc.li/dalton

## Ru and Rh binding sites in the structure of human serum transferrin with Fe<sup>3+</sup> bound at the C-lobe

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**Ru and Rh binding sites in the structure of human serum transferrin with Fe<sup>3+</sup> bound at the C-lobe were identified by X-ray crystallography. Several His side chains and one Met are involved in the recognition of the metal ions by the protein.**

### Introduction

Serum transferrin (hTF) is an abundant human plasma glycoprotein of about 80 kDa that binds Fe<sup>3+</sup> and transports it throughout the human body, playing a critical role in regulating its biodistribution and homeostasis.<sup>1</sup> The protein is recognized by the cells through the TF receptor (TFR).<sup>2</sup>

hTF carries two lobes (N- and C-) of about 330 residues, that are connected by a short flexible peptide constituted by residues 331–339 and contain equivalent clefts with the Fe binding site.<sup>1</sup> Each lobe can be further divided into two subdomains: N1 (residues 1–92, 247–330), N2 (residues 93–246), C1 (residues 340–425, 573–679) and C2 (426–572). Upon Fe<sup>3+</sup> binding, the subdomains undergo a conformational variation that reduces solvent accessibility of the Fe binding sites.<sup>3</sup> Thus, the apo (iron-free) conformation is described as “open”, while the holo (Fe<sub>2</sub>-hTF) form adopts a “closed” conformation. Beyond the apo- and holo-forms, hTF exists in the monoferric form with Fe<sup>3+</sup> bound at the C-lobe only (Fe<sub>C</sub>-hTF) or at the N-lobe only (Fe<sub>N</sub>-hTF).<sup>4</sup>

Apart from iron, hTF can bind and transport other metal ions, including Cu(II), Al(III), Co(III), Cr(III), Ga(III), In(III), Ti(IV), Zn(II), Bi(III), Sm(III), Ru(II), Ru(III), Pu(IV), Cm(III), V(III), V(IV), V(V) and Th(IV).<sup>5–10</sup>

Given this capability the protein could play a role in detoxification process.<sup>5,11</sup> Furthermore, since TFR amount is significantly increased in cancer cells,<sup>12</sup> hTF has been proposed as a

potential metallodrug delivery system,<sup>13,14</sup> although this possible role is still controversial.<sup>15</sup> In this frame, it has been demonstrated that the apo- and Fe<sub>C</sub>-hTF forms bind the anticancer agent cisplatin,<sup>16,17</sup> that the apo-form binds the antiarthritic gold-based drug aurothiomalate<sup>18</sup> and the anticancer agent KP1019,<sup>15</sup> and that Fe<sub>C</sub>-hTF binds a divanadate formed upon reaction of the antidiabetic drug [VO(acac)<sub>2</sub>] (acac = acetylacetonate) with the protein.<sup>19</sup> These metal-bound forms of hTF remain able to bind TFR, at least *in vitro*. Cisplatin bound form of apo-hTF selectively delivers the anticancer agent to cancer cells *in vitro* and *in vivo*.<sup>20–24</sup> Similarly, it has been shown that hTF enhances the cytotoxicity of titanocene dichloride.<sup>25</sup> Gallium and indium bound forms of hTF inhibit the growth of malignant carcinoma cell lines at least 10 times more than their free salts.<sup>26</sup> hTF has been also used to deliver Gd-contrasting agents to the brain.<sup>27,28</sup> On the other hand, it has been shown that if Ru(III) compounds bind hTF in the iron binding site, Ru uptake by cells is not mediated by hTF, while if it binds close to protein surface residues hTF can promote the transport of the metallodrug to cells, but this process is unlikely in the presence of serum albumin.<sup>15</sup>

Crystallographic studies carried out on Fe<sub>C</sub>-hTF adducts formed with Os and Ru compounds revealed that the protein binds Ru<sup>3+</sup> close to side chain of His14, the side chains of His14 and His289, and that of His578,<sup>29</sup> and Os<sup>3+</sup> close to His14/His289, His273, His349/His350, Lys489-Lys490/Glu507, His578/Arg581.<sup>29</sup> Structural analyses of the apo-form and Fe<sub>C</sub>-hTF adducts with cisplatin have identified platinum binding sites near Met499 and Met256.<sup>16,17</sup> Gold ions, in contrast, interact with the side chains of multiple residues in apo-hTF, including His25, Asp63, His207, Tyr238, His249, His273, His289, His300, His473, His606, His642, and His858.<sup>18</sup>

For Cr(III), Ti(IV), Bi(III), and Yb(III), binding occurs primarily at the iron-binding site of the protein, near residues such as Tyr426, Tyr517, His585, and Asp392, or alternatively Tyr188, Tyr95, Asp63, and His249.<sup>30</sup> Notably, residues involved in iron recognition also coordinate V(IV) and V(V)-containing species.<sup>19,31</sup>

In 2022, by using FT-IR and UV-vis absorption spectroscopy it has been shown that hTF strongly binds Rh<sup>3+</sup> ions with a

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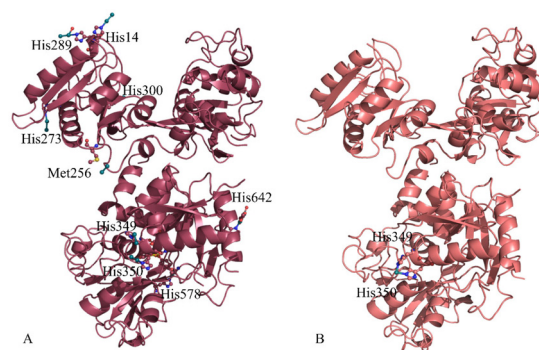
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change in the microenvironment of aromatic residues.<sup>32</sup> However, the exact Rh binding sites are unknown. Therefore, the aim of this work is to identify the specific Rh binding sites on human serum transferrin. To achieve this goal, we have solved X-ray structure of the Rh/Fe<sub>C</sub>-hTF adduct formed upon reaction of the paddlewheel Rh(II) compound [Rh<sub>2</sub>(CH<sub>3</sub>COO)<sub>4</sub>] with the protein. These results were compared with those obtained when Fe<sub>C</sub>-hTF reacts with the analogous Ru(II/III) paddlewheel complex [Ru<sub>2</sub>(CH<sub>3</sub>COO)<sub>4</sub>Cl] to the form the Ru/Fe<sub>C</sub>-hTF adduct. Both these compounds and some of their analogues have been already studied in the interaction with aminoacids,<sup>33</sup> peptides,<sup>34,35</sup> proteins<sup>36–44</sup> and nucleic acids,<sup>45–47</sup> due to their biological activity.<sup>6,48–50</sup>

## Results and discussion

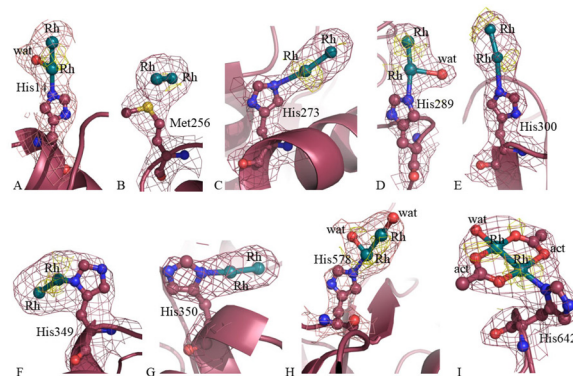
Crystals of the Rh/Fe<sub>C</sub>-hTF and Ru/Fe<sub>C</sub>-hTF adducts were obtained by soaking Fe<sub>C</sub>-hTF crystals, grown by hanging drop vapour diffusion method using 15% (w/v) PEG 3350, 16% (v/v) glycerol, 8 mM disodium malonate, and 150 mM Na-PIPES pH 6.5 as a reservoir, in a solution of the reservoir containing the dimetallic compound in <10% DMSO. The presence of DMSO increases the solubility of the compounds and reduces their ability to form oligomeric species.<sup>40,51</sup> It has been verified using UV-visible absorption spectroscopy that under the investigated experimental conditions both compounds remain stable for days (data not shown). X-ray diffraction data on several crystals of each adduct were collected at 100 K on different European Synchrotrons (SOLEIL and ESRF, see SI for details and Table S1 for data collection statistics). As expected, the crystals of the adducts of Fe<sub>C</sub>-hTF with Ru and Rh are isomorphous to those of Fe<sub>C</sub>-hTF. Soaking with dirhodium tetraacetate significantly deteriorates the crystals, reducing their diffraction power. The best crystal of Rh/Fe<sub>C</sub>-hTF diffracts X-ray at 2.37 Å resolution and was obtained by exposing Rh-free Fe<sub>C</sub>-hTF crystals to a solution containing 1.9 mM dirhodium tetraacetate for 2 hours. On the contrary, crystals of Ru/Fe<sub>C</sub>-hTF diffract X-ray at high resolution, up to 2.26 Å, even when treated with a reservoir solution containing 5 mM diruthenium tetraacetate for 72 h. The structures were solved by molecular replacement using the program Phaser MR<sup>52</sup> and the coordinates of Fe<sub>C</sub>-hTF structure recently obtained in our group<sup>19</sup> as the search model. Final models for the structures of Rh/Fe<sub>C</sub>-hTF and Ru/Fe<sub>C</sub>-hTF refine *R*-factors of 0.182/0.186 (*R*<sub>free</sub> = 0.244/0.234) using REFMAC5<sup>53</sup> (Table S2). Notably, the binding of metal-containing fragments to the protein does not significantly alter its overall structure (Fig. 1). Indeed, the C $\alpha$  root mean square deviation of the Rh/Fe<sub>C</sub>-hTF adduct from the starting model and from the structure of Ru/Fe<sub>C</sub>-hTF are 0.305 Å and 0.299 Å, respectively. Pairwise comparisons performed by calculating the rotation angle ( $\chi$ ) needed to superimpose N2 or C2 subdomains after the best fitting of N1 or C1 subdomains of Rh/Fe<sub>C</sub>-hTF or Ru/Fe<sub>C</sub>-hTF when compared to apo-hTF further confirm this result. Indeed, data reveal that N-lobes of both Rh/Fe<sub>C</sub>-hTF and Ru/Fe<sub>C</sub>-hTF structures are



**Fig. 1** Overall structures of Rh/Fe<sub>C</sub>-hTF (panel A) and of Ru/Fe<sub>C</sub>-hTF (panel B) adducts. Rh and Ru binding sites are shown in balls and sticks. Coordinates and structure factors of Rh/Fe<sub>C</sub>-hTF and Ru/Fe<sub>C</sub>-hTF adducts were deposited in the PDB under the accession codes 28MS and 28MR.

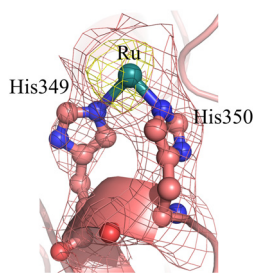
similar to that of apo-hTF ( $\chi$  values to superimpose N2 subdomains after the best fitting of N1 subdomains between 2.22 and 2.95°) and thus adopt the “open” conformation, while the C-lobes appear “closed” ( $\chi$  values to superimpose C2 subdomains after the best fitting of C1 subdomains are between 41.9 and 42.5°).

DiRh centers are found close to His14, Met256, His273, His289, His300, His349, His350, His578, and His642 (Fig. 2), while a single Ru atom was found close to the side chains of His349 and His350 (Fig. 3). Identification of Rh binding sites is based on the inspection of Fourier difference ( $2F_o - F_c$  and  $F_o - F_c$ ) and anomalous difference electron density (e.d.) maps (Fig. 2). The Ru binding site has been identified analyzing  $2F_o - F_c$  and  $F_o - F_c$  e.d. maps and inspecting isomorphous difference ( $F_o - F_o$ ) e.d. maps (Fig. 3) calculated using the data of the reported structures and those of the Fe<sub>C</sub>-hTF structure that we have previously refined (negative control with DMSO).<sup>19</sup> The assignments are also further supported by refinements of duplicated structures at slightly lower resolution (data not shown).



**Fig. 2** Rh binding sites in the structure of the adduct that Fe<sub>C</sub>-hTF forms with dirhodium tetraacetate.  $2F_o - F_c$  electron density map is reported at  $1.0\sigma$  (ruby). Anomalous difference map is reported in yellow at  $2.0\sigma$ .





**Fig. 3** Ru binding site in the structure of the adduct that  $\text{Fe}_C$ -hTF forms with diruthenium tetraacetate.  $2F_o - F_c$  electron density map is reported at  $1.0\sigma$  (ruby). Isomorphous difference ( $F_o - F_c$ ) electron density map is reported at  $4.0\sigma$  (yellow).

DiRh centers are bound to His side chains at the axial binding site, with the only exception of the dimetallic centre anchored to His349 (Fig. 2F). Binding to Met256 occurs at the equatorial site (Fig. 2B). Close to His642, two acetate ligands were modelled (Fig. 2I). At the other metal binding sites, metal ligands were not assigned, if one excludes equatorial water molecules bound to the Rh atoms coordinated to His14 (Fig. 2A), His289 (Fig. 2D) and His578 (Fig. 2H). This is common in the low-resolution structures of adducts of metalodrugs with proteins.<sup>54,55</sup> His has been frequently found as a potential diRh binding site.<sup>39</sup> Met256 has been identified as Pt binding site in the  $\text{Fe}_C$ -hTF adduct with cisplatin.<sup>17</sup>

Binding of a monometallic Ru-containing fragment close to the side chains of His349 and His350 should be due to degradation of the diruthenium complex.

The binding of  $\text{Ru}^{3+}$  to His350 was hypothesized in the structures of the  $\text{Ru}/\text{Fe}_C$ -hTF adducts that  $\text{Fe}_C$ -hTF forms upon reaction with the Ru compounds  $\text{Ru}(\text{NTA})_2$  (NTA = nitrilotriacetate) and  $\text{Ru}(\text{bpy})\text{OHCl}_2(\text{DMSO})_2$  (bpy = 2,2'-bipyridine) but not modelled in the final structures.<sup>29</sup>

Occupancy values for Rh atoms are within the range 0.30–0.50, while the Ru atom in the structure of  $\text{Ru}/\text{Fe}_C$ -hTF has occupancy = 0.70. On the contrary,  $\text{Fe}^{3+}$  ion has occupancy equal to 1.00. B-factors for the metal centers are high (69.92–88.35  $\text{\AA}^2$  in the case of Rh atoms and 122.47  $\text{\AA}^2$  in the case of Ru), but this is not surprising considering the resolution, partial occupancy and conformational disorder associated with the paddlewheel dimetallic compound fragment binding to protein atoms. Average value for the  $\text{N}\cdots\text{Rh}$  distances is  $2.44 \pm 0.16$   $\text{\AA}$ , while the observed  $\text{N}\cdots\text{Ru}$  bond is 2.28  $\text{\AA}$  in the case of His349 and 2.80  $\text{\AA}$  in the case of His350. These values are not far from those expected (Rh–N distance should be = 2.23  $\text{\AA}$ , while Ru–N distance should be within the range 2.18–2.25  $\text{\AA}$ ).  $\text{Rh}\cdots\text{S}(\text{Met256})$  distance is 3.10  $\text{\AA}$ , larger than expected (Rh–S distance should be = 2.57  $\text{\AA}$ ).

## Summary and conclusion

In conclusion, inspired by previous studies on the interaction of metalodrugs with hTF,<sup>17</sup> we have investigated the reactivity

of the isostructural dirhodium and diruthenium tetraacetate complexes with  $\text{Fe}_C$ -hTF and have identified diRh and Ru binding sites in the structure of the adducts formed by the protein with these two complexes. Our results reveal that:

(i) DiRh binding sites are found close to His14, Met256, His273, His289, His300, His349, His350, His578, and His642, with dirhodium center that prefers axial rather than equatorial binding mode. This is the first structural observation of binding of Rh atoms to hTF. This is in line with previous reactivity of this compound with proteins.<sup>44,56</sup>

(ii) A Ru binding site has been identified close to the side chains of His349 and His350. This result supports previous finding indicating that Ru ligands play a role in defining the final Ru binding site.<sup>57,58</sup> Indeed, the structures of  $\text{Ru}/\text{Fe}_C$ -hTF adducts formed when the protein reacts with the Ru compounds  $\text{Ru}(\text{NTA})_2$  and  $\text{Ru}(\text{bpy})\text{OHCl}_2(\text{DMSO})_2$  are different from each other<sup>29</sup> and from the one obtained here with diruthenium tetraacetate.

Results (i) and (ii) suggest that the Ru and Rh ions bind protein surface residues, although the iron binding site of the N-lobe is unoccupied.

(iii) The binding of Ru and Rh ions to  $\text{Fe}_C$ -hTF does not significantly affect the overall conformation of the protein that adopts a closed conformation of the C-lobe and an open conformation of the N-lobe. This agrees with what has been previously observed for cisplatin binding to both apo- and  $\text{Fe}_C$ -hTF forms<sup>16,17</sup> and with what has been found in the case of adducts with Os<sup>29</sup> and V.<sup>19</sup> However, this result differs from previous observations obtained comparing the apo-hTF structure with that of the adducts formed with gold ions from aurothiomalate<sup>18</sup> and with  $\text{Bi}^{3+}$ .<sup>59</sup>

(iv) Ru and Rh-containing fragments from dimetallic paddlewheel complexes compete for the same binding sites on the hTF structure. Under the same experimental conditions, the dirhodium complex is much more reactive with hTF than its diruthenium analogue.

Previous investigations by P. Lay and coworkers have demonstrated that Ru binding to the surface His side chains does not affect the hTF cycle.<sup>31</sup> While the  $\text{Ru}/\text{Fe}_C$ -hTF and Rh/ $\text{Fe}_C$ -hTF adducts could theoretically mediate cellular uptake of Ru or Rh, this mechanism might be disrupted by competing proteins like serum albumin. However, further studies are necessary to verify these hypotheses and test the possible use of these hTF adducts as a Trojan Horse to transport and deliver metalodrugs to cancer cells.

## Author contributions

All authors have given approval to the final version of the manuscript. A.-S. B. and G. F. equally contribute to this work.

## Conflicts of interest

There are no conflicts to declare.



## Data availability

Data are all available in literature or deposited in the PBD.

Supplementary information (SI): Tables S1 and S2. See DOI: <https://doi.org/10.1039/d6dt00205f>.

## Acknowledgements

Antonello Merlino thanks MIUR PRIN 2022 - Cod. 2022JMFC3X, "Protein Metalation by Anticancer Metal-based Drugs" for financial support. A.-S. B., A. P. and I. C. thank the ALPX lab operators for their support with crystallization and the HTX Lab at EMBL Grenoble for additional lab assistance. The authors thank Tatiana Isabet for the data collection support at Proxima2A (SOLEIL), as well as Romain Talon and Matthew Bowler for the data collection support at MASSIF-1 (ESRF).

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