



# Quantum chemical studies of structures and spin Hamiltonian parameters of iron transferrin using isolated and embedded clusters models

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**Abstract.** Density functional theory (DFT) based calculations using large cluster models are used to elucidate the ground state electronic structure of iron bound transferrin. Explicit incorporation of second coordination amino acid residues and crystallographic water molecules anchors the active site. Our calculations clearly suggest that tyrosine amino acid (Tyr188) residue is bound to iron when the structures are optimized within the continuum solvation model. However, in the gas phase optimized structure, we note that Tyr188 is unbound to Fe (by more than 3 Å). The Mössbauer isomer shift ( $\delta$ ) and quadrupolar splitting ( $\Delta E_Q$ ) of iron transferrin are in line with the experimental data only when Tyr188 is bound to Fe(III). Further, the computed oxygen hyperfine coupling constant value is very large ( $-14.5$  MHz) when bound to iron which can be verified through  $^{17}\text{O}$  NMR experiments. We propose that Tyr188 is strongly bound to Fe(III) at physiological pH, which needs to be protonated (acidic pH) to weaken this bond, thus the metal release pathway can be possible only in acidic conditions.

**Keywords.** Transferrin; electronic structure; DFT; amino acid; modelling.

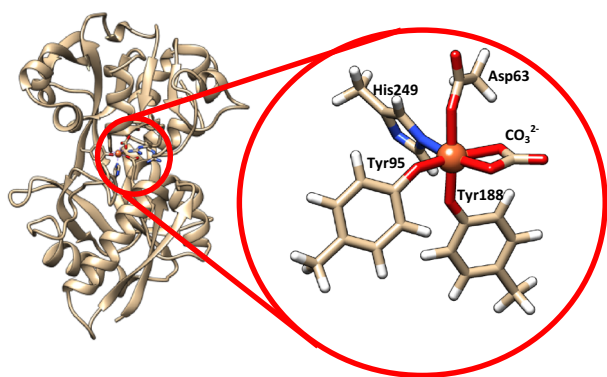
## 1. Introduction

Serum transferrin (sTf) is the protein that transports iron through the blood between sites of uptake, utilization, and storage. The major function of these proteins is to control the level of free iron in physiological fluids by binding and sequestering Fe(III) cations.<sup>1–3</sup> The active site iron is octahedron geometry coordinated by two tyrosines (Tyr95, Tyr188), a histidine (His 249), an aspartate (Asp63) and an inorganic carbonate ( $\text{CO}_3^{2-}$ ) (Figure 1).<sup>4–6</sup> Further, there are a number of amino acid residues in the second coordination shell such as arginine (Arg124), salt-bridged lysine (Lys206 and Lys296), threonine (Thr120) and several water molecules anchors the first coordination shell. It is now well-known that at physiological pH the  $\text{Fe}^{3+}$  binding strengths to the protein is very strong ( $K_a = 10^6 \text{ M}^{-1}$ ). Only 30% of the sTf is occupied by  $\text{Fe}^{3+}$ , whereas the remaining vacant sites

can be accommodated by a variety of metal ions such as other 3d transition metal ions (e.g.,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Co}^{3+}$  and  $\text{Ni}^{3+}$  to name a few) and inner transition elements (e.g.,  $\text{Eu}^{3+}$ ,  $\text{Am}^{3+}$ ,  $\text{Cm}^{3+}$ ,  $\text{Th}^{4+}$ ,  $\text{Np}^{5+}$ ,  $\text{UO}_2^{2+}$ ).<sup>7–20</sup>

A number of experimental studies have been carried out to understand the geometric and electronic structure and the ion release pathway of sTf.<sup>4,5,7,21,22</sup> As the magnetic anisotropy is very small ( $<0.5 \text{ cm}^{-1}$ ) due to half-filled  $d^5$  iron,<sup>23–27</sup> the isomer shift and quadrupolar splitting of Fe derived from Mössbauer spectroscopy (MB) are often used to elucidate the electronic structure of the active site.<sup>28–30</sup> As far as ion transmission pathways are concerned, several molecular dynamics simulations are carried out by varying the protonation state of the nearby amino acids such as salt bridged Lys, Tyr188, and carbonate.<sup>31</sup> A number of computer studies are carried out on the geometric structures of iron-transferrin using small cluster models.<sup>10,31,32</sup>

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**Figure 1.** Active site structure of sTf.

Rinaldo and Field<sup>33</sup> carried out the very first gas phase density functional theory (DFT) based calculations and suggested that salt bridged Lys–Lys could be vital for ion transmission due to the automatic proton transfer from Lys296 to the bound Tyr188 which weakens the Fe–Tyr188 interaction. However, recent molecular dynamics (MD) and QM/MM/MD simulations in physiological and in acidic pH suggest that Fe–Tyr188 interaction weakens only in acidic pH.<sup>31–34</sup>

A plethora of spin Hamiltonian parameters (SH) derived from MB spectroscopy and EPR spectroscopy are available which can directly reveal the electronic structure of iron sTf in physiological pH.<sup>15,23–26,28,29,35–38</sup> Computing these SH parameters can directly reveal the coordination environment of Fe(III) in sTf which was never attempted before.

Thus, the question arises what is the geometric and electronic structure of iron bound sTf in physiological pH? We have attempted to solve the important question through the computation of SH parameters derived from EPR and MB spectroscopy.<sup>39–43</sup> Particularly, we propose that the oxygen (<sup>17</sup>O) hyperfine coupling constant (HFCC) values of Tyr188 are very large (>13MHz) when bound to Fe(III) and very small (<2MHz) when it is unbound. These variations can be used as fingerprint values to distinguish the binding nature of Tyr188 to Fe(III) ion.

## 2. Experimental

We have taken the N lobe structure of sTf for our computational study (PDB Code: 1A8E, 1.6 Å resolution).<sup>5</sup> In addition to the first coordination shell (Asp63, Tyr95, Tyr188, His249, and carbonate), we have incorporated the salt bridge (Lys206 and Lys296), hydrogen bonded residues such as Arg124 (to carbonate), Thr120 (to carbonate) and two crystallographic water molecules. It should be noted that the salt bridge (Lys296 and Lys206) is strongly hydrogen bonded to Tyr188 (Figure 2). In our calculations, we have truncated

all amino acids between C<sub>β</sub>–C<sub>α</sub> positions. The C<sub>α</sub> carbons are fixed to crystallographic positions to mimic the steric effect of the protein. We have used large size cluster models which can be treated as an alternative to QM/MM models in line with our earlier studies and in the pioneering works by Siegbahn *et al.*<sup>44–46</sup> We have earlier used large cluster models to understand the electronic structure of several metalloproteins which are comparable to QM/MM simulations.<sup>47–49</sup>

The choice of density functional is very crucial for the accurate prediction of geometry. In this area, both B3LYP and BP86 functionals are commonly applied to model metalloproteins. The structures are optimized in both vacuum (gas phase) and in protein dielectric ( $\epsilon = 5$ ) using dispersion corrected (D3BJ) BP86 functional<sup>47,48</sup> in conjunction with TZVP<sup>49,50</sup> basis set for all atoms. We have successfully used this functional to model the electronic structure of several iron and copper containing metalloproteins.<sup>39–42</sup> We have also carried out additional calculations with neutral and anionic histidine's. The protein dielectric calculation is carried out using the COSMO continuum solvation model as implemented in TURBOMOLE 6.0.<sup>51</sup>

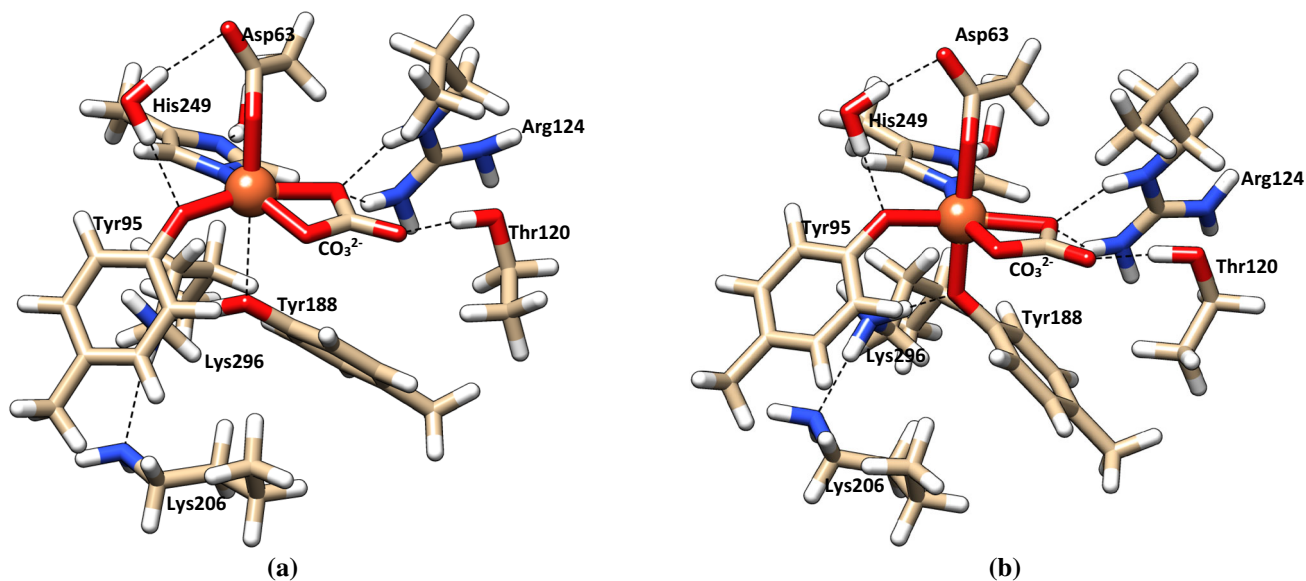
EPR and MB calculations are carried out at the DFT level using the B3LYP functional.<sup>52,53</sup> The choice of using this functional is well-known that functionals containing HF-exchange tend to predict the spin Hamiltonian parameters better than the pure functionals. Particularly for MB parameters, the standard deviation of B3LYP functional is smaller than the BP86 functional. For these calculations, we used a CP(PPP)<sup>54</sup> (for Fe) and TZVP (for all atoms) basis set. The technical details on the computation of SH parameters are discussed in detail in references.<sup>55,56</sup> The isomer shift ( $\delta$ ) and the quadrupolar splitting ( $\Delta E_q$ ) are closely related to the electron density distribution of the iron nucleus.

To validate the computed SH parameters, we have computed the EPR parameters for copper transferrin, where the experimental data are known<sup>39–41</sup> (ESI). Our predicted geometries and the g-tensor and hyperfine coupling constants (HFCC) of Cu and N (His249) are in excellent agreement with the experimental data.<sup>25</sup>

## 3. Results and Discussion

### 3.1 Gas phase vs. embedded optimized geometries

The optimized structures of sTf in isolated (gas phase) and in embedded models (optimized within the continuum solvation model) are very different (Figure 2 and Table 1). Significant geometric changes within the first coordination shell are observed within the two optimized structures. Particularly, a five-coordinated species is found in the optimized isolated model with a very long Fe–OTyr188 (more than 3 Å, Figure 2a). The longer Fe–OTyr188 is attributed to the direct proton transfer from the neighboring salt bridge (Lys296) during optimization which leads to long Fe–OTyr188 bond and consequently, the Fe–OAsp63 bond (1.927 Å) is



**Figure 2.** Optimized structures of (a) isolated and (b) embedded models.

**Table 1.** Optimized first coordination parameters (in Å) around the iron center of sTf.<sup>a</sup>

Models	X – O <sub>Asp63</sub>	X – O <sub>Tyr95</sub>	X – O <sub>Tyr188</sub>	X – N <sub>His249</sub>	X – O1 <sub>CO3</sub>	X – O2 <sub>CO3</sub>
Isolated	1.927	1.919	5.026	2.042	2.032	2.227
Embedded	2.043 (2.032)	2.110 (1.957)	2.078 (2.058)	2.110 (2.194)	2.072 (2.035)	2.212 (2.197)
X-ray	2.02	1.99	1.90	2.10	2.06	2.24

<sup>a</sup> Values given in the parenthesis are for an optimized embedded structure with neutral His249.

shortened. These geometric variations are also observed in the gas phase geometry optimizations of Rinaldo and Field.<sup>33</sup> Further, the carbonate is asymmetrically bound to Fe(III) (2.032 to 2.227 Å) due to strong hydrogen bonding from Arg124 and Thr120 (Figure 2). We note that one water molecule is anchoring Tyr95 and Asp63 through a hydrogen bond, whereas the second water molecule is hydrogen bonded to anionic His249 ( $\delta$  or  $\epsilon$ ).

However, in the optimized embedded model, we find that Tyr188 is strongly bound to Fe(III) (2.078 Å) and the proton is strongly intact with Lys296. Further, there is no proton transfer from salt bridged Lys296 to Tyr188 (1.663 Å) which lead to conclude the hexacoordinated Fe(III) active site geometry. We find that even if we start with Tyr188 as protonated, we find that the proton simultaneously transferred back to Lys296 which is  $\sim 9$  kcal mol<sup>-1</sup> lower in energy than when Tyr188 being protonated. These variations are consistent with the recent MD simulations study of Mujika *et al.*,<sup>31</sup> where they have suggested that only at low pH, the metal release is possible. Finally, the Fe-OAsp63 bond length in the embedded model is elongated as compared to the isolated model by 0.12 Å. The other Fe-ligand bond lengths in the embedded and isolated models are very similar (Table 1). Further, we have also considered

neutral His249 which weaken the Fe–N bond and alters the geometry significantly. Optimized first coordination parameters (in Å) are given in brackets for an embedded structure with neutral His249. Indeed, Rinaldo *et al.*, reported that His249 should be protonated.<sup>33</sup> Other protonation sites such as Asp63 are beyond the scope of the work as we are focused only on physiological pH.

Within the two clusters, we find that the embedded model (CPCM-B3LYP/TZVP//CPCM-BP86//TZVP) is more stable than the isolated cluster (CPCM-B3LYP/TZVP//BP86//TZVP) by 6.97 kcal mol<sup>-1</sup>.

### 3.2 MB and EPR spin Hamiltonian parameters

We have computed the isomer shift ( $\delta$ ), quadrupolar splitting ( $\Delta E_q$ ) and HFCC of the coordinating ligands (<sup>14</sup>N and <sup>17</sup>O) of both optimized structures. The trivalent oxidation state of iron is a high spin state (<sup>6</sup>A<sub>1g</sub>) with half-filled d-orbitals. The computed spin Hamiltonian parameters using B3LYP functional for both isolated and embedded models are compared with experimental data (Table 2). We have also computed the SH parameters for the embedded cluster with neutral His249 as well.

**Table 2.** Computed MB (isomer shift ( $\delta$ ), quadrupolar splitting ( $\Delta E_q$ ) ( $\text{mm s}^{-1}$ ) and superhyperfine coupling constants ( $A_{\text{iso}}$ , MHz).

Models	$\delta$	$\Delta E_q$	$\text{Fe } A_{\text{iso}}$	$\text{OAsp63 } A_{\text{iso}}$	$\text{OTyr95 } A_{\text{iso}}$	$\text{OTyr188 } A_{\text{iso}}$	$\text{NHis249 } A_{\text{iso}}$	$\text{O1CO3 } A_{\text{iso}}$	$\text{O2CO3 } A_{\text{iso}}$
Isolated	0.478	1.241 (1.145)	-17.0	-17.3	-15.2	-1.5	-8.3	-7.9	-7.7
Embedded	0.519	-0.578 (-0.587)	-18.5	-14.0	-15.3	-14.5	-8.2	-7.7	-7.3
Expt <sup>30</sup>	0.38	0.75							

The computed  $\delta$  of both isolated ( $0.478 \text{ mm s}^{-1}$ ) and embedded ( $0.519 \text{ mm s}^{-1}$ ) models are in agreement with the experimental value ( $0.38 \text{ mm s}^{-1}$ ) which is characteristic of high spin ferric species. However, the computed  $\Delta E_q$  is very different for the two species. It should be noted that for high spin ferric systems, the  $\Delta E_q$  should be minimal due to the absence of an electric field gradient. Between the two models, we find that  $\Delta E_q$  is very large ( $1.241 \text{ mm s}^{-1}$ ) for the isolated model, whereas for the embedded model this value is small ( $-0.578 \text{ mm s}^{-1}$ ) and in line with experimental value ( $0.75 \text{ mm s}^{-1}$ ). The major component in the  $\Delta E_q$ , is the electric field gradient which may be positive or negative. This depends on the type of asymmetry of the electronic charge distribution. These variations are perhaps due to large geometric variations in the isolated model (penta-coordinated iron) as compared to the embedded model (hexa-coordinated iron) which will be more prominently reflected in the HFCC of the ligands.

To our knowledge, the HFCC parameters derived from EPR spectroscopy in both experiment and in theory of native sTf was never measured or computed before. However, some insights on Cu bound sTf are known at least experimentally.<sup>25</sup> To gauge our computational protocol, we have computed the EPR SH parameters of Cu-sTf using the embedded model as described for Fe-sTf. The optimized structural parameters and geometry are shown in Table S1 and Figure S1 (Supplementary Information). The Cu-OTyr188 is found to be weak in both X-ray ( $\sim 2.8 \text{ \AA}$ ) and in optimized structure ( $\sim 3.2 \text{ \AA}$ ) which is expected due to the Jahn-Teller nature of Cu(II) ion.<sup>43</sup>

An axial type g-tensor is predicted in our calculations ( $g^{\parallel} = 2.225$  and  $g^{\perp} = 2.071$ ) in line with the experimental estimates of ( $g^{\parallel} = 2.312$  and  $g^{\perp} = 2.062$ ).<sup>7,15,36-38,57</sup> It is now well-known that metal HFCC is very hard to predict due to the limitations of DFT itself. Nevertheless, we have corrected the Cu HFCC as discussed in the detailed investigation of Sinnecker and Neese.<sup>58</sup> Our predicted Cu HFCC is now in excellent agreement with the experimental data with 10 MHz which shows the reliability of our computational tool employed here even for challenging biomolecules.

As far as Fe-sTf are concerned, the computed HFCC of the directly bound amino acid ligands to the Fe(III) center is shown in Table 2. For both isolated and embedded models, the  $^{17}\text{O}$  HFCC of carbonates ( $\sim -7$  to  $8 \text{ MHz}$ ), Tyr95 ( $\sim -15 \text{ MHz}$ ) and  $^{14}\text{N}$  of His249 ( $\sim -8.2 \text{ MHz}$ ) are very similar. However, the computed HFCC of  $^{17}\text{O}$  Tyr188 in the isolated model is very small ( $-1.5 \text{ MHz}$ ) due to very long Fe-OTyr188 (more than  $3 \text{ \AA}$ ) thus the oxygen interaction with high spin Fe(III) is rather weak. However, the Fe-OTyr188 bond is very strong ( $\sim 2.1 \text{ \AA}$ ) in the embedded model which lead to very large HFCC ( $-14.5 \text{ MHz}$ ) due to stronger Fe-OTyr188 interaction. Conversely,  $^{17}\text{O}$  HFCC of Asp65 in the embedded model is smaller ( $-14.0 \text{ MHz}$ ) as compared to the isolated model ( $-17.3 \text{ MHz}$ ). These variations are due to stronger Fe-OAsp bond in the isolated model ( $\sim 1.9 \text{ \AA}$ ), whereas in the embedded model this distance is slightly longer ( $\sim 2.1 \text{ \AA}$ ). These variations in super-HFCC of oxygens can be estimated through high field EPR and NMR studies.<sup>59-62</sup>

The quantum chemical study presented here provide valuable insights to understand the electronic structure of iron-transferrin at physiological pH. Incorrect modeling such as neglecting the second coordination aminoacid residues, solvation and explicit incorporation of water molecules can lead to biased conclusions is clear from our study. Further, from a biochemistry perspective, our study suggests that at physiological pH,  $\text{Fe}^{3+}$  remain strongly bound and an external perturbation such as lowering of pH. In acidic conditions, Ty188 aminoacid will be protonated and can weaken its interaction with  $\text{Fe}^{3+}$ , thus releasing the iron.

#### 4. Conclusions

In this paper, we have carried out electronic structure calculations to understand the debatable geometric structure of Fe-sTf. We find that the use of gas phase geometry optimization predicts a wrong penta-coordinated geometric structure and thus an incorrect electronic structure, whereas the geometry predicted within the continuum solvation model is hexacoordinate. We predict that Fe in sTf is hexa-coordinated with

a bound OTyr188 at physiological pH. Our computed MB parameters are in line with the experimental data only when OTyr188 is bound to Fe. Further,  $^{17}\text{O}$  HFCC of Tyr188 is very large ( $-14.5$  MHz) when bound to Fe as compared to unbound state ( $-1.5$  MHz) which can be measured with paramagnetic NMR experiments.

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