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EXAFS simulation refinement based on brokensymmetry DFT geometries for the Mn(IV)–Fe(III) center of class I RNR from *Chlamydia trachomatis*

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Ribonucleotide reductases (RNRs) catalyze the reduction of ribonucleotides into deoxyribonucleotides necessary for DNA biosynthesis. Unlike the conventional class Ia RNRs which use a diiron cofactor in their subunit R2, the active site of the RNR-R2 from *Chlamydia trachomatis* (*Ct*) contains a Mn/Fe cofactor. The detailed structure of the Mn/Fe core has yet to be established. In this paper we evaluate six different structural models of the *Ct* RNR active site in the Mn(v)/Fe(w) state by using Mössbauer parameter calculations and simulations of Mn/Fe extended X-ray absorption fine structure (EXAFS) spectroscopy, and we identify a structure similar to a previously proposed DFT-optimized model that shows quantitative agreement with both EXAFS and Mössbauer spectroscopic data.

Introduction

Ribonucleotide reductases (RNRs) are crucial enzymes. All organisms rely on these enzymes to produce deoxyribonucleoside triphosphates (dNTPs) - the building blocks of deoxyribonucleic acid (DNA).¹ The structure and assembly of RNRs have been investigated by a variety of experimental²⁻¹¹ and theoretical¹²⁻¹⁸ methods. Three main classes of RNRs have been discovered that display a common reaction mechanism using metals and free radical chemistry.¹⁹ Although these classes differ in composition and cofactor requirements, they all possess a conserved cysteine residue at the active site that is converted (during the catalytic cycle) into a thiyl radical (Cys-S'), which initiates substrate turnover by abstracting a hydrogen atom from the ribose ring of the substrate.²⁰⁻²² There is a dinuclear metal center in class I RNRs, a cobalt containing cobalamin cofactor (adenosylcobalamin) in class II RNRs, and a 4Fe-4S cluster in class III RNRs.²² Each of these cofactors generates a radical that transfers to produce Cys-S'. Class I RNRs are found in all eukaryotes as well as in some microorganisms like Escherichia (E.) coli and have two dissimilar protein subunits R1 (α_2 -homodimer) and R2 (β_2 -homodimer).

R1 contains the substrate binding site and the conserved cysteine residue, and acts as a catalyst for the dehydroxylation of the 2'-hydroxyl group of the ribose ring. R2 contains the dinuclear metal cluster that generates a stable radical (except for class Ic RNRs where the cluster itself is the oxidant). This radical then transfers (through a long-range proton-coupledelectron-transfer propagation mechanism) to create Cys-S' which initiates the ribonucleotide-to-deoxyribonucleotide reaction in R1. In E. coli class Ia RNR, a tyrosine residue (Tyr122 in R2) is the radical bearer closest to the diiron center in R2.^{21,23,24} The fairly stable tyrosyl radical is generated by an Fe(m)Fe(m) intermediate state $X^{2,5,6,8,10,11,25-32}$ following the reaction of the reduced $Fe(\pi)Fe(\pi)$ center with molecular O_2 .²⁵ The active form of class Ia R2 is described as an Fe(III)Fe(III)-Tyr state.1-10,19,20,33-38 The radical bearing tyrosine is conserved among more than 200 sequenced R2s. Mutants with a phenylalanine in this position are enzymatically inactive^{39,40} with the exception of native RNR-R2 from the pathogenic bacteria Chlamydia trachomatis (Ct). There, a phenylalanine (Phe127) resides in place of the tyrosine residue which carries the radical in conventional RNRs.¹ Later Bollinger's group discovered that the Ct-RNR-R2 contains a Mn-Fe center rather than a diiron center in its functional form, and it uses the Mn(IV)-Fe(III) cofactor (intermediate X) directly for radical initiation instead of the tyrosine radical.41,42

Currently, a variety of Mössbauer, hyperfine, and EXAFS data are available for the *Ct*-R2 center in different oxidation states.^{11,41,43–46} However, explicit structures of the Mn/Fe active site in each oxidation state are incompletely known. The first X-ray structure (pdb entry 1SYY) obtained for *Ct*-R2 was in the

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Fig. 1 Ct RNR active site model **3**. In **3**, Mn(v) occupies site 1, the metal position closer to Phe127, and Fe(w) occupies site 2, which is the metal position further from Phe127. The protein environment surrounding the Fe(w)/Mn(v) cofactor is the same in each model. The differences between the active site cores are shown in Fig. 2.

Fe(III)-Fe(III) diiron inactive form.⁴⁷ This diiron center is very similar to the diferric center of the hydroxylase component of soluble methane monooxygenase (MMOH).⁴⁸

While we were preparing the current manuscript, two relevant experimental papers on *Ct*-R2 were published.^{49,50} Andersson *et al.*⁴⁹ performed X-ray crystallographic anomalous diffraction experiments and concluded that Mn occupies site 1 (the metal site which is closer to Phe127, see Fig. 1) in *Ct*-R2. Dassama *et al.*⁵⁰ also studied the location of Mn(rv) *vs*. Fe(m) by correlating X-ray crystallographic anomalous scattering intensities with catalytic activity for *Ct*-R2 protein samples reconstituted *in vitro* by two different procedures. They found that Mn occupies site 1 preferentially to site 2 with Fe in the alternate site, but with some heterogeneity in Mn and Fe sites. With diminished Mn(n) loading in the reconstruction, no evidence for Mn occupancy of site 2 was found.⁵⁰

Multiple theoretical studies have been performed in order to characterize the *Ct*-R2 active site structures and the positions of the Mn and Fe sites.^{11,16,18} For the Mn(iv)–Fe(iii) state, it is now commonly agreed that the active site likely contains a bridging hydroxo- and a bridging oxo-ligand.^{11,16,18} In ref. 16, two μ -oxo- μ -hydroxo structures (see Fig. 1 for a full active site structure, and models 3 and 4 in Fig. 2 for first coordination shell) for the Mn(iv)–Fe(iii) state of the *Ct*-R2 active site have been examined in addition to two di- μ -oxo structures (see structures 1 and 2 in Fig. 2). In these μ -oxo– μ -hydroxo models, oxygen O2 (compare Fig. 1) on the side of the negatively charged carboxylates of Glu89 and Glu227 is protonated



Fig. 2 *Ct* RNR active site model cores of structures **1–4** as presented in ref. 16. The subscripts 1 and 2 on the metal atoms indicate which site these atoms occupy. Site 1 is closer to Phe127 (not shown, see Fig. 1) than site 2. The subscripts 1 and 2 on the bridging oxygen atoms simply differentiate the two positions (*i.e., cis* and *trans* to His123, respectively).

because protonation of the oxygen atom O1 nearer to the neutral His123 and His230 is energetically less favourable.¹⁸ Structures **1** and **2** differ principally in the positions (site 1 or 2) of the Mn/Fe metals in the cofactor relative to the position of Phe127 (see Fig. 1). This difference in the positions of the metal atoms also differentiates structures **3** and **4**.

This paper evaluates the quantitative agreement with EXAFS of the four *Ct* RNR active site model structures (1–4) examined by Han *et al.*¹⁶ as well as of models 5 and 6 (see Fig. 3) which have been derived from structures 3 and 4, respectively, through additional fitting of interatomic distances comparing simulated and experimental¹¹ Mn and Fe extended X-ray absorption fine structure (EXAFS) spectra. Calculated Mössbauer parameters for the Mn(rv)–Fe(m) states are also compared with experimental parameters for Fe isomer shift (δ) and quadrupole splittings (ΔE_Q). There are several possibilities to change the atomic positions for a good fit to the experimental EXAFS data. We have found optimally fit structures with reasonably close atomic distances starting



Fig. 3 *Ct* RNR active site model core of structures **5** and **6**. The subscripts 1 and 2 on the metal atoms indicate which site these atoms occupy. Site 1 is closer to Phe127 (not shown, see Fig. 1) than site 2. The subscripts 1 and 2 on the bridging oxygen atoms simply differentiate the two positions (*i.e., cis* and *trans* to His123, respectively).

from structures 3 and 4 whose computed Mössbauer parameters are in good agreement with experimental data.

Experimental EXAFS and Mössbauer spectroscopy data

Experimental EXAFS and Mössbauer spectroscopy data are from Younker *et al.*¹¹

EXAFS calculations

All EXAFS spectra were computed using the program FEFF version 8.30, and all fits were performed using the program IFEFFIT version 1.2.11, which optimizes the fitting parameters with a least-squares fit to the experimental data.^{51,52} For each structure, unfitted FEFF EXAFS spectra were generated. Fitting of these EXAFS spectra to experimental data was performed in reduced distance (*r*)-space after Fourier transformation over a range of $k = 3.05-11.6 \text{ Å}^{-1}$ (*k* is the photoelectron wave number) using the program IFEFFIT. For the Fourier transformation, a Hanning window with $\Delta k = 1 \text{ Å}^{-1}$ was applied to the k^3 -weighted EXAFS data. The grids of *k* points, which are equally spaced at 0.05 Å⁻¹, were then used for the Fourier transformation to *r*-space.

As in ref. 11, three shells of Debye–Waller factors σ^2 , which account for thermal disorder in the structure,⁵³ were considered, whereby one shell is used for the Mn-Fe distance and the other shells account for the six nearest atomic neighbours around the scattering centers (coordination numbers of 2 and 4 for the first and second coordination shell, respectively, were employed). The negligibly small contribution of hydrogen atoms was not considered. In each fit, the passive electron reduction factor S₀ for each shell was held constant while the K-edge energy E0 and each shell's Debye-Waller factor σ^2 were varied. As in ref. 11, fits were performed using values of 0.8, 0.9, and 1.0 for So in case of Mn and Fe K-edge EXAFS, respectively. The initial guess values for E_0 have been chosen as 6551.6 eV for the Mn EXAFS and 7122.1 eV for the Fe EXAFS calculations, also based on Ct RNR EXAFS fitting data from Younker et al.11

The fitted EXAFS spectra presented in the following have been chosen since they show positive and reasonable low Debye–Waller factors and give rise to the smallest *R*-factors. The *R*-factor, which measures the absolute misfit between the computed and experimental spectrum, is in general given as follows⁵³ (for details, we refer to ref. 54):

$$R = rac{\sum\limits_{i=1}^{N_{ ext{fit}}} \left[x_i^{ ext{exp}} - x_i^{ ext{fit}}(p)
ight]^2}{\sum\limits_{i=1}^{N_{ ext{fit}}} \left[x_i^{ ext{exp}}
ight]^2}$$

 $N_{\rm fit}$ is the number of data points, $x^{\rm exp}$ is the experimental, and $x^{\rm fit}$ the calculated EXAFS function which depends on the set of fitting variables p. The fit of the calculated data to the experimental one is the closer, the smaller the *R*-factor value is.

For the derivation of structures 5 and 6, an iterative procedure was used: two or three interatomic distances were fitted in addition to the Debye–Waller factors and E_0 for one metal scattering center. The structures were chosen such that the interatomic distances are close to the density functional theory (DFT)-optimized ones of structures **3** and **4**, respectively, and chemically reasonable. As additional criteria, a good agreement between the experimental and calculated Mössbauer parameters and a good match between the experimental and computed EXAFS spectrum with the other metal as scattering center were considered.

DFT calculations

The calculations were performed using the Amsterdam Density Functional (ADF) package^{55,56} with the Vosko, Wilk, and Nusair (VWN) parametrization⁵⁷ for the local density approximation and the OPBE density functional⁵⁸⁻⁶⁰ for the generalized gradient (GGA) exchange and correlation. In analogy to ref. 16, the triple-zeta TZP basis set with frozen core as implemented in ADF has been employed in all calculations except of the ones for the Mössbauer properties where no frozen core has been applied. The COSMO conductor-like screening model⁶¹⁻⁶⁴ has been used with the dielectric constant set to 4 and van der Waals radii for the atoms Fe, Mn, C, N, O, and H have been chosen as 1.5, 1.5, 1.7, 1.55, 1.4, and 1.2 Å, respectively.¹⁶ A value of 2.0 Å has been applied for the probe radius of the contact surface between the molecule under study and the solvent. Single-point energy calculations have been performed for models 1-4, whose optimized geometries as presented in ref. 16 have been used. The structures obtained using the EXAFS optimizer starting from the DFToptimized models 3 and 4 by best fit to the experimental EXAFS spectra produce models 5 and 6. At this stage, the six nearest atomic neighbours around the Mn and Fe centers and the Mn and Fe positions are fixed from the EXAFS optimization calculations. The remaining atoms of the residues Glu89, Glu227, Glu193, His230, Glu120, and His123 as well as the hydrogen atoms of the water molecule bound to the Mn/Fe atom and of the µ-hydroxo bridge are optimized by DFT for the purpose of comparative energy calculations. All other residues, which are further away from the metal centers, have been frozen. The energies given in this paper are spinprojected energies for the whole model shown in Fig. 1 and calculated as described in ref. 16. The Mössbauer quadrupole splitting, isomer shift, and asymmetry parameter η were calculated for each structure with the same computational settings as those described in ref. 16.

Results

The active site cores of the structures studied in this paper are shown in Fig. 2 and 3, while an example of a core and the local protein environment is shown in Fig. 1. A terminal water ligand at metal site 1 has been chosen since previous results based on EXAFS fitting¹¹ and quantum chemical calculations^{11,18} showed that a water ligand is more reasonable than a hydroxo ligand. The *xyz* coordinates of model structures **1–4** were taken from ref. 16. Structure **5** has been derived from



Fig. 4 Mn K-edge EXAFS spectra in k-space (left-hand side) and r-space (righthand side) for model structures **1–4**. A passive electron reduction factor S_0 of 0.8 was used as well as the K-edge energy shifts and Debye–Waller factors given in Table 1.

structure 3 by fitting of interatomic distances to obtain a better consistency of calculated and experimental EXAFS data (for details, see above). In an analogous way, structure 6 has been obtained from structure 4. The computed Mn K-edge EXAFS spectra in k-space and r-space for structures 1–4 are shown in Fig. 4 and the analogous spectra for structures 5 and 6 in Fig. 6.

Values of 0.8, 0.9, and 1.0 for S_0 were employed where $S_0 =$ 0.8 gave the smallest deviation of the calculated Mn EXAFS spectrum for each model structure compared to the experimental data. This is consistent with fitting results from Younker et al.¹¹ The Fe K-edge EXAFS spectra for structures 1-4 are shown in Fig. 5, and the analogous ones for structures 5 and 6 in Fig. 6. In accordance to ref. 11, an S_0 value of 1.0 gave the closest agreement of the calculated Fe EXAFS spectra to the corresponding experimental ones for each model structure, compared to other S_0 values of 0.8 and 0.9. As expected, the simulated spectra of structures 5 and 6 match the experimental Ct RNR EXAFS spectra the best. The Mn and Fe EXAFS fitting data confirms this conclusion (compare Tables 1 and 2) since structures 5 and 6 have the lowest R-factor. The data also show that the next best models are structures 3 and 4.

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Fig. 5 Fe K-edge k-space (left-hand side) and r-space (right-hand side) EXAFS spectra for model structures **1–4**. The passive electron reduction factor S_0 was set to 1.0 and the K-edge energy shifts and Debye–Waller factors employed are presented in Table 2.

Table 3 shows computed Mössbauer properties for each model (the results for structures 1–4 are taken from ref. 16) as well as experimental values from ref. 41 and 42. The model structures showing best agreement between the calculated and experimental isomer shifts (δ) are 2, 3, and 5. The smallest deviation between calculated and experimental Mössbauer quadrupole splitting (ΔE_Q) values has been obtained for structure 5, followed by models 4 and 3. The uncertainty in the calculated Mossbauer parameters can be estimated by considering the training sets used in ref. 65 which contain isomer shift fits for 30 Fe sites and quadrupole-splitting calculations for a corresponding but larger set of 59 Fe sites, all using the OPBE exchange-correlation potential.

The calculated standard deviation (SD) for the isomer shift test set was 0.072 mm s^{-1} and for the quadrupole splitting 0.25 mm s⁻¹. Using these SD's in Table 3, we find that the quadrupole splittings are more diagnostic in separating the best models from the rest, since model 6 differs from model 5 by more than 1 SD (also the sign of the quadrupole splitting of model 6 is wrong), while both models 3 and 4 are acceptable. Models 1 and 2 are poor because their quadrupole-splitting values deviate at least 2 SD compared to experiment.



Fig. 6 Mn (upper part) and Fe (lower part) K-edge k-space (left-hand side) and r-space (right-hand side) EXAFS spectra for model structures **5** and **6**. A passive electron reduction factor S_0 of 0.8 and 1.0 for the Mn and Fe EXAFS calculations, respectively, was used. The K-edge energy shifts and Debye–Waller factors employed are given in Tables 1 and 2.

Table 1 Mn EXAFS data for *Ct* RNR model structures **1–6**. In each fit the passive electron reduction factor $S_0 = 0.8$ was held constant while the K-edge energy E_0 shift (in eV) and each coordination shell's Debye–Waller factor σ^2 (in 10^{-3} Å^2) were varied. Shell 3 corresponds to the Mn–Fe distance, shells 1 and 2 to Mn–O/N distances. The dimensionless *R*-factor (*R*) is the absolute misfit between the experimental and the computed data

Structure	E_0 shift	Shell 1 σ^2	Shell 2 σ^2	Shell 3 σ^2	R
1	-27.2	8.3	5.0	1.4	0.88
2	-38.0	8.9	9.8	6.8	1.03
3	10.4	0.4	2.8	1.7	0.20
4	13.2	8.5	3.1	0.0	0.31
5	5.1	0.8	3.7	0.7	0.08
6	3.2	6.2	3.4	1.1	0.07

The experimental line width $\Gamma = 0.3 \text{ mm s}^{-1}$ (Table 3)^{41,42} should approximately correspond to the experimental SD of the quadrupole splitting. For Table 3, the experimental uncertainty (SD) is thus similar to the calculated SD. In Table 4, the experimental SD is larger, since the line with Γ is approximately 0.5 mm s^{-1,41} while the SD obtained with the OPBE density functional remains the same (SD = 0.25 mm s⁻¹).

Table 2 Fe EXAFS data for *Ct* RNR model structures **1–6**. In each fit the passive electron reduction factor $S_0 = 1.0$ was held constant while the K-edge energy E_0 shift (in eV) and each coordination shell's Debye–Waller factor σ^2 (in 10^{-3} Å^2) were varied. Shell 3 corresponds to the Mn–Fe distance, shells 1 and 2 to Fe–O/N distances. The dimensionless *R*-factor (*R*) is the absolute misfit between the experimental and the computed data

E_0 shift	Shell 1 σ^2	Shell 2 σ^2	Shell 3 σ^2	R
-2.8	4.2	7.3	8.5	0.53
7.3	4.3	6.8	8.9	0.73
6.4	4.1	8.6	4.9	0.40
11.5	4.6	6.7	6.1	0.27
3.6	1.3	3.7	4.4	0.02
4.6	4.3	4.8	4.5	0.03
	-2.8 7.3 6.4 11.5 3.6 4.6	$\begin{array}{cccccccccccccccccccccccccccccccccccc$	-2.8 4.2 7.3 7.3 4.3 6.8 6.4 4.1 8.6 11.5 4.6 6.7 3.6 1.3 3.7 4.6 4.3 4.8	-2.8 4.2 7.3 8.5 7.3 4.3 6.8 8.9 6.4 4.1 8.6 4.9 11.5 4.6 6.7 6.1 3.6 1.3 3.7 4.4 4.6 4.3 4.8 4.5

Discussion

Our objective of this paper is to determine which structure among models 1-6 agrees best with experiment based on evaluation of their simulated EXAFS and Mössbauer spectroscopy data. Structures 1 and 2, which both have di-µ-oxo bridging between Mn(IV) and Fe(III), can be eliminated immediately because their Mn and Fe EXAFS spectra give rise to the highest R-factors (see Tables 1 and 2 and Fig. 4 and 5). These EXAFS fits were obtained by constraining the Debye-Waller factors to be positive and less than 0.010 Å² because otherwise negative or unreasonably large Debye-Waller factors were computed. The high values of the K-edge energy E_0 shifts also indicate that structures 1 and 2 are not good models for fitting the experimental EXAFS data. In addition, the calculated quadrupole splitting values of structures 1 and 2 deviate significantly from the experimental one leading to an overall better agreement of the μ -oxo- μ -hydroxo models (3-6) with experiment. This is consistent with results from Han et al.¹⁶ Therefore, the remainder of this discussion will evaluate the relative accuracy of the µ-oxo-µ-hydroxo models 3, 4, 5, and 6.

Comparison of the R-factors from the Mn EXAFS fits for each structure shows that structure 6 has the lowest value, closely followed by structure 5. In case of the Fe EXAFS fits, structure 5 leads to a slightly lower R-factor compared to model 6. The calculated Fe-Mn distances of 2.92 and 2.91 Å in models 5 and 6, respectively, are in agreement with the findings in ref. 11. One short Mn-O distance (1.68 Å for structure 6 and 1.71 Å for structure 5) is obtained, similar to a single oxygen scatterer at 1.74 Å found in ref. 11. In addition, structures 6 and 5 contain four scatterers around the Mn atom with distances between 1.85 Å and 1.96 Å, close to the result in ref. 11 where four scatterers at 1.95 Å have been determined. Since models 3 and 4 are not EXAFS-refined structures, their *R*-factors are significantly larger compared to the ones of structures 5 and 6: the R-factors of models 6, 5, 4, and 3 are 0.07, 0.08, 0.31, and 0.20 in the Mn EXAFS fits and 0.03, 0.02, 0.27, and 0.40 in the Fe EXAFS fits. The EXAFS data for structure 3 agrees better with experiment than the one corresponding to structure 4 in case of the Mn EXAFS fit but the situation is reversed in case of the Fe EXAFS fit. Thus, based on analysis of the Fe and Mn computed EXAFS data alone, structure 3 cannot be preferred to structure 4.

Table 3 Computed Mössbauer properties [isomer shift δ (in mm s⁻¹), quadrupole splitting ΔE_Q (in mm s⁻¹), and asymmetry parameter η] of Ct-R2 model structures **1–6** with oxidation state Mn(ν)–Fe(\parallel).^a The experimental data is taken from ref. 41 and 42

	1	2	3	4	5	6	Exp.
$\delta \Delta E_{ m Q} \eta \Gamma^b$	ref. 16 0.58 -0.82 0.93	ref. 16 0.53 -0.78 0.74	ref. 16 0.54 -1.23 0.24	ref. 16 0.49 -1.38 0.83	Calc. 0.54 -1.29 0.45	Calc. 0.47 1.63 0.91	ref. 41, 42 0.52 -1.32 0.11 0.3

^{*a*} Computed results for models 1–4 are taken from ref. 16. ^{*b*} Line width Γ (in mm s⁻¹).

Table 4 Computed Mössbauer properties [isomer shift δ (in mm s⁻¹), quadrupole splitting ΔE_Q (in mm s⁻¹), and asymmetry parameter η] of *Ct*-R2 model structures containing Mn(III)–Fe(III) and Mn(IV)–Fe(IV) oxidation states,^a "1" and "2" refer to the site the metal occupies (see Fig. 1 for details)

	Mn1(III)-Fe2(III)	Fe1(III)-Mn2(III)	Exp. ^b
δ	0.56	0.53	0.53
ΔE_{Ω}	0.37	0.60	0.73
η	0.66	0.74	0.40
•			
	Mn1(IV)-Fe2(IV)	Fe1(IV)-Mn2(IV)	Exp.
δ	Mn1(rv)-Fe2(rv)	Fe1(IV)-Mn2(IV)	Exp. ⁶ 0.17
$\delta \Delta E_{\Omega}$	Mn1(rv)-Fe2(rv) 0.24 0.47	Fe1(IV)-Mn2(IV) 0.23 0.76	Exp. ⁶ 0.17 0.75

^{*a*} Calculated Mössbauer properties are taken from ref. 16. ^{*b*} Taken from ref. 41; the experimental quadrupole splitting $\Delta E_{\rm Q}$ was determined at T = 190 K, large line width $\Gamma \sim 0.5$ mm s⁻¹. ^{*c*} Taken from ref. 43, experimental isomer shift δ and quadrupole splitting $\Delta E_{\rm Q}$ were not precise because the magnetic spectrum persists up to T = 120 K.

Considering the Mössbauer calculations, for the isomer shifts, the errors for structures **3** and **5** relative to experiment are 3.8%; for structures **4** and **6**, 5.9% and 9.6%, respectively. For the calculated quadrupole splittings, we mainly focus on the absolute values when comparing with experiment, since our previous studies show that if η is close to **1**, the sign of the calculated quadrupole splitting can change with the chosen atomic basis sets, computational methods, and the size of the quantum cluster.⁶⁶ The computed quadrupole-splitting values of 1.29, 1.38, 1.23, and 1.63 mm s⁻¹ for structures **5**, **4**, **3**, and **6** give rise to an error of 1.5%, 4.5%, 6.8%, and 23.5% compared to experiment, respectively.

Comparing structures with the same metal site specificity, the most significant change in structure **5** compared to model **3** occurs for the distance of the Fe center to the oxygen atom of the μ -hydroxo group, which is shortened by 0.13 Å, as well as the bond length of Fe to an oxygen atom of the Glu120 carboxy-late group, which is elongated by 0.20 Å. Structure **6** contains a bond between the Fe center and the oxygen of the μ -hydroxo bridge which is elongated by 0.13 Å compared to structure **4**. Another remarkable modification is the distance between the Mn center and the nitrogen atom of His230, which is 0.20 Å longer in structure **6** than in structure **4**.

The major structural difference between structures 3 and 5 vs. 4 and 6 is the position of the metal atoms in the cofactor. In models 3 and 5, Fe(m) occupies the metal site 2 that is

further away from Phe127, while in model structures 4 and 6 Fe(III) occupies the metal site 1 that is closer to Phe127 (see Fig. 1). Considering the EXAFS data, model 3 cannot be favored over model 4. Based on the simulated Mössbauer data alone, Han et al. concluded that model 4 is a better structure than model 3 because its Fe1(III)-Mn2(III) and higher oxidation state Fe1(IV)-Mn2(IV) models' calculated ⁵⁷Fe quadrupole splitting values (Table 4) agreed better with experiment.¹⁶ The DFT calculations performed by Younker et al. showed that placing Fe(III) in site 1 gave better isomer shifts for the mixed valence Fe(III)-Mn(IV) state.¹¹ Nevertheless, the computed isomer shift of structure 3 is somewhat closer to experiment than that of structure 4. Thus, considering the EXAFS, Mössbauer isomer shift, and quadrupole splitting results, it cannot be decided clearly if structure 3 or 4 is in better agreement with experiment, and no statement about the exact position of the metal atoms can be made based on these results. This issue is also not resolved if spin-projected energies are taken into account since they solely differ by about 0.9 kcal mol⁻¹ for structures 3 and 4 with structure 3 lower in energy.¹⁶ The situation is different for models 5 and 6. Although the EXAFS fitting results of course favor structures 5 and 6 compared to models 3 and 4, they do not clearly favor either model 5 or 6 based on the R-factor alone. We note, however that, the Debye-Waller factors are mainly smaller for model 5 than for model 6 or any of the other models 1-4. Since Debye-Waller factors are treated as free fitting parameters, and the experimental sample temperature is low (near that of liquid N_2), the fit with the smaller Debye-Waller factors, model 5 is preferred. Also the computed Mössbauer properties (δ , $\Delta E_{\rm O}$, η) of model 5 deviate less from the experiment values than those of structure This leads to the conclusion that model 5 is the one in best agreement with the experimental EXAFS and Mössbauer data used in this study. The spin-projected energy of model 5 is found to be 1.7 kcal mol⁻¹ higher than the corresponding energy of model 3 but it is 2.7 kcal mol^{-1} lower than that of model 6.

In earlier work,¹⁶ we proposed that a model where Fe is nearer Phe127, and Mn is farther from Phe127 was likely based on calculated mono-Mn(II) site binding energies, and on comparisons of calculated *versus* experimental Mössbauer parameters for two oxidation states Mn(III)–Fe(III) and Mn(IV)– Fe(IV) (see Table 4). In particular, the predicted quadrupole splittings ΔE_Q are in better agreement with experiment for the Fe1–Mn2 site arrangement than for the opposite Mn1–Fe2 ordering. However, for experimental reasons, the accuracy of Mössbauer parameter determination has significant limitations for these two oxidation states (see Table 4, footnotes b and c for more details) in comparison with the mixed valence Mn(w)-Fe(m) state. The latter has a much sharper line width $\Gamma = 0.30$ mm s⁻¹, and is well resolved at low temperature T = 4.2 K.

Turning to the question of metal ion binding to form an Fe–Mn heterodinuclear center, a close reading of Younker *et al.*¹¹ and the very recent paper by Dassama *et al.*⁵⁰ shows that our earlier calculations and analysis used an overly simple model. One standard protocol (used to prevent formation of Fe(II)–Fe(II) and Fe(III)–Fe(III) after reaction with O_2) is to start with an excess of Mn(II) in the solution in air and then to add Fe(II) slowly. Like Fe(II)–Fe(II), Mn(II)–Fe(II) reacts with O_2 , while Mn(II)–Mn(II) is labile and does not react with O_2 . It is clear that the Mn–Fe complex formation is complicated and involves kinetic as well as thermodynamic issues. The rationale for the site specificity of the Mn(II)–Fe(II) complex and for the higher oxidation states leading to Mn(IV)–Fe(III) cannot be solved by considering the mono-Mn(II) site binding affinity in isolation.

Conclusions

Based on comparison of experimental data with simulated Mn and Fe EXAFS spectra as well as calculated Mössbauer properties, we find that structure 5 is the model out of the six models considered in this paper that agrees best with experimental data. The EXAFS simulations of models 5 and 6 show that these models are, in contrast to the ones of structures 3 and 4, consistent with the experimental EXAFS data. A significant difference is observed in case of the computed Mössbauer properties where model 5 agrees better with experiment than model 6. In addition, structure 5 is found to be lower in energy than model 6.

Structure 5, like structure 6, has been derived by fitting of interatomic distances to experimental EXAFS data. The calculated Mössbauer data further support this structure as a good model for the Ct-R2 Mn(iv)–Fe(in) active site.

The metal site binding affinity calculations on Ct-R2 for $Mn(\pi)$ -binding¹⁶ and on *E. coli*-R2 for Fe(π)-binding⁶⁷ show that site 2 in both proteins has higher metal-binding affinity. A single-Fe containing mouse R2 structure was crystallized at pH 4.7 (PDB code: 1XSM), and Fe was also found bound at site 2.68 Current experimental X-ray49,50 and EXAFS data11 and our DFT calculated energies⁶⁷ versus structure leads to the proposal that, for RNR-R2 structures the metal site 2 has higher metal-binding affinity. In Ct-R2, site 2 has higher Fe(II)binding affinity than Mn(II)-binding based on X-ray anomalous diffraction data.^{49,50} Even if site 2 is occupied by Mn(II) when Mn(π) is added to apo-R2 prior to the addition of Fe(π),⁵⁰ the Mn(II) on site 2 will be replaced by Fe(II) when a smaller amount of Fe(n) is subsequently added. If more Fe(n) is added, the Mn(II) on site 1 will also be replaced by Fe(II)—forming the inactive diiron form of Ct-R2. The relative $Mn(\pi)$ - and $Fe(\pi)$ -

binding affinities for sites 1 and 2 in Ct-R2 and O₂ reaction merit further work. Our current EXAFS calculations support the recent experimental observations that Fe mainly occupies site 2 and Mn site 1 in the Ct-R2 Mn/Fe active site. Further, the combined DFT-EXAFS simulation refinement methodology we have developed should prove valuable for analyzing structures and metal site specificity in metalloenzymes.

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