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Insights into the different dioxygen activation pathways of methane and toluene monooxygenase hydroxylases

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Abstract

The methane and toluene monooxygenase hydroxylases (MMOH and TMOH, respectively) have almost identical active sites yet the physical and chemical properties of their oxygenated intermediates, designated P^{*}, H_{peroxo}, Q and Q^{*} in MMOH and ToMOH_{peroxo} in ToMOH, are substantially different. We review and compare the structural differences in the vicinity of the active sites of these enzymes and discuss which changes could give rise to the different behavior of H_{peroxo} and Q. In particular, analysis of multiple crystal structures reveals that T213 in MMOH and the analogous T201 in TMOH, located in the immediate vicinity of the active site, have different rotatory configurations. We study the rotation energy profiles of these threonine residues with the use of molecular mechanics (MM) and quantum mechanics/molecular mechanics (QM/MM) computational methods and put forward a hypothesis according to which T201 and T213 play an important role in the formation of different types of peroxodiiron(III) species in MMOH and ToMOH. The hypothesis is indirectly supported by QM/MM calculations of the peroxodiiron(III) models of ToMOH and the theoretically computed Mössbauer spectra. It also helps explain the formation of two distinct peroxodiiron(III) species in the T201S mutant of ToMOH. Additionally, a role for the ToMOD regulatory protein, which is essential for intermediate formation and the protein functioning in the ToMO system, is advanced. We find that the low quadrupole splitting parameter in the Mössbauer spectrum observed for a ToMOH_{peroxo} intermediate can be explained by protonation of the peroxo moiety, possibly stabilized by the T201 residue.

Introduction

Soluble methane monooxygenase and toluene o-xylene monooxygenase belong to the family of bacterial multicomponent monooxygenases (BMMs) that specialize in the transformation of C–H chemical bonds into the C–OH groups for a variety of hydrocarbons.^{1–4} Methane monooxygenase hydroxylase (MMOH) has been extensively studied,^{5–12} and the toluene monooxygenase hydroxylase (TMOH) has also generated considerable interest^{13–16} after its three-dimensional structures became available.^{15,17–21} Soluble MMOH enzymes are found in two phylogenetically related proteins, MMOH-Mc and MMOH-Mt, purified from two related organisms, *Methylococcus capsulatus* (Bath) and *Methylococcus trichosporium* OB3b, respectively. A toluene monooxygenase hydroxylase enzyme TMOH similarly occurs in a variety of organisms, two of which will be discussed in this work: toluene/o-xylene monooxygenase hydroxylase (ToMOH) and toluene 4-monooxygenase hydroxylase (T4moH), expressed in *Pseudomonas stutzeri* OX1 and *Pseudomonas mendocina* KR1,

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respectively. These particular variants of MMOH and TMOH have been characterized crystallographically and are most accessible for theoretical analysis. The active sites in each pair are almost identical.

Despite having very similar active sites, the MMOH and TMOH enzymes differ in chemical behavior, something that has puzzled researchers. Not only do they have reactivity specific for distinct types of hydrocarbons, but the properties and structures of their oxygenated intermediates also differ.^{4, 13, 14} Some light has been shed on the problem after the identification of an extended channel connecting the surface of ToMOH with its active site, a channel that is absent in MMOH.¹⁸ Another advance was the preparation and spectroscopic study of ToMOH mutant forms in which the residue threonine-201, located near the active site, was substituted by other amino acids, serine in particular.¹⁴ Important implications for the kinetics of the reaction and for the catalytic cycle emerged as a result of the substitution.

In the present study we reprise the structural differences between MMOH and TMOH active sites and investigate the energetic consequences of rotation about the threonine $C_{\alpha}-C_{\beta}$ bond (T213 in MMOH and T201 in TMOH) by molecular mechanics (MM) and quantum mechanics/molecular mechanics (QM/MM) computational methods. Studying threonine-201 in TMOH and its analog in MMOH is important because understanding their respective abilities to influence the chemistry of the diiron sites may be key to the explanation of differences in the intermediates formed by the two proteins. There are several reasons to ascribe a principal role to this threonine residue: (i) it is strictly conserved in the BMM family and located at the active site; (ii) its mutations have a measurable effect on the catalytic mechanism; (iii) its rotational configurations are markedly different in the available crystal structures of MMOH and TMOH forms; (iv) it is involved in an extended hydrogen bonding network that stretches from the active site to the surface of the protein and may provide a mechanism for proton delivery during the catalytic cycle; and (v) as we demonstrate here, the threonine potential energy profiles correlate strongly with experimentally observed trends in the formation of the hydroxylase intermediates. Based on our theoretical analysis, we formulate a hypothesis that can explain the differences in the behavior of the intermediates and provide further insight into the role that the threonine plays in tuning the chemistry of the BMM enzyme active sites.

Computation details

Three computational methods were used in this project to compute energies: molecular mechanics (MM), quantum mechanics/molecular mechanics (QM/MM), and Monte-Carlo (MC).

The MM method was applied to generate rotational profiles of serine and threonine in several protein structures. Except when the QM/MM-optimized geometries were available (for the ToMOH protein only), all the crystal structures were obtained from the Protein Data Bank and subsequently treated by the ICDA method²⁶ to assign the positions of the hydrogen atoms and resolve certain misassignments that could be present in the crystal structure. The correct charges of atoms and protonation states of the ligands around the active site were specified manually. As an energy function the OPLS-AA⁴⁶ molecular mechanics force field was used.

The QM/MM method realized in the program QSite²⁷ and based upon the frozen orbital approach²⁸⁻³⁰ for the quantum/classical interface was used to compute high-quality energies of the oxygenated intermediates as well as to minimize their structures. The QM part was treated with the B3LYP DFT functional (unrestricted orbitals) while the MM part was handled with the OPLS-AA force field. For the geometry optimizations we utilized a

smaller basis set (LACVP**^{31–33} on iron atoms, and 6–31G* on all other atoms in the QM region) than that with which the single point energy was subsequently computed (LACV3P** on iron atoms, aug-cc-pVTZ without the f-functions on the active site waters and the dioxygen, and cc-pVTZ without the f-functions on all remaining QM atoms). All the QM/MM energies given in this work correspond to the single point energies obtained with the larger basis set.

In Monte Carlo sampling of model structures we used the combination of MC torsion sampling (MCMM) and large-scale low-mode sampling (*ALMOD*).^{34,35} During exploration of the configurational space of the models the distance between two Fe atoms and the O-O distance in the dioxygen-derived ligand were constrained.

The DFT optimization of protonated model structures utilized the B3LYP functional, Wachters basis set on Fe atoms and cc-pVDZ basis set on all the other atoms.

In computing the Mössbauer parameters of the model structures we followed the methodology of isomer shift parametrization and functional/basis set selection from our recent work.⁴⁰ The B3LYP functional was used to compute the isomer shift whereas O3LYP was the functional of choice for the quadrupole splitting parameter. The completely uncontracted Partridge-1 basis set was placed on the iron atoms, and the conventional cc-pVDZ basis resided on the rest of atoms of the model. Our Mössbauer benchmarking study⁴⁰ indicates that these are currently the best functional/basis set combinations for the prediction of the isomer shift and quadrupole splitting parameters. The molecular model systems for Mössbauer calculations were produced from the corresponding active sites by truncation and hydrogen-capping.

Comparison of MMOH and TMOH active sites

To date, almost thirty MMOH and over ten TMOH crystal structures have been deposited in the Protein Data Bank (PDB). The active sites of all the crystallized MMOH and TMOH forms share a considerable degree of similarity. The identical elements of the immediate coordination environment (the so-called first shell), regardless of the oxidation state of the iron atoms, comprise two histidine and four glutamine side chains, with the histidine residues in MMOH and TMOH using different nitrogen atoms to coordinate to the iron atoms.¹⁸ The amino acid residues are fixed elements of the structures whereas the water molecules, hydroxide ions, and molecules from the crystallization solution that are located in immediate proximity to the iron atoms are variable elements. Their presence fluctuates from structure to structure and depends on the oxidation state of the iron atoms, crystallization conditions, and complex formation with the regulatory protein. The positions of all groups coordinated to iron, including side chains, are subject to translational or rotational modifications. Many amino acids surrounding the coordinating ligands (the second shell) are conserved between MMOH and TMOH, which makes the architecture of their active sites nearly identical.

In any study of the differences in chemistry between MMOH and TMOH intermediates, it is the fixed elements of their active site structures (amino acids) that we have to focus on. As a starting point, it is important to investigate deviations within the MMOH and TMOH structure groups, in order to understand whether we can use one structure as representative of the entire group. MMOH-Mc and MMOH-Mt show almost no variation in the surroundings of the diiron center. Out of the 58 residues, located in the active site-forming helices, MMOH-Mc and MMOH-Mt differ only by 3 (see Fig. 1). Only one of these three differences (V ↔ I) is close to the diiron center; however, it involves an inessential residue. A more detailed overall comparison of the two MMOH proteins has been undertaken earlier.^{24,25} The mutations differentiating ToMOH from T4moH are more numerous and include 9

out of the analogous 58 residues, but all of them are located relatively far from the diiron center and most of them are inessential for our study (see Fig. 1). The number of mutations around the active site agrees with the phylogenetic placement of these proteins²³ based on the entire α -subunit: MMOH-Mc is closer to MMOH-Mt than ToMOH is to T4moH. Notwithstanding these differences, we may conclude that, as far as the chemistry of the active site is concerned, any of the two members of each group may be considered as representative of the group as a whole.

Next, we turn to the differences between the MMOH and TMOH active site environments. Fig. 1 reveals a great number of differences: 35 residues out of the 58 do not match when MMOH-Mc and ToMOH sequences are aligned. It is clear that the majority of the changes occur in residues relatively distant from the active site. All residues coordinated to the iron atoms are conserved, but there are several key changes in the 'second', and the 'third' shells. Ones of particular interest, being closest to the active site, are listed in Table 1. Residues not directly coordinated to the diiron center, but with side chains that are not obstructed from the diiron center by other residues, are considered second shell. This shell may play a direct role by interacting with the variable elements of the active site, such as the dioxygen or water molecules. Residues having some other amino acid chains between them and the diiron center are considered third shell. This shell is considered to be of relatively low importance, although in the catalytic cycle it could still play a role in stabilizing second shell conformations.

From Table 1, we can identify important differences in the active sites of MMOH and TMOH. Substitutions 1, 4, 7, and 8 are not very interesting. The first is not even conserved across both variants of the TMOH family. The side chains of mutations 4 and 7 are relatively distant (about 7 Å) from the diiron center. Substitution 8 deserves somewhat more attention because A235 of the TMOH family is located in the second shell. However, its importance is not clear. The other four mutations are more significant. Substitution 2 is not conserved in both variants of the MMOH family but the residues associated with this substitution in MMOH-Mc and MMOH-Mt have a functionally similar side chain: threonine and serine, respectively, so that we may consider it to be effectively conserved. Alteration T121 (MMOH) $\rightarrow$ E111 (TMOH) is unusual. The side chain of E111 is apparently hydrogen-bonded to a water molecule, which is in turn hydrogen bonded to the critical residue E134 serving as a ligand for the diiron center (see Fig. 2). T121 of MMOH does not form hydrogen bonds with G144, and therefore substitution T121 (MMOH) must play a role different from $\rightarrow$ E111 (TMOH). Interestingly, substitution T121 (MMOH) $\rightarrow$ E111 (TMOH) is complimentary to substitution number 3 in Table 1: Q140 (MMOH) $\rightarrow$ G130 (TMOH). The backbone of Q140 (MMOH) is aligned with the backbone of G130 (TMOH). But G130 (TMOH) has only a hydrogen atom for a side chain and cannot carry out the apparently important function similar to that of Q140 in MMOH. This function in TMOH is accomplished by the side chain of E111, even though its backbone is not aligned with that of MMOH Q140. Backbones of MMOH Q140 and TMOH E111 are not aligned but their side chains have the same function in the stabilization of the active site. The role and significance of this hydrogen bond are currently not understood. Substitution 6 changes the aliphatic hydrophobic group of isoleucine into the aromatic hydrophobic group of phenylalanine in the second shell of both protein subfamilies. Whether the presence of the phenyl ring in the second shell of TMOH might lead to a π - π interaction with the aromatic substrate is unknown but should not be discounted. The analogous residue in aromatic-oxidizing phenol monooxygenase is also phenylalanine.²²

It is unlikely that any single substitution of those just discussed will explain the profoundly dissimilar scenarios by which dioxygen is activated in MMOH and TMOH. It is more probable that the effect of these substitutions is cumulative and that they all contribute to the

observed differences. Moreover, studying the changes provides just one window on understanding differences in the MMOH and TMOH catalytic cycles. At least two additional resources remain. The first is the effect of the regulatory protein on the secondary structure of helix E and the presence of an additional ordered water molecule in the vicinity of the diiron core. The ability to observe catalytic intermediates in the MMOH and ToMOH reaction cycles requires the presence of the respective regulatory proteins. The second resource is the rotatory conformation of the conserved threonine T213 in MMOH and T201 in TMOH, *vide infra*.

We now briefly discuss the effects of the regulatory protein binding on structure before moving to the rotameric forms of the threonine, which comprise the key subject of this work. Out of the four proteins that we compared only one in complex with its regulatory protein, T4moH,²¹ has been crystallized (hereafter referred to as T4moHD. There are two noticeable modifications from the structure of the hydroxylase in the absence of the regulatory protein. The first is a distortion of E helix, which affects the secondary structure of two residues, T201 and N202, involved in a hydrogen bond network leading from the surface of the protein to the active site and thought to be important for the proton shuttling.²² As a result of this distortion, T201 and N202 no longer reside in the α -region of the E helix but belong to a transitional structure between α and π helices. As a further consequence, T201 recedes from the diiron center: the distance from its backbone oxygen atom to Fe₂ increasing from 5.36 Å to 6.26 Å. The regulatory protein thereby creates some extra space between the diiron core and residues T201, Q228, and F205 located just beside it. The other modification is the presence of a new water molecule apparently hydrogen-bonded to the water molecule coordinated with Fe₁. It is located in the extra space just mentioned. The presence of this additional water molecule may have important implications for the hydrogen bond network extending to the surface of the protein and for the role it may play in the formation of a peroxo species; the latter point is discussed below. These changes brought about by the regulatory protein in T4moH are noteworthy, but whether they are mirrored in ToMOH and, most importantly, the MMOH proteins, is currently unknown. When the corresponding MMOH/B crystal structure becomes available for one of the MMOH proteins it will be clear whether the regulatory protein has an identical structural role, or, perhaps, induces a distinct set of changes that might contribute to factors that distinguish the chemical properties of the MMOH and TMOH intermediates.

Threonine conformations in MMOH and ToMOH crystals

In this section we describe the rotatory conformations of the threonine residues observed in MMOH and TMOH crystal structures and discuss the consequences that these conformations might have for stabilizing the catalytic intermediates. The next section will focus on the rotational energy profiles.

In order to quantify the rotational configurations we refer to the dihedral angle Fe1–Fe2–C _{β} –O illustrated in Figs. 3 and 4, hereafter 2Fe-THR. Positive values of the dihedral angle represent the situation when the threonine hydroxyl group turns towards the diiron center, and negative values correspond to the opposite orientation. Because the C _{α} backbone carbon atom to which the threonine hydroxymethyl group is attached may shift slightly from structure to structure, the value of the dihedral angle does not necessarily define the distance of the oxygen atom to the diiron center, which would correlate with the possibility of threonine participation in dioxygen activation. We therefore require additional geometrical information to describe this structural element. Tables 2 and 3 contain this additional information and include the rotatory geometry of threonine with respect to the diiron center. These tables clearly reveal that positive 2Fe-THR dihedral angles signal a significantly shorter (about 1 Å) distance from the threonine hydroxyl group to the diiron core. An

interesting caveat is the set of distances for T4moHD. For this structure, a positive dihedral angle corresponds to $d_{\text{Fe1-O}}$ and $d_{\text{Fe2-O}}$ distances that are about the same as those in other proteins where the dihedral angle is negative. This result occurs because the regulatory protein distorts the secondary structure of helix E and drives T201 away from the diiron site by about 1 Å. Presumably, the structures of MMOH and/or ToMOH in complex with their respective regulatory proteins would have similarly negative dihedral angles, with distances that are larger than those corresponding to the positive angle.

Another observation that follows from the tables is the striking difference in dihedral angles for the MMOH and TMOH structures. If we disregard soaked structures, which are likely to have distorted and unnatural geometries, we can conclude that in MMOH the threonine hydroxyl is rotated in a direction away from the diiron center whereas in ToMOH it always faces the iron atoms. As discussed below, we believe that the difference has far-reaching implications for the stability of the peroxo intermediates with respect to conversion to the hydroxylating intermediate Q in MMOH. The distance between the threonine hydroxyl oxygen atom and the diiron core, even in the case of a positive dihedral angle, is too large to allow a direct interaction between the two. But when we consider that a peroxo ion O_2^{2-} can be positioned between the threonine and the iron atoms, the distance appears just right. Our quantum mechanics/molecular mechanics (QM/MM) optimizations on ToMOH predict that a strong hydrogen bond will form between the threonine OH group and the coordinated peroxo ion. Such an interaction helps make the peroxo structure 4.72 kcal/mole more stable than Q-type intermediate, a putative di(μ -oxo)diiron(IV) unit in which the O atoms are too far away from the threonine to form a hydrogen bond.

Fig. 5 shows the active site geometries of the two lowest energy peroxo intermediates and a Q-type intermediate predicted by the QM/MM optimization starting from the ToMOH 2INC crystal structure. The relative energies of these structures are also shown. One other peroxo intermediate found in the course of optimization did not form a hydrogen bond with T201 and was several kcal/mole higher in energy than the Q structure. We therefore ascribe to this hydrogen bond the stabilization effect that helps make the peroxo more favorable than Q. In contrast, our previous QM/MM study of MMOH predicted almost identical energies for the peroxo and Q structures.¹⁰ The peroxo structure in that study had no possibility to form a hydrogen bond with T213, which was pointed away from the active site, and therefore could not be stabilized by such an interaction. The present findings agree well with experimental observations. In MMOH, the peroxo intermediate transforms spontaneously into Q, which serves as the principal substrate oxidizing agent.⁶ In ToMOH, intermediate Q is not observed, and oxidation is performed by a peroxo species.¹³ Our conclusion is further supported by the fact that the peroxo intermediates of MMOH and ToMOH have very different quadrupole splittings in their Mössbauer spectra, 1.51 mm/s in both MMOH-Mc and MMOH-Mt and 0.67 mm/s in ToMOH. Such different quadrupole splittings indicate a different geometry of the active site or a different protonation state. The observation that the threonine may stabilize the peroxo intermediate in TMOH but not in MMOH is thus in accord with the thermodynamics and compatible with the Mössbauer measurements.

During the course of our QM/MM studies of the ToMOH protein using the 2INC crystal structure as the starting geometry, the crystal structure of T4moH in complex with its regulatory protein (3DHH) became available. The latter structure is valuable for theoretical modeling of the TMOH catalytic cycle because the regulatory protein is essential for the efficiency of the oxidation, and it is also complexed to either T4moH or ToMOH in most experiments. As we have already mentioned, and as evident from the data in Table 3, the regulatory protein increases the distance between the threonine side chain and the diiron center by about 1 Å. This change makes it much more difficult for a strong hydrogen bond $\text{OH}\cdots\text{O-O}$, such as that observed in the peroxo structures based on the 2INC geometry, to

occur. However, formation of the complex between T4moH and its regulatory protein brings an additional water molecule to the active site cavity that is situated between the diiron center and the threonine hydroxyl. It is conceivable, therefore, that stabilization of the peroxo intermediate in the presence of the regulatory protein occurs through this water filling in the extra 1 Å of space between the threonine OH group and the peroxo ion. Currently we are investigating this hypothesis by studying the intermediates by the QM/MM method starting from the T4moHD crystal structure. In addition, the peroxo ion may be protonated,^{14,36} which permits other patterns for the hydrogen bond network between the peroxo ion and the threonine hydroxyl group, as is discussed next in section V.

The threonine rotational energy profiles

The mutation of ToMOH T201 into a very similar residue, serine, leads to significant changes in the oxidation chemistry of the reduced hydroxylase.¹⁴ Judging from the Mössbauer and optical spectra, when in complex with the regulatory protein (ToMOD), the mutant produces two types of peroxo structures upon reaction with O₂: the ToMOH_{peroxo} and T201S_{peroxo} species. The ratio of the concentrations of the two types of the diiron intermediates is approximately 1:1.¹⁷

The hypothesis set forth in the previous section proposes that an MMOH-like peroxo, T201S_{peroxo}, can be formed when the threonine points its hydroxyl group away from the diiron center. According to this hypothesis, the Thr→Ser mutation must facilitate the rotation around the C_α-C_β bond. An evaluation of this suggestion and possibly to provide a verification of our hypothesis, we carried out a straightforward computational study in which a series of single-point energies were generated for different dihedral angles 2Fe-THR and in which all other atoms not participating in the rotation were frozen. We also considered whether the regulatory protein would have any effect on the barrier to rotation, because in T4moHD it notably affects the position of the threonine compared to wild-type T4moH.

Threonine rotation was studied by two computational methods: molecular mechanics (MM) and QM/MM. The first is more suitable for faster but less accurate work, whereas the second is good for more accurate, quantitative analysis albeit computationally much more expensive. We applied the MM method to probe the problem and formulate initial conclusions, and then repeated the most interesting rotational profiles by using the QM/MM method, to ascertain the quality of the MM results.

Fig. 6 superimposes threonine or serine rotational profiles for the oxidized states of six different proteins: MMOH-Mc, ToMOH, ToMOH-T201S, T4moH, T4moH-T201S, and T4moHD-T201S. The energy is plotted as a function of the dihedral angle 2Fe-THR rather than the χ (N-C-C-O) angle around which the rotation was actually performed. The functional transformation of χ into Fe1-Fe2-C_β-O is important because the value of χ does not necessarily relate to the distance of the threonine hydroxyl with respect to the diiron center, and this position is key in our discussion. Moreover, the backbone deformation effected by the regulatory protein in T4moHD makes the same values of the χ angle (in, say T4moH vs. T4moHD) correspond to substantially different positions of the hydroxyl with respect to the diiron core, so that comparing χ angles in different proteins for the sake of evaluating our idea about the threonine rotation becomes problematic. Note, for example, different regions to which the experimental χ angles belong in the non-deformed TMOH and TMOHD crystal structures.

As indicated in the diagram of Fig. 4, positive values of the 2Fe-THR angle in Fig. 6 reflect the situation when the OH group is directed toward the iron atoms whereas negative angles are for the OH group pointing away from the iron atoms. In MMOH and ToMOH the

rotation energy minima are located approximately at 139.2 and -102.9 corresponding to χ angles of -40 and 85.0. The change of the minima compared to the 2Fe-THR angles observed in the corresponding crystal structures is most likely due to the crystal structure relaxation during the theoretical modeling plus the inherent limited accuracy of the MM method, although the latter is expected to have a small contribution to the error. The change in shift is less pronounced in terms of the χ angle, which suggests that the relaxation affects the diiron core more than it does the side chain of the threonine.

Fig. 6 clearly demonstrates several important points about the structural properties of the threonine in MMOH and TMOH proteins. First, it confirms the pattern observed earlier for the crystal structures of the wild-type MMOH and TMOH: the hydroxyl group in MMOH strongly prefers negative angles whereas in TMOH it assumes a different configuration, with positive angles. Even though ToMOH (green curve) has high-energy rotational minima corresponding to the negative angle, the very high barriers separating negative angle from positive angle minimums do not leave the C_{α} - C_{β} bond much kinetic possibility to rotate. The rotation in the T4moH (magenta curve) is much less restricted. One of the barriers gets significantly smaller and the energy of the MMOH-type Thr rotational configuration is higher than the absolute minimum by over 6 kcal/mole (compare with roughly 38 kcal/mole in the case of ToMOH). Nevertheless, the TMOH-type configuration should be highly predominant even in T4moH.

Second, the association of the regulatory protein with T4moH changes the rotational energy landscape dramatically (compare the magenta and grey curves). The regulatory protein totally prohibits rotation of the hydroxyl by imposing an enormously high barrier, which essentially freezes the rotation to adopt the configuration where the OH group faces the active site. Possibly, in this manner it imposes a certain specificity for the formation of only one type of peroxo intermediate, compared with the case of the ToMOHD-T201S mutant discussed below. Because the crystal structure of ToMOHD is unavailable, we were not able to check what effect the regulatory protein has on ToMOH, but we expect it to be very similar to the effect observed in T4moHD.

Third, the mutation of the threonine into serine in the TMOH proteins produces another dramatic change in the rotational energy curves. While preserving the absolute minima at the positive angles, the energies of the local minima at the negative angles are drastically reduced. Additionally, the barriers to rotation fall sharply in magnitude so that rotation around the C_{α} - C_{β} bond and the population of both configurations (MMOH-like and TMOH-like) becomes possible. The effect is most pronounced when the regulatory protein associates with T4moH (black curve). The MMOH-configuration in this case is separated from TMOH by only 9.9 kcal/mole of a barrier and lies higher only by 0.2 kcal/mole. This energy profile qualitatively agrees with the experimental observation that ToMOHD-T201S mutant forms two peroxoforms in comparable proportion. The energy difference of 0.2 kcal/mole is close to the ratio of 1:1 that would be predicted using the $\exp(-\Delta H/kT)$ formula if we assumed that $\Delta E \approx \Delta H$ and took the temperature 277 K at which the experiment was performed. However, beside the $\Delta E \approx \Delta H$ approximation, we have to remember that the two forms with comparable concentrations were observed on ToMOHD-T201S, and not T4moHD-T201S, the energy profile of which we are currently discussing. Additionally, we do not currently know the structure of peroxo TMOH, and so it would be difficult to model its Thr rotation profile. Our preliminary calculations based on the ToMOH (a) peroxo model from Fig. 5 indicate that the presence of O_2^{2-} in the active site and even the formation of the hydrogen bond between O_2^{2-} and T201 does not significantly alter the general shape of the ToMOH (Ox) curve. These several approximations allow us to conclude that the energy separation of 0.2 kcal/mole obtained from the T4moHD-T201S curve and the ratio of the

comparable peroxo populations in the experiment are in an excellent qualitative agreement. Overall, the rotational energy profiles in Fig. 6 reveal the major influence of the regulatory protein and also explain how the threonine mutation into serine can lead to a formation of both peroxo structure types.

Fig. 7 compares several MM rotational energy curves with QM/MM curves for the same structures. The QM/MM method, based on the B3LYP DFT functional, should be considered significantly more accurate than MM. We should not, however, forget that B3LYP itself is not without defects, especially when metal atoms are present, and so its predictions must also be viewed with caution. Because the QM/MM method is much more computationally expensive than MM, it would be wasteful to apply QM/MM if the accuracy of MM is sufficient. Therefore, the goal of the comparison in Fig. 7 is to ascertain whether the MM energy profiles can be trusted without the recourse to the QM/MM method. From Fig. 7 we note that MM and QM/MM agree almost quantitatively in the regions of the 2Fe-THR from -180 to -140 and from 100 to 180 of the 2Fe-THR angle. The agreement in the region from -140 to -100 is only qualitative, with the differences in energy between QM/MM and QM in some cases over 30 kcal/mole but the shapes of the corresponding QM/MM and MM curves are similar throughout. This region of qualitative agreement contains the MMOH-like minima and is thus a potentially important span of angles. These or other QM/MM curves that we computed (not shown) did not alter our qualitative conclusions regarding the role of the regulatory protein and the effect of the mutation but should more quantitative calculations be made in the future (especially with the goal of computing ΔG , and not only ΔE), the QM/MM method should be chosen.

Mössbauer spectra and proposed structures of the peroxo intermediates

The remaining issue of interest is the nature of the geometrical structures of the peroxo intermediates for MMOH and TMOH proteins. In the previous section we showed that the appearance of one or another peroxo intermediate, or both, as in the case of the T201S mutant, in the experiment correlates well with the position of the energetic minima on the threonine rotation energy profile. In an earlier section, we showed that the QM/MM optimization predicted two $\text{ToMOH}_{\text{peroxo}}$ structures stabilized by a hydrogen bond formed between T201 and the peroxo ion, a geometry that would be impossible for MMOH. There has been a disagreement in the past over the structure of MMOH, in particular, whether it has the η^2 , η^2 or $\text{cis-}\mu\text{-1,2}$ active site geometry.^{10,37–39} In any case, neither of these geometries can form a hydrogen bond with T213 because the distance is too long and therefore must be different from the types of structures we show in Fig. 5.

The geometrical differences between our $\text{ToMOH}_{\text{peroxo}}$ structures and their lower energy with respect to Q are precisely the features that we would expect from the species occurring in nature. However, the peroxo structures shown in Fig. 5 cannot be *exactly* correct for at least two reasons. Firstly, they were optimized starting from a ToMOH structure not associated with To-MOD. The crystal structure of T4moHD, an excellent model for ToMOHD, revealed that there is additional space and an extra water molecule separating T201 and the diiron center, by comparison with T4moH, so that the hydrogen bond formation *directly* between T201 and the peroxo ion coordinated to the iron atoms would be problematic. Secondly, the Mössbauer characteristics that we computed for these structures deviate widely from the experimentally observed values (see Table 4). At the same time, the theoretically predicted Mössbauer spectra of MMOH peroxo candidates agree well with the experiment.^{37,40} Nevertheless, we still present and discuss these peroxo structures because for the first time they illustrate the kinds of structural elements that might explain the different chemical behavior of the MMOH and TMOH intermediates and serve as a first approximation for forthcoming modeling. Importantly, through certain modifications in the

geometries it is possible to eliminate both obstacles mentioned above and reconcile theory with experiment. The rest of this section explains how.

As the rotational energy profiles of T201 suggest, and as proposed in the literature^{14,36} based on experimental studies, T201 must participate in the stabilization of the TMOH peroxo intermediate. The water molecule that accompanies binding of the regulatory protein can serve as a hydrogen bonding bridge between the peroxo ion and the threonine side chain. Possible hydrogen bond networks between O_2^{2-} and T201 through the water molecule are shown in Fig. 8. Such networks may involve protonation, as, for example, models (a) and (b), and this proton can be carried by the extra water molecule as H_3O^+ . In Fig. 8 we placed the proton on the peroxide anion as intuitively the most basic species. However, in the proximity of two Fe(III) ions the basicity of the oxygen-containing species will change. Other modes of coordination of O_2^{2-} with the diiron center are possible. A detailed study of the relative stabilities of such structures will be the subject of a separate study. Here we are only concerned with proposing how peroxo structures such as those in Fig. 5 could be modified in the presence of the regulatory protein so that T201 can be involved in the stabilization of the peroxo structure despite the increased distance between the O_2^{2-} and the hydroxyl group.

The next task is to explain why the Mössbauer spectra of the MMOH- and TMOH-type peroxo species are different from one another. The MMOH peroxo intermediate displays only one signal with an isomer shift δ and quadrupole splitting ΔE_Q equal to 0.66 mm/s and 1.51 mm/s, respectively.⁶ The Mössbauer parameters of the TMOH-type peroxo are: $\delta = 0.55$ mm/s and $\Delta E = 0.67$ mm/s, also comprising only one signal.¹³ Although there is a noticeable difference in the isomer shift, it is the change in the quadrupole splitting parameter that is the most striking. The transition from $\Delta E = 1.51$ mm/s in MMOH to $\Delta E = 0.67$ mm/s in ToMOH may mean a significant alteration in the structure of the active site, most probably involving charged atoms or altered electron effects of the bound ligands, because such effects typically have a marked influence on the quadrupole splitting.^{42–44}

As we noted before, and as Table 4 reports, the computed Mössbauer characteristics of the per-oxo structures shown in Fig. 5 are totally incompatible with the experimental values. There may be several explanations for this discrepancy. Peroxo structures (a) and (b) may be artifacts of the TMOH active site because the TMOHD active site, involving an additional water molecule, might be essential for a proper modeling of peroxo intermediates and, consequently, reproduction of their Mössbauer spectra. However, this explanation appears improbable, because the substitution of the $O_2^{2-} \cdots HO$ -Thr hydrogen bond by the $O_2^{2-} \cdots H_2O \cdots HO$ -Thr hydrogen bond as a result of the complexation with the regulatory protein should not cause a significant change in the quadrupole splitting. Another, more plausible, explanation is that we did not sufficiently explore the configurational space in the course of the QM/MM optimization. Although we started from eight different peroxo models that converged to four distinct coordinations, many other geometries corresponding to local energy minima might exist which either lie far from the starting geometries or simply have not been found by the optimization algorithm. This problem is a common one of theoretical optimizations that involve several essential degrees of freedom and where the targeted geometry is not known for certain from experiment. Finally, the peroxo structure may be protonated, which may result in a significant change of the quadrupole splitting. From a number of studies^{41,45} it can be inferred that certain protonated peroxo diiron structures have much lower quadrupole splittings (below 1 mm/s) than their unprotonated analogs. This observation has a direct relevance to our predicament of matching the low quadrupole splitting of a peroxo species.

In order to address the two latter possibilities, we generated over sixty possible model peroxo diiron(III) structures within ToMOH active site with the help of Monte-Carlo sampling. We presumed that sampling the T4moHD active site at this stage of the investigation would be undesirable because it would involve several extra degrees of freedom due to the additional water molecule in the active site, significant presence of which would probably not contribute to significant changes in the quadrupole splitting parameters. Then, we computed the Mössbauer characteristics of all these structures and none of the quadrupole splittings was close to the experimental value of $\Delta E = 0.67$ mm/s for both iron atoms. We usually got quadrupole splittings having large values for both iron atoms, larger than 1–3 mm/s, or one iron with low value (less than 1 mm/s) while the other iron was characterized by a large value (over 2 mm/s), similarly to the situation observed for peroxo structures (a) and (b) in Fig. 5. It is only when we began to place protons on our model structures that we observed a significant drop in the quadrupole splittings of the resulted protonated species.

Fig. 9 presents a DFT-optimized protonated model structure of the TMOH active site along with the corresponding Mössbauer characteristics which are quite close to the experimental values. The isomer shift is 0.58, 0.59 mm/s (two signals) and the quadrupole splitting is 0.60, 0.29 mm/s. Even though this quadrupole splitting does not qualify as providing quantitative agreement with the experimental value (0.67 mm/s) it is qualitatively reasonable. The isomer shift is quite close (the corresponding experimental value is 0.54 mm/s), and both computed characteristics of the protonated structure are in sharp contrast with the computed Mössbauer characteristics of the unprotonated peroxo models from Fig. 5 (see Table 4), which clearly contradict the experiment. We stress that the structure shown in Fig. 9, with two bridging carboxylate groups and two water molecules, each coordinated to an iron atom, is only an approximate model obtained from a DFT minimization in the absence of the protein environment. We are not suggesting that it is necessarily representative of the true ToMOH peroxo structure, especially in the coordination mode of the dioxygen, carboxylates, and water molecules. A proper prediction of the ToMOH peroxo structure should involve a QM/MM modeling starting from the crystal structure with the regulatory protein attached. What the structure in Fig. 9 illustrates is that, according to DFT, there exist stationary points of the protonated models which, at the same time, have Mössbauer characteristics that are quite close to the experimental ones. A meticulous study of similar protonated structures (also involving double protonation), both structural and spectroscopic, should be performed in the future following QM/MM optimization. Since the protonation is the only way we found to reconcile the low experimental quadrupole splitting with the theoretical predictions, the generation and analysis of the protonated structures holds great promise for finding the right structure model for the ToMOH peroxo.

Recently, an X-ray structure of T4moH with H_2O_2 putatively bound to the active site was published¹⁵ (PDB 3I63). Its examination reveals a H_2O_2 molecule loosely bound with the apparently ferric ions. The O–O distance (1.5 Å) in H_2O_2 is very similar to the distance in free H_2O_2 , and the shortest distances between the iron atoms and the peroxide oxygen atoms (2.2 and 2.4 Å) are too long to indicate a true peroxo species. Additionally, our Mössbauer calculations based upon the model of this X-ray structure yield the isomer shifts (IS) equal to 0.83 and 0.85 mm/s and quadrupole splittings (QS) equal to 1.75 and 1.48 mm/s, which is in obvious disagreement with the experimental data (IS = 0.56 mm/s, QS = 0.67 mm/s). All these details make us regard of this structure as T4moH co-crystallized with H_2O_2 rather than a protonated peroxo T4moH. As such, it probably has little relevance for the actual geometry of the peroxo species.

Conclusion

Nature surpasses human imagination in how it can utilize small modifications in biomolecules in order to fine-tune biochemical processes or produce drastic changes in the phenotype. Countless examples are known when the difference of one hydrogen bond or a single amino acid cause macro-effects. Bacteria with a metabolism that depends upon BMMs seem to exploit small variations in these protein structures for better adaptation to their biological niche. In this work we have examined the structural differences between the two classes of BMM enzymes, MMOH and TMOH, the active sites of which are almost identical and yet their chemistry is markedly divergent. We focus on the process of dioxygen activation, which produces different types of peroxo species in MMOH and TMOH, according to spectroscopic measurements.

After listing and discussing some notable amino acid differences in the vicinity of the MMOH and TMOH active sites, we cannot find a clear explanation as to how any of these changes can bring about the disparate chemistry of the dioxygen activation of the two enzymes. Then, we narrowed our analysis to the strictly conserved threonine residue in the BMM family (T213 in MMOH and T201 in TMOH) and demonstrated that, in all the non-perturbed crystal structures of MMOH, T213 has a rotational configuration along its $C_{\alpha}-C_{\beta}$ bond that is qualitatively distinct from the configuration of T201 in all the crystal structures of TMOH. A series of rotational profiles around the $C_{\alpha}-C_{\beta}$ bond of the threonine side chain that we generated using both MM and QM/MM methods are in good agreement with experimental observations and help explain the formation of two peroxo species in the case of the T201S mutant. According to the rotational energy profiles, the regulatory protein imposes a very tall barrier on rotation of the threonine side chain, perhaps controlling specificity in the formation of the peroxo species of the wild-type proteins. A more detailed study involving other mutants, both theoretically and experimentally, is reserved for the future, to either confirm or refute the hypothesis that the configuration of the threonine is directly related to the formation of one or another type of peroxo species.

Our QM/MM geometry optimizations of the peroxo and Q intermediates of TMOH starting from the 2INC crystal structure (not involving the regulatory protein) demonstrate that the most stable peroxo form, of the μ -1,2 type coordination of the oxygen and stabilized by the hydrogen bond that the peroxoanion forms with the threonine hydroxyl, is nearly 5 kcal/mol more stable than the Q structure. This result is in contrast to our recent investigation of the MMOH intermediates¹⁰ in which it was shown that the peroxo and Q structures have an almost equal energy. The present computational result is in agreement with experiment, which does not detect a Q form for ToMOH. The computed Mössbauer parameters of the optimized peroxo structures are in disagreement with experiment, and we propose a number of explanations for this fact. In particular, we show that protonation of the peroxo unit can reconcile theoretical and experimental Mössbauer data. Finally, we argue that it will be important to undertake a QM/MM study based on the T4moHD crystal structure, since this structure should be relevant to the intermediates observed in experiment.

More work is required to clarify several remaining issues: the structure of the $\text{ToMOH}_{\text{peroxo}}$ intermediate, its possible protonation and stabilization through the hydrogen bonding network involving T201, the relative stability of the peroxo with respect to the Q structure, and the further analysis of the hypothesis (through the studies of the mutant forms) that T201 in TMOH and T213 in MMOH play a key role in the formation of the peroxo species. However, we believe that this work, through the insights into the function of the threonine residue, the role of the regulatory protein, and the possibility of protonation at a suitable location in the active site to explain the $\text{ToMOH}_{\text{peroxo}}$ quadrupole splitting, paves the way

towards future progress in understanding the chemistry of the TMOH and MMOH intermediates.

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Figure 1.

Comparison of the residues around the diiron core in MMOH-Mc (PDB 1MTY), MMOH-Mt (PDB 1MHY), T4moH (PDB 3DHG), and ToMOH (PDB 2INC) crystal structures. The height of the bars above and below the residue codes signifies the approximate distance between the closest atoms belonging to the residue and the diiron core. The substitutions are highlighted in bold.

Figure 2.

The hydrogen bonds (traced with the dashed green lines) formed by the bridging carboxylate and preserved in MMOH-Mc and ToMOH crystal structures despite the mutations in the second shell residues. The color-coding is as follows: carbons are grey, oxygens are red, nitrogens are blue, and irons are cyan. Hydrogen atoms are omitted for clarity.

Figure 3.

The locations of the two iron atoms and C β and O atoms of threonine (or serine in the mutant) relative to one another. These atoms form the Fe1-Fe2-C β -O dihedral angle denoted 2Fe-THR in the text. The arrow shows a rotation around the C α -C β bond used to construct plots in Figs. 6 and 7.

Figure 4.

A schematic projection down the C_{α} - C_{β} bond of the threonine (or serine) C_{β} -O bond onto the plane in which two iron atoms lie and which is perpendicular to the C_{α} - C_{β} bond. This projection reveals that when dihedral angle Fe1-Fe2- C_{β} -O changes (due to rotation around C_{α} - C_{β} bond of Thr), the distance between the hydroxyl oxygen and the diiron center also changes. The positions and the values of four Fe1-Fe2- C_{β} -O angles (0, +/-90, +/-180 degrees) in the projected picture are shown. Because Thr is allowed to move parallel to the line connecting the iron atoms, a particular dihedral angle does not necessarily indicate the proximity of the oxygen atom to the diiron center. For this reason, the distances Fe1-O and Fe2-O are used in addition to the Fe1-Fe2- C_{β} -O angle in Table 2 to indicate the nearness of the threonine hydroxyl group to the diiron center.

Figure 5.

The two lowest-energy ToMOH peroxo structures (left, *cis-μ-1,2* and center, *μ-1,1*) and the Q structure (η^2 , η^2) optimized with the QM/MM method (B3LYP/OPLS2001); the only unlabeled residue is GLU231. Their relative energies computed in the larger basis set are given below. The dashed green lines indicate the hydrogen bonds formed between the T201 residue and the peroxide ion. The color-coding is as follows: hydrogens are white, carbons are grey, oxygens are red, nitrogens are blue, and irons are cyan.

Figure 6.

Rotation profiles of Thr in various MMOH and TMOH proteins computed with the MM method. In MMOH, Thr corresponds to T213, and in TMOH it stands for T201. The T201S mutant curves were produced by replacing the methyl group in the corresponding Thr crystal structures with a hydrogen atom.

Figure 7.

Rotational profiles of Thr in various MMOH and TMOH proteins computed with the QM/MM method. In MMOH, Thr corresponds to T213, and in TMOH it stands for T201.

Figure 8.
Possible hydrogen bond networks stabilizing the peroxo structure of μ -1,1 coordination in the TMOHD complex. Models (a) and (b) are protonated.

Figure 9.

The geometry and the Mössbauer characteristics of one of the protonated model peroxo structures optimized with B3LYP (the $\mu - 1, 1$ type coordination of the dioxygen molecule). The structure is shown in perspective in order to avoid atoms blotting one another; the only unlabeled residue is GLU231. The protonated distal oxygen atom is indicated. The color-coding is as follows: hydrogens are white, carbons are grey, oxygens are red, nitrogens are blue, and irons are cyan.

Table 1

The mutated residues closest to the diiron center of MMOH-Mc (IMTY) and ToMOH (2INC). The column ‘Conserved?’ indicates whether the shown mutation is shared by all the members of the groups MMOH and TMOH.

Substitution No.	MMOH-Mc	Comment	ToMOH	Location	Conserved?
1	G113	2 nd shell	E103	3 rd shell, points away from diiron	No ^a
2	T121	3 rd shell	E111	2 nd shell, H-bond through water with E134	No ^b
3	Q140	2 nd shell, H-bond with E144	G130	3 rd shell	Yes
4	T148	2 nd shell, points away from diiron	G138	3 rd shell	Yes
5	Q205	3 rd shell	T193	2 nd shell	Yes
6	I217	2 nd shell	F205	2 nd shell	Yes
7	E240	3 rd shell, points away from diiron	Q228	3 rd shell, points away from diiron	Yes
8	M247	3 rd shell, points away from diiron	A235	2 nd shell	Yes

^aE103 in ToMOH and G103 in T4moH.

^bT121 in MMOH-Mc and S121 in MMOH-Mt.

Table 2

The rotational conformations of T213 in MMOH with respect to the diiron center in a variety of crystal structures. The distances $d_{\text{Fe1-O}}$ and $d_{\text{Fe2-O}}$ are measured between the corresponding iron atom and the oxygen of the threonine hydroxyl group; the dihedral angle 2Fe-THR ($\text{Fe1-Fe2-C}_\beta\text{-O}$) is illustrated by Figs. 3 and 4. The dihedral angle $\text{N-C-C}_\alpha\text{-O}$ characterizing the internal rotation of the threonine is also given and denoted χ . The two values given for each entry correspond to the two different protomers of MMOH-Mc. The crystal structure notation corresponds to that of the Protein Data Bank.

Structure	Protein	Oxidation state	2Fe-THR , degrees	$d_{\text{Fe1-O}}$, Å	$d_{\text{Fe2-O}}$, Å	χ , degrees	Comment
1XU3	MMOH-Mc	+3, +3	109.0 163.1	6.02 6.66	5.61 5.42	113.8 75.2	Soaked in bromophenol
1XU5	MMOH-Mc	+3, +3	-89.4 164.7	7.13 6.56	6.65 5.54	-48.2 72.3	Phenol soaked
1XVB	MMOH-Mc	+3, +3	-153.8 -155.6	7.82 7.86	6.55 6.53	-68.9 -67.1	Soaked in 6-bromohexanol
1XVC	MMOH-Mc	+3, +3	-78.0 -77.5	7.08 6.91	6.63 6.37	-58.8 -61.0	Soaked in 8-bromooctanol
1XVD	MMOH-Mc	+3, +3	-76.8 160.6	6.76 6.53	6.45 5.45	-59.4 77.5	Soaked in 4-fluorophenol
1XVE	MMOH-Mc	+3, +3	-77.9 -100.9	7.29 7.71	7.10 7.23	-61.1 -42.3	Soaked in 3-bromo-3-butenol
1XVF	MMOH-Mc	+3, +3	-81.1 -109.8	7.16 7.38	6.80 6.81	-56.6 -26.3	Soaked in chloropropanol
1XVG	MMOH-Mc	+3, +3	155.6 168.8	6.36 6.55	5.22 5.63	78.8 69.4	Soaked in bromoethanol
1XMF	MMOH-Mc	+3, +3	-75.3 -89.6	6.80 7.09	7.09 7.07	-70.7 -60.0	Mn replaces Fe; soaked
1XMH	MMOH-Mc	+2, +2	-87.0 -92.4	7.28 7.27	6.95 6.82	-59.4 -50.9	Co replaces Fe
1FZ0	MMOH-Mc	+2, +2	-74.7 -80.7	7.32 7.37	6.76 6.62	-46.7 -53.9	Mixed valent
1FZ1	MMOH-Mc	+3, +3	-89.2 162.1	7.19 6.65	6.80 5.60	-48.4 73.5	Oxidized
1FZ2	MMOH-Mc	+2, +3	-69.8 -71.7	7.21 7.28	6.61 6.64	-56.2 -54.3	Mixed-valent; soaking
1FZ3	MMOH-Mc	+3, +3	169.0 -84.9	6.62 6.96	5.66 6.29	68.3 -55.9	Soaked at pH 6.2 (0.1 M PIPES)
1FZ4	MMOH-Mc	+3, +3	-82.7 -82.7	6.94 7.10	6.45 6.42	-53.4 -54.6	Soaked at pH 8.5 (0.1M TRIS)

Structure	Protein	Oxidation state	2Fe-THR, degrees	d _{Fe1-O} , Å	d _{Fe2-O} , Å	χ, degrees	Comment
1FZ6	MMOH-Mc	+3, +3	-77.8 -112.9	7.09 7.22	6.81 6.60	-16.7 -59.0	Soaked in 1M methanol
1FZ7	MMOH-Mc	+3, +3	-68.8 161.9	6.69 6.56	6.53 5.58	-70.3 70.6	Soaked in 0.9M ethanol
1FZ8	MMOH-Mc	+3, +3	21.7 176.6	6.00 6.72	6.13 5.72	-171.1 65.5	Soaked in bromoethane
1FZ9	MMOH-Mc	+3, +3	-85.2 100.1	7.31 6.59	6.61 7.20	-53.1 -54.2	Soaked in iodoethane
1FZH	MMOH-Mc	+3, +3	-70.6 -178.9	6.58 6.36	6.24 5.50	-66.4 54.8	Pressurized with xenon
1FZI	MMOH-Mc	+3, +3	-68.1 -58.9	6.85 7.00	6.97 6.91	-62.0 -75.7	Pressurized with xenon
1MHY	MMOH-Mt	+3, +3	-87.6	6.77	6.23	-46.0	
1MHZ	MMOH-Mt	+3, +3	-97.5	6.75	6.03	-43.6	
1MTY	MMOH-Mc	+3, +3	-72.5 -58.2	7.10 6.91	6.80 6.80	-61.9 -75.7	
1MMO	MMOH-Mc	+3, +3	-90.1 -90.5	6.99 7.03	6.41 6.45	-48.5 -48.0	

Table 3

The rotational conformations T201 in TMOH with respect to the diiron center in a variety of crystal structures. The distances d_{Fe1-O} and d_{Fe2-O} are measured between the corresponding iron atom and the oxygen of the threonine's hydroxyl group; the dihedral angle $2Fe-THR$ ($Fe1-Fe2-C_{\beta}-O$) is illustrated in Figs. 3, 4. The dihedral angle $N-C-C_{\alpha}-O$ characterizing the internal rotation of the threonine is also given and denoted χ . The crystal structure notation corresponds to that of the Protein Data Bank.

Structure	Protein	Oxidation state	2Fe-THR, degrees	d_{Fe1-O} , Å	d_{Fe2-O} , Å	χ , degrees	Comment
3I5J	T4moHD	+3, +3	141.2	7.37	6.24	58.9	
3I63	T4moHD	+3, +3	139.4	7.32	6.19	56.2	H ₂ O ₂ is loosely bound to Fe atoms
3DHG	T4moH	+3, +3	156.0 135.3	6.45 6.48	5.36 5.27	66.5 78.5	
3DHH	T4moHD	+3, +3	136.6	7.37	6.26	57.9	
3DHI	T4moHD	+2, +2	137.6	7.33	6.64	58.9	
2RDB	ToMOH	+3, +3	157.4	7.03	5.67	64.6	1100W mutant
2IND	ToMOH	+2, +2	164.9	7.13	5.98	64.9	Mn replaces Fe
2INC	ToMOH	+3, +3	161.8	6.71	5.62	64.0	
1T0Q	ToMOH	+3, +3	164.4	6.88	5.74	66.0	
1T0R	ToMOH	+3, +3	147.8	6.67	5.45	78.5	With azide bound
1T0S	ToMOH	+3, +3	179.7	6.84	5.65	57.6	Soaked in 4-bromophenol

Table 4

The computed Mössbauer characteristics of some geometrical models of the ToMOH intermediates whose structures are shown in Fig. 5.

Form	Fe	Oxidation state	Spin	δ_{exp} , mm/s	$ \Delta E _{\text{exp}}$, mm/s	δ , mm/s	$ \Delta E $, mm/s
peroxo (a)	Fe1	+3	+5	0.54	0.67	1.02	3.02
	Fe2	+3	-5	0.54	0.67	0.65	0.44
peroxo (b)	Fe1	+3	+5	0.54	0.67	1.00	2.71
	Fe2	+3	-5	0.54	0.67	0.59	0.34