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The active site structure of methane monooxygenase is closely related to the binuclear iron center of ribonucleotide reductase

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Methane monooxygenase (MMO) catalyses the biological transformation of methane to methanol at a binuclear iron site. Guided by the three-dimensional structure of the R2 protein of *E. coli* ribonucleotide reductase (RNR), we have aligned the sequences of two different MMOs with the sequences of the iron coordinating four helix bundle in R2. The model suggests that the central four helix bundle of R2 is present also in MMO. The iron coordination is similar in MMO and R2 with two histidine ligands and four carboxyl ligands in both cases. The residues lining the proposed oxygen binding site in MMO are significantly smaller in MMO than in R2 allowing binding of both molecular oxygen and methane at this site. This binding site is lined by residues Cys^{151}, Thr^{213}, Ile^{217} and $Ile(Val)^{239}$.

Active site model; Binuclear iron protein; Gene duplication; Methane monooxygenase; Ribonucleotide reductase

1. INTRODUCTION

Methane monooxygenase (MMO) is an iron-dependent enzyme from bacteria which catalyses the NAD(P)H- and O_2-dependent oxidation of methane to methanol [1]. The enzyme is made up of the different protein components A (containing α, β, and γ subunits), B and C and the active site is located at a binuclear iron centre on the α-subunit of protein A. Protein C contains an iron sulphur cluster and a NADP-flavin-dependent reductase, while protein B appears to be a regulatory protein.

Current interest in the enzyme stems from its broad substrate specificity [2] such that it may be used in vivo for the biotreatment of halogenated hydrocarbon pollutants [3]. In the long term it may also be of significant value to the petrochemical industry in the design of new low temperature catalysts for the direct oxidation of methane to methanol.

Previous evidence for the nature of the active site of MMO is based on spectroscopic studies which have revealed the presence of a dinuclear iron centre which activates dioxygen [4,5]. A high valent ferryl species is believed to abstract hydrogen from methane to form a methyl radical [6] and ultimately methanol [7].

Chemical models have been synthesized to mimic the low temperature conversion performed by the binuclear iron cWntre of the hydroxylase component [8,9] but with limited success to date, largely due to the nature of the ligand environment being unknown.

Recently the cloning and sequencing of MMO proteins from two methanotrophs [10,11] revealed a remarkably high degree (94%) of conservation in the α-subunit of the hydroxylase, the protein which is known to interact with the hydrocarbon substrate [12,13]. Stainthorpe et al. [10] identified two places in the sequence of the MMO α-subunit to contain a sequence motif: Glu-X-X-His, known to coordinate the iron ions in the binuclear iron-binding site of the R2 protein of ribonucleotide reductase (RNR) [14]. (Earlier, protein R2 was labelled differently for each species, e.g. protein B2 for the *E. coli* protein. At a recent ribonucleotide reductase meeting it was recommended that it should be called protein R2 for all species).

Active ribonucleotide reductase is formed by two dimeric proteins R1 and R2 where the substrate binds to the R1 protein. The R2 protein, which functions as an initiator of a radical based reaction, is essential for the activity of ribonucleotide reductase [15,16]. The protein carries a stable free tyrosyl radical at Tyr^{122} which is generated by dioxygen and a binuclear iron center. The X-ray structure of protein R2 has recently been solved [14], and the basic structural motif of the R2 subunit is a barrel of a-helices where the binuclear iron centre is buried between the four helices B, C, E and F (Fig. 1). This four helix bundle displays a pseudo two-fold symmetry where helices B and C can be superimposed on helices E and F with an rms difference of 1.2 A;. The helix pairs show sequence homology to each other indicating that they have evolved through a gene

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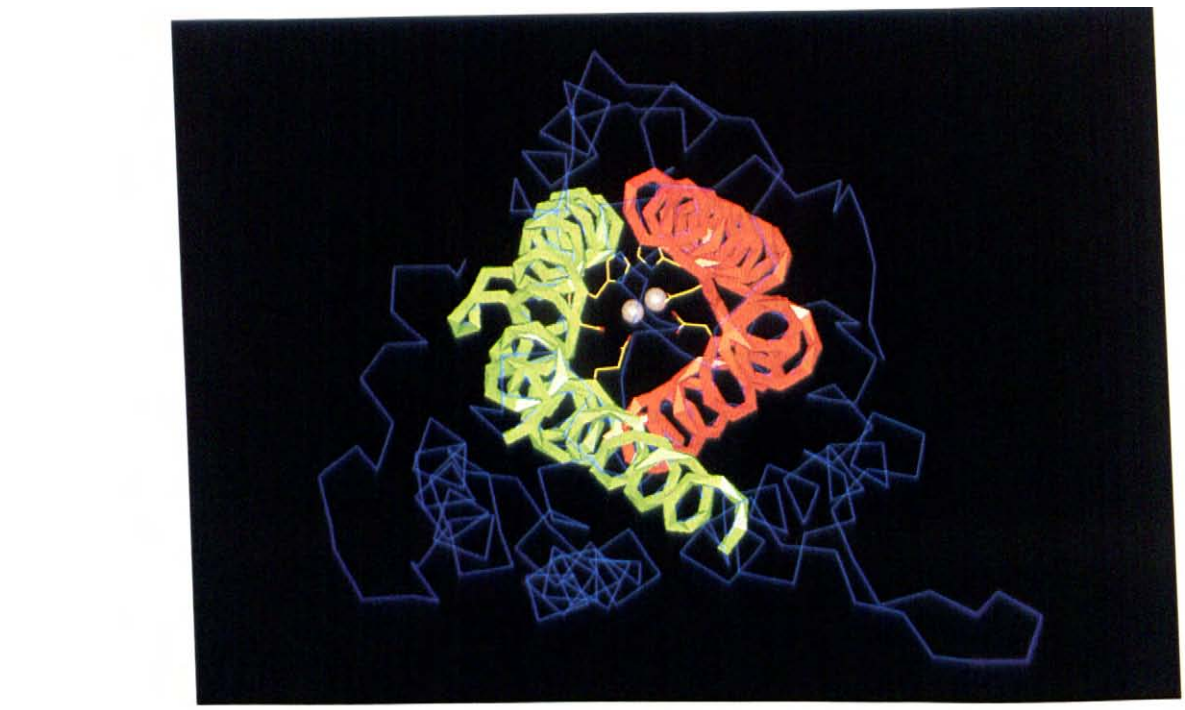

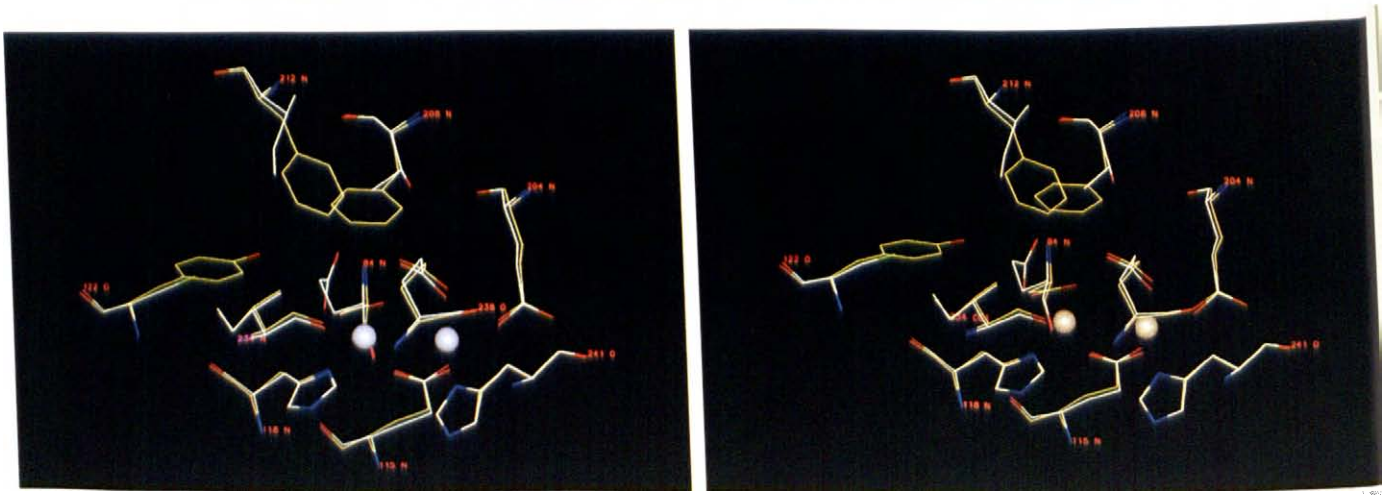

Fig 1. The iron binding motif of protein R2 in the center of the subunit, with the iron ions in light blue. Helix B and C (in orange) are pseudo symmetrically related to helix E and F (in green) and can be superimposed through a two-fold rotation perpendicular to the helices, giving an rms deviation of 1.2 Å; for Cα atoms. The iron ligands contributed by the helices are as follows: αB: Asp^{84}, αC: Glu^{115} and His^{118}, αE: Asp^{204} and αF: Glu^{238} and His^{241}. The picture was taken from an Evans and Sutherland workstation using the program O [23].

Fig 5. The active site of *M. capsulatus* MMO (white carbon atoms) as modeled from the iron binding motif of RNR R2 (yellow carbon atoms). The active site of MMO can be regarded as a widening of the oxygen binding site close to the R2 iron binding structure where Asp^{84} (R2)→Glu^{114} (MMO), Glu^{115} (R2)→Glu^{144} (MMO), His^{118} (R2)→His^{147} (MMO), Tyr^{122} (R2)→Cys^{151} (MMO), Glu^{204} (R2)→Glu^{209} (MMO), Phe^{208} (MMO), Phe^{212} (R2) →Ile^{217} (MMO), Ile^{234} (R2)→Ile^{239}(MMO), Glu^{238}→Glu^{243} (MMO), His^{246} (R2)→His^{246} (MMO). Two different sets of main chain atoms (from two different cycles of refinement) have been used for the case of interpretation of the picture. The picture was taken from an Evans and Sutherland workstation using the program O [23].

duplication [14]. The coordination of the two ferric ions in the structure of R2, in which each helix pair contributes two carboxylate ligands and one histidine ligand, is shown in Fig. 2. In addition the two ferric ions are bridged by an oxide ion.

The chemistry performed by the metal centres in MMO and protein R2 is closely related; both proteins activate oxygen and act by abstraction of a hydrogen atom to generate radicals [7,15,17,18]. In addition, both proteins depend on electrons provided by their own flavin-NADP reductases, Protein C in the case of MMO and a free protein component in the R2 system [19–21]. We have recently found also these two reductases to be homologous to each other (cited in [22]). his communication discusses the extension of the alignment of the MMO sequence to protein R2 which supports an evolu-

tionary relationship between the two proteins. Furthermore this alignment allows, for the first time, the construction of a realistic model of the active site of MMO.

2. MATERIALS AND METHODS

The sequences of MMO from *M. capsulatus* and *M. trichosporium* were aligned to the R2 sequences from 10 species (11 in some parts of the helices) guided by the three-dimensional structure of *E. coli* R2. The deletion between the two iron binding motifs for MMO compared to *E. coli* R2 is similar to that of the mammalian and herpes virus R2s. A one-residue deletion compared to *E. coli* R2 could be positioned at the same place as in the herpes virus sequences between αB and αC.

Model building was performed using the program O [23]. The side chains of R2 were replaced by the corresponding side chain as derived from the sequence alignment to MMO. As many side chain dihedral angles as possible were left in the same conformation in the MMO structure as in the R2 structure. The model could be built without any severe steric hindrance and most residues surrounding the oxygen binding site are smaller in the MMO model than the corresponding ones in R2.

Coordinates of the model will be deposited at the Brookhaven data bank.

3. RESULTS

We now show that when the alignment of the two short sequence stretches in the MMO hydroxylase subunit, identified as containing the Glu-X-X-His sequence [10], is extended to include the corresponding sequences of helix C and F in R2 (Fig. 2) and further to include helices B and E, a significant part of the MMO sequences aligns to the R2 sequences with similarities comparable to those found between distantly related R2 proteins (Fig. 2). It is most similar to the Epstein Barr virus sequence (about 20% identities). With this alignment, the acidic residues Glu^{114} and Asp/Glu^{209} are identified as the metal ligands corresponding to Asp^{84} and Glu^{204} of helices B and E in R2, respectively (Fig. 3). These carboxylate ligands align without gaps with the Herpes simplex virus (HSV) group of R2 but have a single residue deletion when compared with other R2 sequences.

As for R2, the helices in each of the two iron binding structures of MMO show sequence homology to each other (Fig. 4). The carboxyl ligands in the first helix pair are preceded by a Val-Gly sequence while an Asp and an Arg close to the metal ligands are present in the second helix pair.

A model of the four helix bundle of MMO containing the metal ligands demonstrate that the two proteins in all probability have very similar structures in these parts and all the substitutions of the side chains are compatible with a conserved structure.

Several of the residues around the proposed oxygen

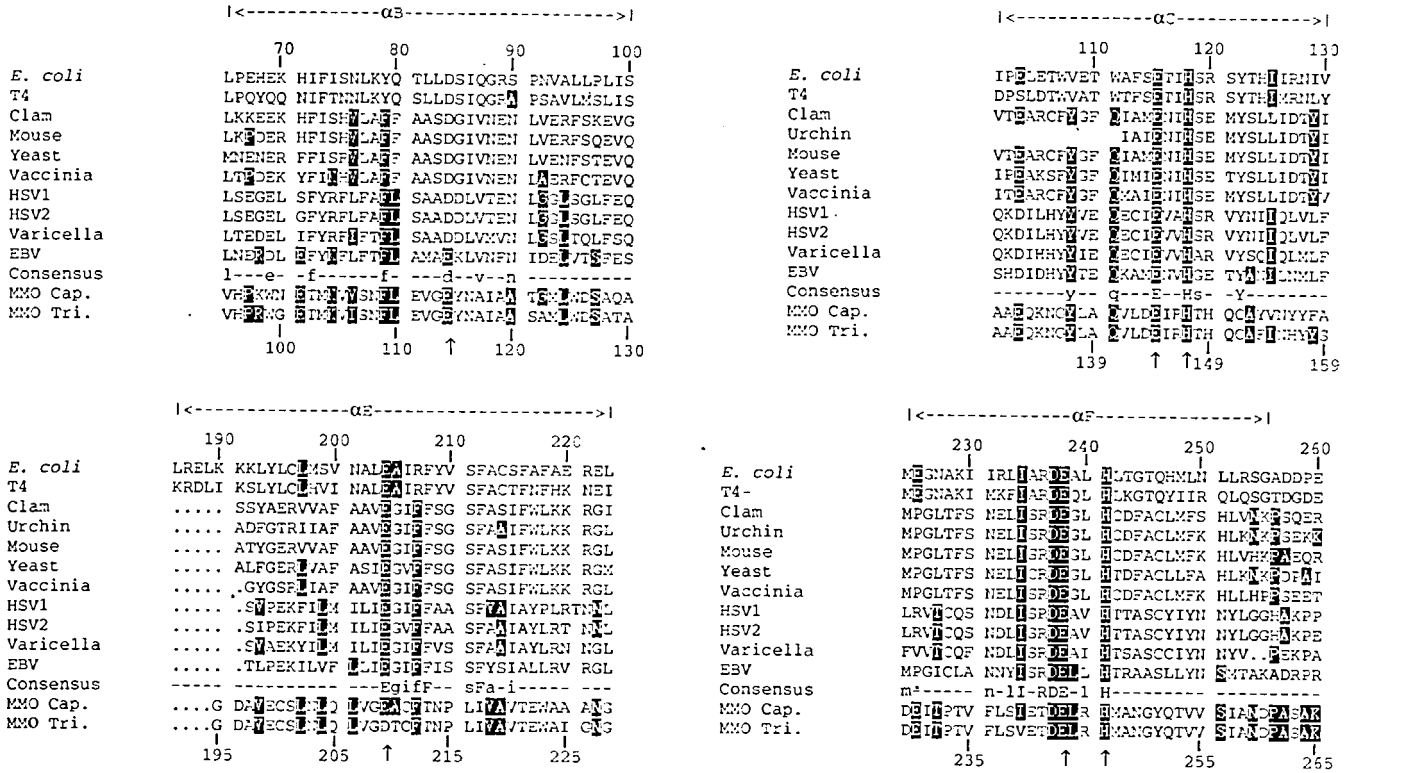

Fig 2. The sequence alignment of the four iron binding helices of protein R2 and the hydroxylase subunit of MMO. One residue is inserted in most of the R2 sequences between helix B and C. The references to the R2 sequences are found in [14]. The iron ligands (arrows below the sequences) are conserved but the terminal ligands at positions 84 (in R2) and 204 (in R2) seem to be able to accommodate both aspartic acids and glutamic acids. Two of the six conserved non iron coordinating residues in protein R2 are also present in MMO, i.e. $Ile^{234}(R2) \rightarrow Ile^{239}$ (MMO *M. capsulatus*) $Asp^{237}(R2) \rightarrow Asp^{242}$ (MMO). In black are the residues present in both MMO and R2 sequences. The consensus sequence show the totally conserved (capital letters) and highly homologous residues (small letters) within the R2 sequences.

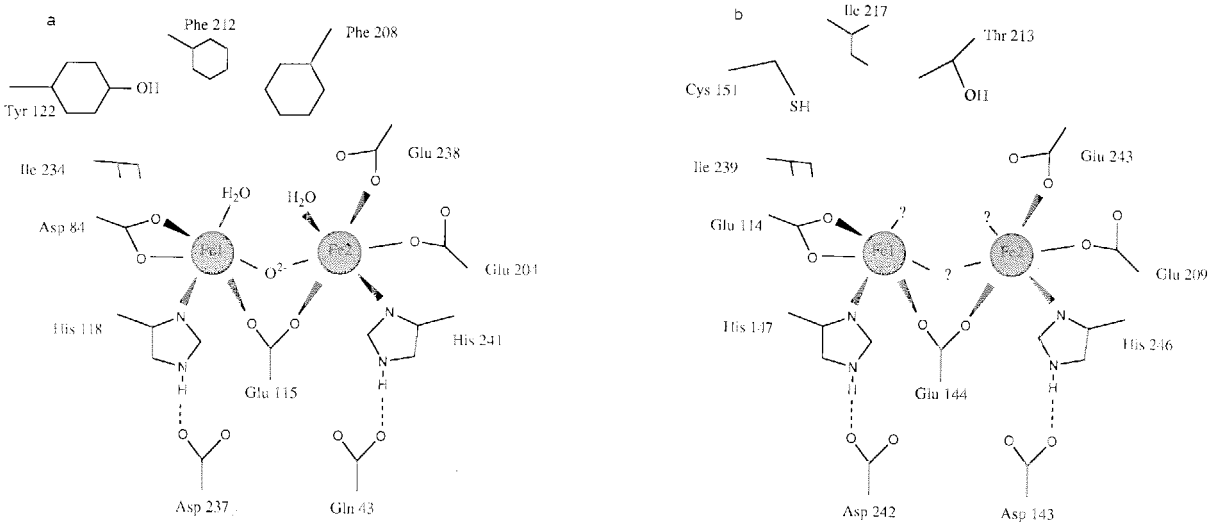

Fig 3. Schematic view of the iron surrounding in protein R2 and the hydroxylase subunit of MMO. (A) The ferric center of R2 is coordinated by two histidines, three glutamic acid, one aspartic acid, a bridging μ-oxo group and two water molecules. In between the radical site Tyr^{122} and the iron ions is a narrow hydrophobic pocket formed by the conserved Phe^{208}, Phe^{212} and Ile^{234}. All residues on this picture are conserved in the R2 proteins except Asp^{84}, that is Glu in Epstein-Barr R2, and Gln^{43} that is Ala, Thr and Ser in other sequences. (B) The active site model of *M. capsulatus*MMO, where the ligands to the binuclear center are of the same type as in protein R2. However, each histidine ligand is linked by an aspartic acid making the pseudo two-fold symmetry stronger in MMO than in protein R2. This is further strengthened in *M. capsulatus* MMO where both terminal carboxylate ligands are glutamic acids. (This is also true for the Epstein-Barr R2 where 84 is a glutamic acid.) Of the residues in protein R2 implicated in forming a pocket for molecular oxygen, one is present in *M. capsulatus* MMO, Ile^{234} (R2)→Ile^{239} (MMO), Val in *M. trichosporium*. Phe^{212} (R2) is changed to Ile^{217} (MMO) and Phe^{208} (R2) is changed to Thr^{213} (MMO). Tyr^{122} which is the radical site in R2, is Cys^{213} in MMO. No conclusions can be made on the details of the iron coordination from our model.

binding site in R2 [14] have been substituted by smaller residues in MMO (Fig. 5). Cys^{151} corresponds to the radical-carrying Tyr^{122}. Thr^{213} and Ile^{217} in MMO have replaced Phe^{208} and Phe^{212} in R2. Ile^{234} in R2 is Ile in *M. capsulatus* MMO and Val in *M. trichosporium* MMO. From the model we suggest that these residues form the substrate binding site in MMO.

4. DISCUSSION

The evolutionary relation between MMO and R2 is very distant and the alignment of the sequences of the two proteins relies heavily on structural information obtained for protein R2. Furthermore, within the family of R2 proteins the sequences of the iron binding helices

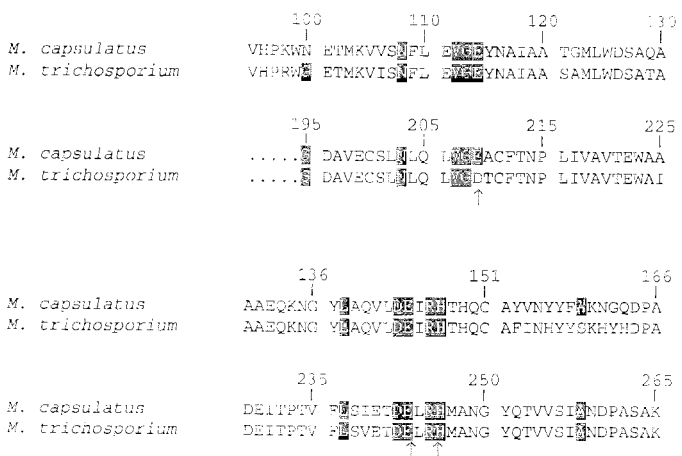

Fig 4. The duplicated motif of the iron binding helices in MMO. The sequence conservation around the iron ligands in the duplication of MMO appears somewhat stronger than the one found in protein R2 [14]. The iron ligands have arrows below the sequences.

have diverged (Fig. 2) and only six residues, in addition to the iron ligands, are conserved in all sequences. Even so, there is little doubt that the peptide fold of the iron binding motif of protein R2 from different species will be virtually identical [24]. This, taken together with the fact that the four helix bundle is a compact structure with a low probability for insertions or deletions within the helices, makes this a favorable case for a model building study of two proteins which have greatly diverged. The appearance of a duplicated motif in both the sequences of R2 and MMO, further supports the view that these proteins are related.

The sequence alignment indicates a somewhat stronger conservation of the duplicated motif in MMO than the one found in R2 [14] and therefore a more symmetric structure of the MMO iron centre might be expected. In R2 Asp^{237} just before the metal ligand Glu^{238} in helix F forms a hydrogen bond to His^{118} of helix C in the first helix pair. The conserved Asp-Glu-X-Arg-His sequence in both duplicated motifs of MMO suggests that both histidines are hydrogen-bonded to negatively charged aspartic acids (Fig. 3) This kind of hydrogen bond give some anionic character to the histidine side chains thereby stabilizing higher oxidation states on the iron. The negative coordination ligands are probably important for catalysis since a highly electrophilic species is required to abstract hydrogen from the very strong C–H bond of methane. The hydrogen bond from additional carboxyl acids to the histidine side chains, may further enhance this property.

No definitive conclusions can be made from our model about the finer details of the coordination of the iron ions in MMO, i.e. the existence of bidentate carboxylate ligands or the mode of bridging of the iron ions [25]. Coordination details like this have been shown to vary between different oxidation states of R2 (Nordlund, manuscript in preparation) and for metal substituted R2 [27].

The replacement of the residues Tyr^{122}, Phe^{208} and Phe^{212} in the proposed oxygen binding site of R2 by the smaller residues Cys^{151}, Thr^{213} and Ile^{217} suggests that the active site of MMO is significantly more open than the narrow oxygen-binding pocket of R2 (Fig. 5). This is consistent with the fact that the hydroxylase subunit of MMO also accommodates hydrocarbon substrates òhereas the R2 subunit of RNR only interacts with O_2. The binding of substrates takes place on the R1 subunit. The MMO active site pocket is mainly hydrophobic as expected for a site that interacts with dioxygen as well as hydrophobic substrates. The pocket in the MMO model is certainly sufficiently large to accommodate a molecule such as methane but model building suggest that it should also be able to accommodate larger substrates which are also known to be oxidized [6].

An intriguing finding is that in the model of MMO, Cys^{151} is found to substitute for the radical site Tyr^{122} in protein R2. Recently a methane radical has been trapped as an intermediate in the MMO reaction cycle [7]. By analogy with protein R2 one could consider an intermediate cysteine radical on Cys^{151} to perform the hydrogen abstraction on methane but to date there is no direct evidence for the involvement of this amino acid in catalysis.

Since both MMO and RNR are enzymes that function via the production of highly active electrophilic species, their active sites ought to contain residues which are able to keep this species in a productive mode. But, in addition they should resist damaging oxidation. In protein R2 the oxygen pocket is formed by two phenylalanines (208 and 212) and one isoleucine (234), residues known to be difficult to oxidize [26]. In MMO the two phenylalanines are substituted by one isoleucine (217) and one threonine (213), while Ile^{234} is Ile also in *M. capsulatus* MMO and Val in *M. trichosporium* MMO.

Thr^{213} at the proposed active site in MMO may have a similar function as a Thr in the heam-containing monooxygenase family of cytochrome P450. Thr^{213} in MMO is located close to Fe1 in MMO at a site where the long E helix in R2 displays a distortion due to a π-turn in the middle of the helix (Fig. 5). Interestingly a threonine is found as one of two conserved residues of the distal I helix at the active site of cytochrome $P450_{cam}$ [27,28]. This Thr and a conserved Gly are located at a position of the very long helix I where a similar distortion to that observed for Helix E in protein R2 is found. The threonine in $P450_{cam}$ makes a bifurcated hydrogen bond to two main chain carbonyls which are in the preceding turn of the I helix [27,28]. Site directed mutagenesis experiments [29,30] on the threonine in cytochrome $P450_{cam}$ have shown that this residue is essential for efficient oxygenation. A recent crystallographic study on a mutant cytochrome $P450_{cam}$demonstrates a significant conformational change of the distorted helix when the threonine is substituted by an alanine residue [31]. Nevertheless, these results do not give a clear answer as to the role of this residue. It may be either a 'catalytic role', to stabilize a peroxide intermediate by donating/transferring a proton (a hydrogen bond), or a structural role, to stabilize the distorted helix, or a combination of these two options. However, it is possible that the threonines and the distorted helices of the two monooxygenase families have similar functions in the oxygenation of carbon substrates.

The present study demonstrates that the principal ligands of the binuclear iron centres are very similar in MMO and R2. This suggests that the mechanism of oxygen activation is very similar in the two proteins and that the branching of the reaction into either an oxygenation reaction (MMO) or an oxidation reaction (R2) is determined by the local environment of the oxygen site. It should therefore be possible both to assess the importance of the active site residues of MMO via site directed mutagenesis and to convert the R2 of RNR into the

functional equivalent of the a-subunit of MMO and vice versa through the expedient of modifying only a few active site residues.

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