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# Carboxylate-Bridged Dinuclear Active Sites in Oxygenases: Diiron, Dimanganese, or is Heterodinuclear Better?

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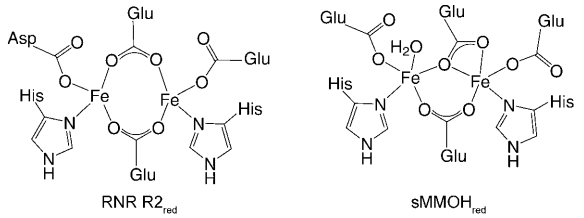
Carboxylate-bridged diiron centers are a frequently observed structural motif in biological systems, and the corresponding enzymes catalyze a wide range of important reactions. Within this class of enzymes, a highly interesting family of diiron proteins is characterized by an eye-catching feature in their secondary structure: The dinuclear metal site is localized between a bundle of four α helices (four-helix bundle proteins) that provide coordinating amino acid residues (histidine, glutamate, and/or aspartate) for the binding of metal ions, which are usually bridged by protein-based carboxylate functionalities and/or oxo ligands (water molecules, hydroxo or oxo ions). It is particularly interesting that the members of this protein family, despite their similarly structured metal sites, catalyze a wide variety of biological processes that appear at first sight to be highly different. However, a closer look at the reactions reveals that most of these processes are based on the common reactivity of the enzymes towards molecular oxygen, which is activated and reduced by the dinuclear metal site. Fascinating and intensely studied examples of this family are the soluble methane monooxygenases (sMMOs),[1] the soluble fatty acid desaturases,[2] and the ribonucleotide reductases (RNRs; Scheme 1).[3]

The similar reactivity towards oxygen, the highly conserved structure of the dinuclear metal site, and a protein environment with four-helix bundles has led to the belief that this family of enzymes has evolved from a common ancient primitive oxidase. The original task of this oxidase might have been the fast and reliable reduction of molecular oxygen, thus protecting the cell against oxidative stress caused by the transition from a reducing to an oxidizing atmosphere about 2.5 billion years ago. This hypothesis is supported by a recent study in which the replacement of a single amino acid in a soluble fatty acid desaturase led to a total loss of desaturase acitivity and a simultaneous increase in oxidase activity.[4]




***Scheme 1.*** Diiron active sites. Left: Protein R2 of a ribonucleotide reductase (RNR); right: hydroxylase of a soluble methane monooxygenase (sMMOH).

Very recently, two new members of this protein family were isolated from certain bacteria and found to be quite different from their prominent relatives in various aspects. These unusual enzymes are the ribonucleotide reductase of *Chlamydia trachomatis*[5] and the N-oxygenase AurF of *Streptomyces thioluteus*.[6] Interestingly, the unambiguous assignment of the identity of the active metal sites in these systems still remains an open question. In particular, it is not yet entirely clear whether these enzymes are homometallic diiron or dimanganese systems or even contain heterometallic MnFe centers as their active sites. The elucidation of the chemical identity and reactivity of these dinuclear metal sites represents a highly exciting and challenging task in the field of modern bioinorganic chemistry.

It is believed that the deoxygenation of ribonucleotides catalyzed by ribonucleotide reductases is initiated by the formation of a protein-based thiyl radical through abstraction of a hydrogen atom.[3] Ribonucleotide reductases are divided into three classes, depending on the metal cofactor used for the formation of this key radical: While a cobalamine or a $\{Fe_4S_4\}$ cluster acts as the hydrogen atom abstractor in class II and III ribonucleotide reductases, respectively, a carboxylate-bridged diiron center is observed in class I ribonucleotide reductases.

Class I ribonucleotide reductases are adducts comprising an R1 subunit, which contains the substrate binding site, and an R2 subunit, in which the metal site resides. The enzyme is activated upon reaction of the reduced $\{Fe_2^{II}\}$ site with dioxygen, presumably resulting in a short-lived mixed-valent $\{Fe^{III}Fe^{IV}\}$ species (intermediate X; Figure 1), which in turn is capable of producing a relatively stable tyrosyl radical in

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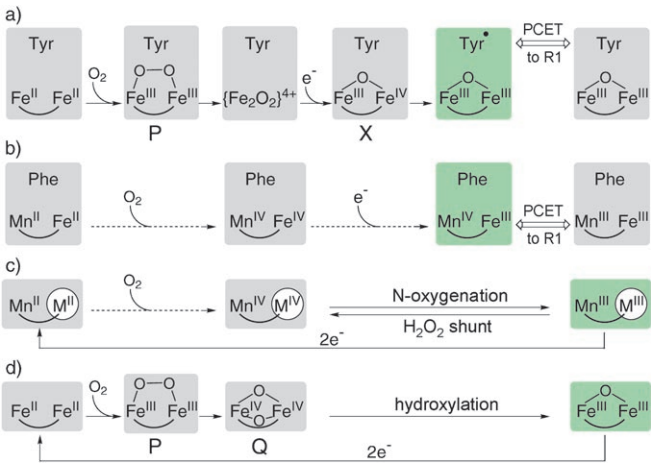



***Figure 1.*** Reaction mechanisms of various dinuclear oxygenases: a) RNR R2 from *E. coli*, b) RNR R2 from *C. trachomatis*, c) AurF from *S. thioluteus*, and d) sMMOH. Stable product states are marked in green. PCET = proton-coupled electron transfer between R1 and R2 subunits, M = Fe or Mn.

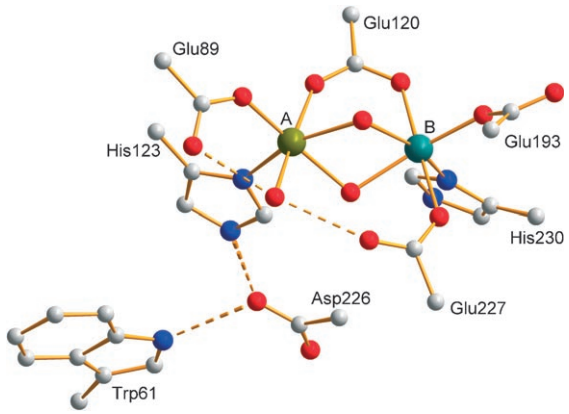



***Figure 2.*** Dinuclear active site of the R2 subunit of *C. trachomatis* RNR; gray C, red O, blue N. His123, Asp226, and Trp61 are part of the suggested radical-transfer pathway to the R1 subunit. For assignment of the A and B sites, see the text.

proximity to the metal center. The radical character of this tyrosine residue is transferred by an electron-transfer pathway to a cysteine residue located near the substrate binding site, thus starting the actual catalytic cycle.

Being located at the key position of the electron-transfer chain and being highly conserved in all systems characterized thus far, the tyrosine residue has always been assumed to be essential for the function of class I ribonucleotide reductases. This assumption has been supported by mutagenesis experiments, where replacement of the tyrosine residue led to a complete loss of activity.[7] On the other hand, it has been revealed in more recent studies on pathogenic *Chlamydia* bacteria that alternative mechanisms for the formation of the initial radical and transfer of the radical character to the substrate binding site must exist. It was demonstrated by genome sequencing that *Chlamydia* contains exclusively class I ribonucleotide reductases, but in contrast to the systems examined so far, *Chlamydia* ribonucleotide reductases lack the commonly observed tyrosyl residue. Instead, the structure of the R2 subunit of *C. trachomatis* contains a phenylalanine residue at the corresponding position. Although phenylalanine is incapable of forming a sufficiently stable radical,[5] this alternative ribonucleotide reductase is nevertheless active (Figure 2).[8]

Initial studies on the catalytic mechanism of this new subclass of class I ribonucleotide reductases revealed a mixed-valent species similar to the known intermediate X (Figure 1 a), and was thus originally described as an $\{Fe^{III}Fe^{IV}\}$ species.[5,9] Based on the analogy between the electron-transfer pathway between the R1 and R2 subunits of the new and the conventional class I ribonucleotide reductases, it has been postulated that this mixed-valent metal site is the initial key species in the radical-transfer chain, and thus takes the role of the tyrosyl radical found in the conventional enzymes. The postulated function of the intermediate X is in accord with its significantly prolonged life time. It is tempting to assume that the observed defect in *Chlamydiae* is actually a crucial advantage for it to act as an intracellular pathogen, since unlike the cases with the tyrosyl radical, the potential key target for the antiproliferative effects of nitrogen monoxide (NO)—produced in the immune response by the host—is missing, which could mean an evolutionary advantage for these pathogenic life forms. This interpretation is supported by the fact that this form of R2 subunit has also been found in other intracellular parasites.[5]

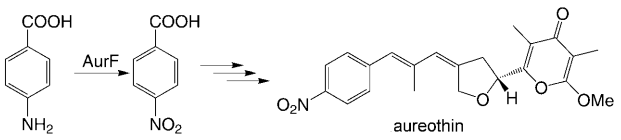
Very recently it has been shown in two independent studies that the active metal site in *Chlamydia* ribonucleotide reductases is in fact not, as believed thus far, a diiron cluster, but rather a heterodinuclear {MnFe} system.[10,11] The highest enzymatic activity was found for an equimolar Mn/Fe ratio and with two metal atoms per protein.[10] A possible reaction sequence—based on these findings and the general catalytic mechanism of class I ribonucleotide reductases—is shown for *Chlamydia* ribonucleotide reductases in Figure 1 b. The postulated oxidation states in this mechanism were confirmed by a series of ESR and Mössbauer spectroscopic studies. The active species was assigned as a $\{Mn^{IV}Fe^{III}\}$ site, which is analogous to the intermediate X of conventional systems. The studies also showed that further reduction of the active species results in the electron transfer taking place at the manganese atom, thereby resulting in a $\{Mn^{III}Fe^{III}\}$ species. Furthermore, the preceding intermediate, a $\{Mn^{IV}Fe^{IV}\}$ species, which arises during the activation of the metal site by dioxygen, could be detected.[12] The formation of such a highly oxidized intermediate is also assumed for the conventional class I ribonucleotide reductases as well as for related diiron proteins such as MMOH and fatty acid desaturases ($\{Fe_2^{IV}\}$, intermediate Q in Figure 1 d).

Despite a concordant mechanistic view based on a heterodinclear {MnFe} site (Figure 1 b), there is a difference of opinion regarding the interpretation of the observed variations in the catalytic activity. This arises in particular because of the possibility of a residual activity of the diiron form of the enzyme. From the unique feature of this new kind of class I ribonucleotide reductases (the lack of an initial tyrosine residue in the electron-transfer path), it seems reasonable that the metal site, with its extended functionality

as radical carrier, must also be altered compared to the active site of conventional class I ribonucleotide reductases. Therefore, the inclusion of a manganese ion can be viewed as an adjustment because of the specific needs of the system, and as a consequence the exclusive activity of the heterometallic species appears to be highly plausible.

In this context, a second highly interesting enzyme is the N-oxygenase AurF. Also being a member of the large four-helix bundle protein family, AurF catalyzes the N-oxygenation of *p*-aminobenzoic acid to *p*-nitrobenzoic acid during the biosynthesis of the nitroaryl-substituted metabolite aureothin in *S. thioluteus* bacteria (Scheme 2).[13] Despite the fact that the activity of isolated AurF has been assessed up to now by using $H_2O_2$ as the oxidizing agent, it is expected that the enzyme uses molecular oxygen under native conditions.[14]




***Scheme 2.*** N-Oxygenation of *p*-aminobenzoic acid by AurF in the biosynthesis of aureothin.

Similar to the R2 subunit of *Chlamydia* ribonucleotide reductases, it was originally assumed on the basis of sequence homologies that the new N-oxygenase AurF of *S. thioluteus* is a carboxylate-bridged diiron protein.[15] It is interesting to note that ESR studies on AurF also indicated the presence of a heterometallic {MnFe} species, but this finding was originally interpreted as contamination of the sample by manganese ions from the medium.

The recently published three-dimensional structure of AurF provided a more detailed insight.[6,14] The structure clearly shows that AurF is a new member of the four-helix bundle protein family, thus confirming the so far assumed relationship between AurF and the diiron enzymes. However, a closer examination of the dinuclear site reveals important differences (Figure 3): It can be clearly seen that the aspartate residues Asp135 and Asp226, which were originally presumed to be part of the metal-binding motifs, are in fact located too far away from the metal centers to contribute to the complexation. Instead, a second histidine residue is found in the coordination sphere of one of the metal atoms, and thus reflects a unique structural feature of AurF compared to other four-helix bundle proteins, which usually have only one coordinated histidine residue per metal atom.

An additional remarkable feature of AurF is the identity of the metal ions. In contrast to the previous assumptions of a diiron site, Hertweck and co-workers could demonstrate from anomalous dispersion effects in the X-ray diffraction analysis that the dinuclear site in the crystallized AurF is composed of two manganese ions. However, the data also indicate the presence of 15% iron in the crystal. In agreement with the crystallographic findings, the authors observed a preferred uptake of manganese over iron by the protein. The authors attribute this observed specificity for manganese to the additional histidine residue present. Furthermore, preliminary ESR measurements indicate that the manganese-specific signal undergoes a significant change upon reaction with $H_2O_2$, thus illustrating the participation of a manganese species in the enzymatic reaction.[14]

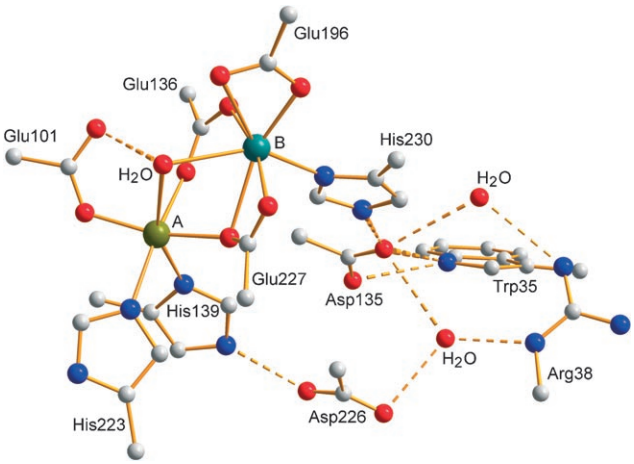



***Figure 3.*** Dinuclear active site of the N-oxygenase AurF of *S. thioluteus*; gray C, red O, blue N. Asp135, Asp226, Trp35, and Arg38 are part of a possible PCET pathway. For the assignment of Mn/Fe to sites A and B, see the text.

This specificity of AurF for manganese is quite remarkable, since there is no apparent chemical feature in the relevant binding pockets that would allow for such a preference for either of the two metal ions. This is consistent with the observation that it is, for example, possible to introduce both iron as well as manganese into bacteria ribonucleotide reductases, although the manganese form is inactive.[16] A possible explanation for the specificity of AurF could be the differentiation between the two metal ions at an early stage of protein assembly, namely a specific protein folding.[17] Interestingly, there are other dimanganese enzymes—a catalase of *Lactobacillus plantarum*[18] and arginases[19] that also contain carboxylate-rich metal-binding sites—but where each manganese ion is only coordinated by one histidine residue. This leads to the question as to whether the additional histidine residue observed in the active site of the N-oxygenase AurF is actually responsible for the specific metal uptake or rather supports the functional variation by adjusting the reactivity of the active site.

Based on the similar spectroscopic properties of these two unusual four-helix bundle proteins—the ribonucleotide reductases R2 of *C. trachomatis* and the N-oxygenase AurF of *S. thioluteus*—Krebs and Bollinger et al. concluded that AurF should also accommodate a heterodinuclear {MnFe} site.[20] A possible reaction sequence for the N-oxygenase AurF arising from this analogy is presented in Figure 1c. The $\{Mn^{IV}Fe^{IV}\}$ intermediate is formed upon activation of the reduced $\{Mn^{II}Fe^{II}\}$ species with dioxygen, as is observed for the R2 subunit of *C. trachomatis* (Figure 1b). This $\{Mn^{IV}Fe^{IV}\}$ intermediate could oxidize the substrate in a two-electron step to give a $\{Mn^{III}Fe^{III}\}$ system. In analogy to bacterial multicomponent monooxygenases, this state could again be converted into the reduced $\{Mn^{II}Fe^{II}\}$ form. The catalytic activity observed in the presence of $H_2O_2$ by Hertweck and co-

workers could be caused by the direct oxidation of the $\{Mn^{III}Fe^{III}\}$ species to form the active $\{Mn^{IV}Fe^{IV}\}$ intermediate. This shunt pathway is also observed for monooxygenases such as sMMO or P450. In addition to mechanistic aspects, the presence of a heterodinuclear {MnFe} site could also account for the iron content of the AurF samples, as observed by Hertweck and co-workers.

From the combined reported results, it is even possible to attempt the assignment of the metal ions in the heterodinuclear active sites located in the crystal structures of the ribonucleotide reductases R2 of *C. trachomatis* and the N-oxygenase AurF of *S. thioluteus*; in both cases position A could correspond to the manganese ion and position B to the iron ion (see Figures 2 and 3). In the case of the ribonucleotide reductases R2 of *C. trachomatis*, this assignment is consistent with the observed change exclusively in the oxidation state of the Mn center ($\{Mn^{IV}Fe^{III}\}\Leftrightarrow\{Mn^{III}Fe^{III}\}$) in the activated state and the spatial proximity of this position to the electron-transfer chain (Asp226 and Trp61). In the case of AurF, the second histidine residue found in one of the two binding pockets could induce the chemical difference needed for the activation of dioxygen by the $\{Mn^{II}Fe^{II}\}$ species. Furthermore, by comparison with the structures of carboxylate-bridged diiron sites of other enzymes one can conclude that a heterodinuclear $\{Mn^{III}Fe^{III}\}$ species should be present in RNR R2 and AurF, since the reduced diiron enzymes with divalent metal ions—in contrast to RNR R2 and AurF—in general contain exclusively bridging carboxylate residues

Based on their hypothesis of a heterodinuclear metal site, Krebs et al. performed a database search and found in more recent entries seven proteins of unknown function where the relevant residues were highly conserved.[20] The sequence homology not only includes the direct coordination environments of the metal ions (His139, His223, His230, Glu101, Glu136, Glu196, and Glu227), but also residues that are known from other systems to participate in the formation of hydrogen bonds (Asp226, Asp135, Trp35, and Arg38). The latter homology is particularly remarkable, since it corresponds to a typical structural motif of an electron-transfer pathway known for class I ribonucleotide reductases. Within this motif, the arginine residue located at the protein surface is also highly conserved, except for one case where arginine is replaced by glutamine. This finding suggests that the hydrogen-bonded residues located near the active site and opposite the substrate channel is part of the electron-transfer chain for the reductive regeneration of the proposed initial $\{Mn^{II}Fe^{II}\}$ state (Figure 1c).

The observed sequence homology of AurF with seven other proteins has led Krebs et al. to postulate that AurF of *S. thioluteus* is the first member of a new family of Mn/Fe-oxygenases.[20] The question of the concrete functions of the related proteins and whether more members of the new family of MnFe enzymes can be found are exciting tasks of future research. These results will definitely stimulate the search for suitable heterodinuclear model systems and reveal interesting new aspects of binuclear enzymes.

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