Oxygen Activation at the Diiron Center of Ribonucleotide Reductase

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RIBONUCLEOTIDE REDUCTASES ARE KEY ENZYMES IN THE biosynthesis of DNA as they catalyze the conversion of ribonucleotides to deoxyribonucleotides (*1, 2*). Amazingly, they use a diverse combination of metal centers and organic cofactors for this transformation. The *Escherichia coli* enzyme has a (μ-oxo)diferric unit together with a tyrosyl radical (*3*); similar enzymes are found in viruses and higher organisms (*4*). The *Lactobacillus leichmannii* enzyme, prototypical for eubacteria, requires adenosylcobalamin (coenzyme B_{12}) as cofactor (*5*). The recently characterized reductase from *Brevibacterium ammoniagenes* is suggested to have a dinuclear manganese center (*6*) that may function like the diiron unit of the *E. coli* enzyme. Finally, another reductase from *E. coli* was identified when the cells were grown under anaerobic conditions; this enzyme appears to require *S*-adenosylmethionine and an as yet unidentified metal cofactor for catalytic activity (7). Despite these differences in cofactor requirement, the mechanism for ribonucleotide reduction appears essentially the same, at least for the well-studied Fe- and Co-dependent enzymes (*2*). In both cases, there is strong evidence for the participation of a protein radical moiety that abstracts the C-3′ hydrogen to initiate the deoxygenation reaction; subsequent steps appear identical. Furthermore, a set of conserved thiols in both enzyme types appears likely to provide the reducing equivalents needed for the deoxygenation.

The mechanism for the formation of the initial radical differs for the enzyme types, however. For the cobalamin-dependent enzyme, homolysis of the adenosyl-cobalt(III) bond generates the adenosyl radical and cobalamin(II) (*8, 9*). It is clear that the adenosyl radical itself does not actually abstract the C-3′ hydrogen, but does so via a protein radical it generates (*5*). For the iron-dependent enzyme, the tyrosyl radical is formed in an O_2-dependent reaction involving the iron centers (*10*). The tyrosyl radical is buried 10 Å deep in the B2 protein (*3*), while the nucleotide binding sites are on the B1 protein (*11*). In this case, the tyrosyl radical must generate a protein radical (presumably via long-range electron transfer) on the B1 subunit that acts as the actual C-3′ hydrogen abstraction agent.

The O_2-dependent formation of the tyrosyl radical on the *E. coli* reductase involves the oxidation of the iron centers from Fe(II) to Fe(III) (*10*). The intermediates observed in this process are the subjects of the papers by Stubbe and her collaborators (*12*). To fully appreciate the significance of these observations, we must consider the results in the context of the chemistry of an emerging subclass of iron proteins with diiron active sites called the diiron-oxo proteins (*13*). Hemerythrin, the dioxygen carrier for certain marine invertebrates, is prototypical of this group. X-ray crystallography (*14, 15*) and extensive spectroscopic investigations (*13*) have established the structures of deoxyhemerythrin and oxyhemerythrin (Fig. 1), the novel features being the diiron site bridged by the (hydr)oxide and two carboxylates. These structural features have now been modeled in a number of synthetic complexes (*13*). In the oxygenation-deoxygenation equilibrium, dioxygen binds to the (μ-hydroxo)diferrous center, engendering a (μ-oxo)diferric hydroperoxide complex; thus the process entails the reversible transfer of two electrons and a proton from the diiron unit to the bound dioxygen (*15*).

On the basis of similarities of spectroscopic and magnetic properties, it has been proposed that the diiron centers of ribonucleotide reductase and methane monooxygenase have structures related to that found for hemerythrin (*13*). The recently solved crystal structure of the B2 protein of ribonucleotide reductase (RRB2) shows a dibridged diiron core with a predominantly oxygen-ligand environment (Fig. 1) (*3*). EXAFS (*16*) and Mössbauer (*17*) data on methane monooxygenase suggest a diiron core bridged by hydroxide and carboxylate in a similarly oxygen-rich ligand environment. The differences in terminal ligands between hemerythrin and the other two proteins may reflect the different functions of the three proteins, with nitrogen-rich ligation promoting reversible oxygen binding in the case of hemerythrin and oxygen-rich ligation favoring oxygen activation in the cases of RRB2 and methane monooxygenase.

A common mechanism for oxygen activation for both enzymes is proposed in Fig. 2 and incorporates many elements found in heme peroxidase and cytochrome P450 chemistry (*18*). For both enzymes, it has been demonstrated that it is the diferrous form that reacts with dioxygen (*10, 19*) and presumably affords a diferric peroxide complex as in hemerythrin. Interestingly, the addition of H_2O_2 to the diferric forms of both enzymes elicits the same reactivity as observed for reactions of the reduced enzymes with O_2, albeit much less efficiently (*20–22*). The availability of this peroxide shunt pathway strengthens the arguments favoring the participation of a diferric peroxide species in the mechanism.

The diferric peroxide complex is then proposed to decompose into high valent species that are diiron analogs of heme peroxidase compounds I and II (*19, 22*). A key observation in support of the involvement of such high valent species is the observation that oxygen atom donors, such as iodosobenzoate and peracids, react with the diferric RRB2 to generate measurable amounts of tyrosyl radical (*22*). The ability of oxygen atom donors to mediate cytochrome P450-catalyzed oxygenations strongly argues for the participation of an iron-oxo species in the catalytic cycle of the latter enzyme (*18*). Thus by analogy to cytochrome P450, the compound

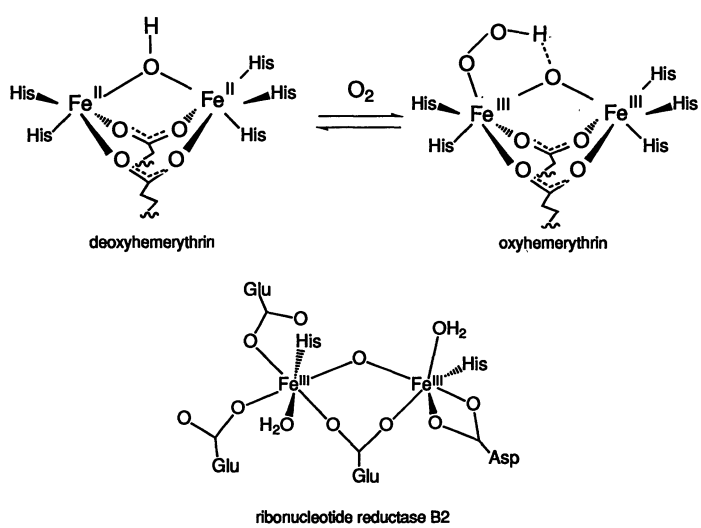

Fig. 1. Iron sites of hemerythrin, in its deoxy and oxy forms, and ribonucleotide reductase in its diferric form.

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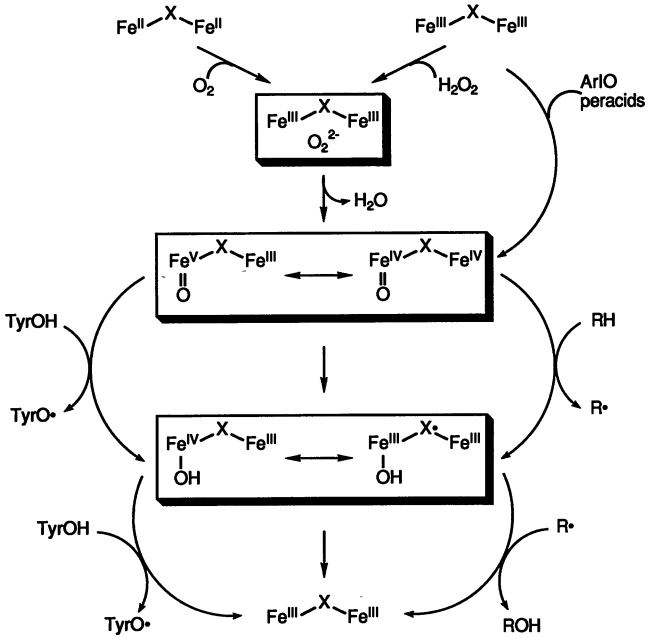

Fig. 2. Proposed mechanism of oxygen activation for ribonucleotide reductase (left) and methane monooxygenase (right).

I analog in the methane monooxygenase cycle is proposed to abstract a hydrogen atom from methane, generating the compound II analog and methyl radical, which in turn react with each other to afford the diferric enzyme and methanol by oxygen rebound (*23*). The RRB2 cycle is analogous to that of horseradish peroxidase (*18*), wherein the analogs to compounds I and II are both capable of oxidizing Tyr^{122} to its radical form. These proposed high valent intermediates, which are extrapolated from established heme chemistry, raise the particularly interesting question of how such high valent species may be stabilized in a nonheme environment.

To what extent is there direct spectroscopic evidence for any of these proposed intermediates? Stubbe's elegant studies on the reaction of diferrous RRB2 with O_2 to assemble the diferric center-tyrosyl radical cofactor reveal the participation of two transient species. The first intermediate is quite short-lived (~0.3 s in the absence of reductant) and is characterized by a visible absorption maximum at 570 nm. Such long wavelength features can be observed in synthetic ferric peroxide complexes (*13*); particularly relevant is the transient complex observed when $[Fe^{(II)}_2(N\text{-Et-HPTB})(O_2CC_2H_5]^{2+}$ (*N*-Et-HPTB is *N*,*N*,*N*′,*N*′-tetrakis(*N*-ethylbenzimidazol-2-ylmethyl)-2-hydroxy-1,3-diaminopropane) is exposed to O_2, which exhibits a visible maximum at 570 nm (*24*). Spectroscopic evidence suggests that O_2 binds in a μ-1,2 mode to the diferrous complex.

In the RRB2 study, the first intermediate is reduced by one electron to produce the second intermediate; the electron can be provided by Tyr^{122} generating the catalytically important Tyr^{122} radical or by other reductants (ascorbate or Fe(II)). This species corresponds in oxidation state to heme peroxidase compound II (with one oxidizing equivalent above Fe(III)). Its longer lifetime (~1 s) allows it to be generated in nearly quantitative yield and has facilitated its characterization by EPR and Mössbauer spectroscopy. Being formally Fe(III)Fe(IV), this intermediate would be expected to show an EPR signal typical of $S = 1/2$, if antiferromagnetic coupling is assumed. Indeed a signal near $g = 2$ is observed, which is split by ^{57}Fe hyperfine coupling, implicating at least one of the iron centers with this signal. Amazingly, the Mössbauer spectrum of this species provides no evidence for an Fe(IV) center but instead two distinct high spin Fe(III) ions in a 1:1 ratio; it is clear from the magnetic hyperfine spectra that the unpaired spin is delocalized onto both iron centers, albeit to different extents. Thus this intermediate must be formulated not as Fe(III)-X-Fe(IV) but as its valence tautomer Fe(III)-X•-Fe(III) with its oxidizing equivalent stored at a ligand (proposed in the scheme as a bridging ligand). There are other instances among metalloproteins where oxidizing equivalents are stored at different sites in a related series. Compound I of horseradish peroxidase, formally Fe(V), is formulated as $[(porphyrin^{+\cdot})Fe(IV)=O]^{+}$ (*18*), while the isovalent species for cytochrome c peroxidase consists of a [(porphyrin)Fe(IV)=O] center with a nearby amino acid radical (*25*).

Stubbe and co-workers have thus been able to observe two of the three intermediates proposed in Fig. 2. In both instances, the diiron unit appears to be in the diferric oxidation state with the oxidizing equivalent in the compound II analog stored on a ligand radical. With respect to the compound I analog, the fact that it is not observed does not necessarily imply its nonexistence, since the kinetics of its formation and reduction may disfavor its being observed. Furthermore, invoking its participation in the mechanism most easily rationalizes the results with oxygen atom donors. Very recently, a synthetic precedent for the formation of a compound I analog in a nonheme iron environment has been provided by Leising *et al.* (*26*). They have found a transient species in the reaction of $Fe_2(TPA)_2O(ClO_4)_4$ [TPA is tris(2-pyridylmethyl) amine] with H_2O_2 capable of hydroxylating alkanes, thus potentially modeling methane monooxygenase activity. Spectroscopic evidence suggests this species to be $[(TPA^{+\cdot})Fe^{IV}=O]^{3+}$, a formulation amazingly similar to that of horseradish peroxidase compound I.

The exciting results reported by Stubbe and co-workers represent the first reactive intermediates observed for this class of enzymes in their reaction with dioxygen. These results, together with relevant biomimetic chemistry that is also emerging, lend strong credence to the mechanisms of oxygen activation proposed for methane monooxygenase and ribonucleotide reductase.

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