Dioxygen Is the Source of the μ-Oxo Bridge in Iron Ribonucleotide Reductase*

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The formation of the iron-radical cofactor in the R2 subunit of ribonucleotide reductase has been monitored by resonance Raman spectroscopy. The dimeric cluster in reduced R2 functions as a tyrosine oxidase; it uses O2 to oxidize Tyr-122 to a stable radical and results in an oxo-bridged dimeric cluster. The Phe-122 mutant produces an identical dinuclear iron center and provides a simplified model for O2 activation. Oxidation with 18O2 results in quantitative incorporation of 18O into the dimeric cluster as evidenced by the 13-cm−1 downshift in the Fe-O-Fe stretching vibration at 500 cm−1. Thus, O2 must be coordinated to the diiron center during O-O bond cleavage. When the Phe-208 adjacent to the dimeric cluster is mutated to Tyr, reaction with O2 results in its oxidation to dihydroxyphenylalanine (DOPA-208) and subsequent coordination to Fe as a catecholate ligand. The Fe-O(catecholate) stretching modes at 512 and 592 cm−1 shift by −13 and −8 cm−1, respectively, when the oxidation is performed in H218O. These isotope shifts indicate that the second oxygen atom of DOPA-208 originates from H2O rather than O2. Taken together, our results are consistent with a μ-1,1-peroxide and a high valent iron-oxo species as reaction intermediates. A common pathway for oxygen activation by the related iron-oxo enzymes methane monooxygenase and fatty acid desaturase is proposed.

Ribonucleotide reductase (RnR)1 catalyzes the conversion of ribonucleotides to deoxyribonucleotides and, therefore, is a crucial enzyme in DNA biosynthesis (1). Iron-containing RnR is found in bacteria and viruses as well as in eukaryotes ranging from yeasts to mammals (2,3). The enzyme consists of an R1 homodimer with two substrate binding and regulatory sites and an R2 homodimer with two dinuclear iron centers and associated tyrosyl radicals. RnR is the first representative of a growing class of protein-radical enzymes (4) containing endogenous amino acid-derived radicals. The x-ray crystal structure of the R2 subunit from *Escherichia coli* shows that the kinetically stable tyrosyl radical, located at position 122 (5), is too far removed from the R1 subunit to participate directly in hydrogen atom abstraction from the substrate (6). The probable role of the tyrosyl radical is to produce a transient radical at the active site via long range electron transfer (7, 8).

The focus of the present study is the mechanism of formation of the iron-radical cofactor within the R2 subunit. The structure of the dimeric site in met R2 (which lacks the tyrosyl radical) is shown in Fig. 1. The two iron atoms are bridged by an oxo and a carboxylate group, and each iron is additionally coordinated to one histidine nitrogen, two carboxylate oxygens, and a water molecule. The radical-bearing residue, Tyr-122, has its phenolic oxygen situated only 5 Å away from Fe-1. The radical is, however, embedded in a hydrophobic pocket consisting of side chains from Phe-208, Phe-212, Ile-231, and Ile-234 that is believed to be responsible for its stabilization.

The major role of the dinuclear iron center in RnR is in the formation of the tyrosyl radical cofactor (9, 10). This reaction is O2-dependent and occurs spontaneously upon addition of Fe(II) and O2 to R2 apoprotein.

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[Tyr-122]+2Fe^{3+}+O_2+H^+\rightarrow [Fe^{3+}-O-Fe^{3+}][Tyr-122'] + H_2O
\]

**Reaction 1**

One proton is released by Tyr-122 as it forms a deprotonated radical (11, 12). This radical can also be produced by reacting met R2 with H218O or single oxygen-atom donors (*e.g.* chloroperoxycarboxylate, monoperophosphorate, or iodosobenzene) (13, 14). These results suggest the involvement of peroxo and high valent iron intermediates similar to those observed with heme-containing enzymes such as peroxidases, cytochrome P-450 monooxygenase, and cytochrome c oxidase (15-17). However, neither of these intermediates has been detected in the R2 subunit by rapid freeze-quench techniques, indicating that, if formed, they are quite short-lived (18, 19).

Our knowledge of the dinuclear iron center in the R2 subunit of RnR has been further enhanced by resonance Raman (RR) spectroscopy. The Fe-O-Fe moiety in met and active R2 gives rise to an intense oxo → Fe(III) charge-transfer band at 370 am (20). Excitation within this absorption band reveals an Fe-O-Fe symmetric stretch at 493 cm−1 which shifts to 480 cm−1 upon incubation in H218O (21, 22). The slow rate of exchange of the bridging oxo group (1/2 ~ 15 min) is indicative of a solvent-inaccessible site (21). We have now performed the oxidation of the *E. coli* R2 subunit in the presence of 18O2 and have determined that the oxygen of the Fe-O-Fe moiety originates from molecular oxygen.

We have also investigated the role of the diiron center and O2 in the oxidation of Tyr-208 to DOPA in the F208Y mutant of R2.
The Tyr at position 208 in the hydrophobic pocket surrounding the iron/radical site (Fig. 1) is oxidized in preference to Tyr-122 (24). The result is an inactive enzyme in which the DOPA-208 product has become iron-coordinated, as determined by UV-visible and RR spectroscopy and x-ray crystallography (23, 24). We have now performed the oxidation of Tyr-208-containing apoprotein in the presence of oxygen isotopes and determined by RR spectroscopy that the second oxygen atom in DOPA-208 originates from solvent rather than molecular oxygen. The oxygen inserted into Tyr-208 could be derived from an Fe(IV)-coordinated terminal OH. These findings are consistent with the known ability of reduced R2 to form a variety of free radical products following, or in conjunction with, O-O bond cleavage (14, 25).

EXPERIMENTAL PROCEDURES

Protein Purification—Wild-type, Y122F, and F208Y R2 proteins were purified according to standard procedures as described earlier (26). ApoY122F R2 was prepared by treating the isolated protein with chelating agent (10). ApoF208Y R2 was isolated by growing E. coli in low iron medium (27).

Oxidation in the Presence of Oxygen Isotopes—ApoR2 protein (~2 nm) in 0.05 M Tris-Cl (pH 7.6) was reacted with ~4 eq of ferrous ammonium sulfate in an anaerobic solution of the same buffer. In order to keep iron reduced, the latter solution also contained ascorbic acid at 25% of the iron concentration. For reaction with 18O2, there was sufficient oxygen in the protein buffer to yield a fully oxidized iron center. For the reaction with 16O2, an anaerobic mixture of iron and ascorbate was added to an anaerobic solution (~100 μl) of the protein and incubated for 10 min. Then 500 μl of 16O2 (95 atom % (Cambridge Isotope Laboratories, Woburn, MA) or 99 atom % (Ion Services, Inc., Summit, NJ) was added via a gas-tight syringe. The color of oxidized protein appeared immediately. In the case of apoY122F, the sample was reacted with 5.3 Fe/R2, then transferred to a capillary and frozen in liquid nitrogen 140 s after 18O2 addition. In the case of apoF208Y, which forms a stable product, the oxidation was carried out on a more dilute sample, and the protein was concentrated using a Microcon-30 (Amicon). RR measurements on the resultant DOPA-208 R2 were performed in the liquid state.

For oxidation of F208Y in the presence of H2 16O, the apoF208Y protein was first exchanged with buffer prepared in H2 18O (97 atom %; Yeda, Rehovot, Israel) by two 5-fold dilution and concentration steps using a Microcon-30 device. The resultant sample was estimated to contain 90% H2 18O. The final concentration of oxidized protein from any of the above procedures was typically 1–2 nm.

Resonance Raman Spectroscopy—Raman spectra were recorded on a computerized Jarrell-Ash spectrophotometer using an RCA C31034 photomultiplier tube and an Ortec model 9302 amplifier-discriminator. The excitation source was a Spectra-Physics 2025-11 (Kr+) laser. For the Y122F R2 mutant, spectra were collected in a 150° backscattering geometry on samples in capillaries inserted into a copper rod in a Dewar flask filled with liquid nitrogen (28). For the R2 F208Y mutant, spectra were obtained in a 90° scattering geometry from samples in capillaries cooled by a copper rod immersed in an ice bath (28). Raman frequencies were corrected using indene as a standard (29) and are accurate to ±1 cm⁻¹.

RESULTS

Origin of Oxo Bridge in R2 Mutant (Y122F)—The active (radical-containing) R2 subunit of RnR exhibits an Fe-O-Fe symmetric stretch at 493 cm⁻¹ in its resonance Raman spectrum (22). The same vibrational frequency is observed for Fe(I1) complex of Y122F R2 was reacted with 16O2 (air) (A) and 18O2 (B). The sample was frozen in liquid N2 within 140 s of exposure to 18O2. The spectra were obtained on ~1.6 nm samples in capillaries at 90 K (160° scattering geometry) using 413.1-nm excitation (15 mW), a scan rate of 0.5 cm⁻¹/s, resolution of 10 cm⁻¹, and accumulations of 16 and 4 scans, respectively.

Previous RR studies have indicated that the p-oxo bridge in active or met R2 exchanges with solvent oxygen with a τ1/2 of ~15 min at 4 °C (21). Thus, in order to determine whether the oxygen atom in the Fe-O-Fe moiety originates from O2, it was necessary to freeze samples in liquid nitrogen immediately after exposure to O2. The Fe(II) complex of Y122F R2 was first oxidized in 16O2 and then frozen in liquid nitrogen. Its resonance Raman spectrum reveals a ν(Fe-O-Fe) mode at 500 cm⁻¹ (Fig. 2A). This frequency is 7 cm⁻¹ higher than that observed at 293 K (22), but close to the 496-cm⁻¹ value obtained for native and met R2 at 90 K (21). Oxidized protein was next prepared by reacting reduced Y122F R2 with 18O2 and freezing of the sample in liquid nitrogen within two minutes of oxygen addition. The RR spectrum of the resulting sample shows that the major vibrational component is now at 487 cm⁻¹ (Fig. 2B). The
isotope shift of -13 cm\(^{-1}\) is similar to the -15 cm\(^{-1}\) value reported previously for active and met R2 in H\(_2\)\(^{18}\)O (22) and, therefore, indicative of a \(ν_1\)(Fe-\(^{18}\)O-Fe) mode. The minor \(ν_2\)(Fe-\(^{18}\)O-Fe) component at 500 cm\(^{-1}\) can be accounted for by back exchange of the Fe-\(^{18}\)O-Fe moiety with solvent (H\(_2\)\(^{16}\)O) during the \(-2\)-min period prior to freezing and by photochemically promoted oxo-bridge exchange during laser irradiation (11). These Raman experiments demonstrate that one atom of \(^{16}\)O has been incorporated into the \(µ\)-oxo bridge in an essentially quantitative fashion and provide the first direct evidence that O\(_2\) can be the source of oxygen in an Fe-O-Fe cluster in a protein.

**Origin of Catecholate Oxygen in R2 Mutant (DOPA-208)—**In the F208Y mutant of R2, the Tyr at position 208 (Fig. 1) is oxidized to DOPA during normal cell growth. The protein, as isolated, is blue in color (\(λ_{\text{max}}\) at 720 nm) due to the presence of an Fe-coordinated catecholate (23, 24). We have been able to investigate the source of oxygen in the DOPA-208 moiety by growing cells in an iron-depleted medium, as described previously (24). The resultant protein obtained from these cells is >90% apoprotein with Tyr at position 208. Reaction of this apoprotein with Fe(II) in air yields the same DOPA-208 species as that produced by the cells (24). The RR spectrum of this species (Fig. 3A) is identical to that reported previously for the F208Y mutant (23). The prominent features at 512, 592, 619, 1143, 1263, 1319, 1475, and 1569 cm\(^{-1}\) are characteristic of a ferric catecholate complex with bidentate ligation of the catechol (31). A similar set of RR modes has been observed in a number of synthetic complexes, as well as in iron-containing proteins such as tyrosine hydroxylase and phenylalanine hydroxylase that have reacted with exogenous catechols (Table I).

Deuteration of the catechol ring causes many of the spectral features between 610 and 1570 cm\(^{-1}\) to undergo shifts (Fig. 4, A, B, and C). A closer examination reveals that iron-catecholate modes at 512, 592, and 619 cm\(^{-1}\) have shifted to lower energy by 13, 8, and 2 cm\(^{-1}\), respectively (Fig. 4, A and B). However, no \(^{18}\)O-dependent shifts could be detected in any of the catecholate ring modes above 1100 cm\(^{-1}\) (Table I). A similar pattern of oxygen-isotope shifts has been observed in the RR spectrum of the non-heme iron protein.
tyrosine hydroxylase, reacted with $^{18}$O-labeled DOPA. These results prove that the new OH group in DOPA-208 is derived from a solvent-exchangeable oxygen.

In order to demonstrate that solvent-oxygen incorporation in DOPA-208 occurs only during the oxidation reaction, a sample of DOPA-208-containing protein isolated from cells grown in iron-rich medium was incubated in H$_2^{18}$O. In this case, the RR spectrum shows no $^{18}$O dependence (Fig. 4C), indicating that neither of the oxygen atoms in DOPA-208 is solvent-exchangeable. In addition, the failure to observe any $^{18}$O-sensitive RR modes that could be attributed to $\nu$(Fe-O-Fe) is in agreement with the absence of a $\mu$-oxo bridge as seen in the x-ray structure of the DOPA-208 protein (24).

### Table I

| Sample* | Raman frequencies (cm$^{-1}$) | Ref. |
|---------|------------------------------|------|
| DOPA-208 | 533 621 1154 1262 1322 1359 1487 1572 | 32 |
| Tyr hydroxylase + cat | 527 604 635 1130 1267 1320 1426 1476 | 34 |
| R2 DOPA-208 | 512 592 619 1143 1263 1319 1350 1475 1569 | This work |
| R2 [3-13ODOPA-208 | 499 584 617 1143 1263 1319 1350 1475 1569 | This work |

* cat, catecholate; cat-d$_4$, perdeuterated catecholate.
| a Isolated as an iron-catecholate complex.

Iron-Catecholate Vibrations of DOPA-208—Oxidation of Tyr-208 to DOPA in the presence of H$_2^{18}$O results in the appearance of three $^{18}$O-sensitive vibrations at 512, 592 and 619 cm$^{-1}$ (Fig. 4), despite the fact that only a single $^{18}$O atom has been inserted at the C-3 position of the tyrosine ring. The crystal structure of the DOPA-208 protein reveals a slight inequivalence of the catecholate oxygens due to the fact that the original oxygen at the C-4 position also serves as a weakly coordinated bridging group to Fe$_2$ (24). If the inequivalence were great enough to destroy the symmetry of the chelate ring, one should observe two separate Fe-O stretching modes with only one showing $^{18}$O sensitivity. Since this is not the case, it is likely that the $^{18}$O-dependent vibrations involve coupled motions of the two Fe-O bonds in the chelate ring. Such coupling is supported by the fact that the observed isotope shifts of -13, -8, and -2 cm$^{-1}$ actually sum to the theoretical value of -23 cm$^{-1}$ for an isolated Fe-O stretch at 512 cm$^{-1}$.

In order to gain a better understanding of the origin of the $^{18}$O-dependent modes at 512, 592, and 619 cm$^{-1}$, we investigated their polarization behavior. All three peaks are strongly polarized with depolarization ratios of 0.34, 0.38, and 0.37, respectively. These values are close to the expected value of $\leq 0.3$ for a totally symmetric vibration in a RR spectrum. The Raman spectra of metal oxalate and acetylated catecholate complexes exhibit only a single intense polarized mode at -580 and -460 cm$^{-1}$, respectively, that has been assigned to $\nu$(M-O) of the chelate ring (37). The occurrence of three vibrations with $\nu$(M-O) character in the ferric catecholate complex of DOPA-208 R2 is most likely due to coupled motions of the 5-membered Fe-chelate ring with the benzene ring of the DOPA ligand. Since the 512 cm$^{-1}$ peak has the greatest $^{18}$O dependence, it is likely to have the greatest $\nu$(Fe-O)$_{catecholate}$ character.

The failure to observe any $^{18}$O-dependent vibration in the high frequency region of the RR spectrum of the DOPA-208 protein (Table I) is surprising. By analogy to ferric phenolate complexes, an in-phase stretching vibration of the two catecholate C-O groups is expected to yield a Raman mode near 1300 cm$^{-1}$ and, in fact, was originally suggested as the origin of the peak at -1260 cm$^{-1}$ (32). However, the large downshift of this mode in response to deuteration of the catechol ring (Table I) makes such an assignment unlikely. It is possible that the $\nu$(C-O) vibration is only weakly resonance-enhanced and, thus, difficult to detect.

**DISCUSSION**

Incorporation of Oxygen into the $\mu$-Oxo Bridge—The data presented in this study clearly demonstrate that one of the oxygen atoms of O$_2$ is retained in the Fe-O-Fe cluster during the oxidation of Y122F R2. A possible mechanism for this reaction is given in Fig. 5. The crystal structure of the iron center...
in reduced R2 (S211A mutant) shows that each of the iron atoms is four-coordinate with two bridging carboxylates (Glu-115 and Glu-238) and one additional histidine N and monodeuterate carboxylate per Fe (38). This structure is similar to that of Mn(II)-substituted R2 (39), except that the Fe(II) form lacks coordinated water molecules. We propose that dioxygen binds in a μ-1,1 fashion, displacing Glu-238. The reorientation of Glu-238 from a bridging position in reduced R2 to a terminal position in met R2 (Fig. 1) is typical of the carboxylate shift seen with model complexes (40). The subsequent reduction of dioxygen to peroxide would be accompanied by the uptake of aqua ligands, making each Fe(III) six-coordinate.

There is, as yet, no direct experimental proof for a peroxy species in RnR. However, previous studies (13, 14) showing that met R2 can react with H₂O₂ to produce the tyrosyl-123 radical strongly argue for its existence. Our Raman result that the μ-oxo of Y122F R2 is quantitatively derived from molecular oxygen (Fig. 2) makes it likely that the peroxy bridges the two Fe(III) atoms in a μ-1,1 fashion (Fig. 5). The other oxygen atom of peroxy could then associate with the hydrogen of a coordinated water, generating a μ-1,1-hydroperoxide. This type of hydroperoxide coordination has been observed in the X-ray crystal structure of the dicobaltic [Co₃(OOH)(NH₃)(OOH)]⁺ complex (41) and has been proposed for the dicupric [Cu₂(OOH)(XYL-O⁻)]²⁺ complex based on its extended X-ray absorption fine structure (42). A similar type of μ-1,1-peroxo intermediate is proposed to form in the O₂-dependent oxidation of the diferrous Fe₂O₂CH₄(BIPPH₃)₂ complex (43). In this system, reaction with ¹⁸O₂ also engenders quantitative incorporation of ¹⁸O into the Fe-O-Fe moiety of the diferric product.

In the proposed mechanism in Fig. 5, the peroxy intermediate undergoes heterolytic O-O bond cleavage, releasing a water molecule and forming a high valent iron intermediate. Although this high valent species is apparently too short-lived to be detected in RnR (18, 19), there are several lines of evidence supporting its existence. First, single-oxygen atom donors such as peracids are capable of reacting with met R2 to generate the tyrosyl radical (14). Second, the oxygen insertion reaction involving Tyr-208 suggests an activated oxygen coordinated to a high valent Fe (see below). Finally, methane monoxygenase, a closely related iron-oxo enzyme (44), has been shown to be capable of forming a Mössbauer-detectable Fe(IV) intermediate (45).

In RnR, any high valent intermediate that forms rapidly accepts one electron from exogenous iron/ascorbate and another electron from an endogenous residue, L, to generate the free radical intermediate II (18, 25). The same intermediate II has been observed in both wild-type and Y122F R2 by rapid freeze-quench EPR and Mössbauer spectroscopy. Its sharp singlet signal at Γ = 2.00 is different from that of the Tyr-122 radical and is broadened by ⁵⁷Fe substitution, suggesting that L is an iron ligand (18, 30). In Y122F R2 (30), intermediate II accepts another electron from exogenous iron/ascorbate, generating met R2 (Fig. 5). In wild-type R2 (18), intermediate II instead accepts an electron from Tyr-122, generating the stable tyrosyl radical.

**Incorporation of Oxygen into Tyr-208**—In the present study, we have shown that the conversion of Tyr-208 to DOPA is accomplished by the insertion of an oxygen atom derived from solvent. A proposed mechanism for this reaction is given in Fig. 6. The initial steps proceed via a peroxy intermediate that undergoes heterolytic cleavage to a high valent iron intermediate (as in Fig. 5). The water ligands that are added during the oxidation process would be solvent-exchangeable and would be isotopically labeled when the reaction is carried out in H₂¹⁸O.

The high valent intermediate should have the ability to oxidize the nearby Tyr-208 to a tyrosyl radical. Recombination of this tyrosyl radical with an Fe(IV)-bound OH would generate DOPA.

Since there are three relatively equivalent oxygens associated with the high valent intermediate (Fig. 6), any one of them could potentially be an oxygen donor to Tyr-208. Our results for the Y122F mutant (see above) show that the bridging oxygen of the proposed high valent intermediate does not undergo significant exchange with solvent during the course of reduction to met R2. We assume that neither the Y122F nor the F208Y mutation affects the rate of bridge exchange and that the bridging oxygen is derived from O₂ in both cases. Thus, it is likely that one of the terminal OH groups is activated by being bound to Fe(IV) and, thus, reacts as an oxygen radical with Tyr-208. This mechanism explains the finding that the second oxygen atom of DOPA-208 is derived from solvent. Even though the reaction is proceeding through a high valent intermediate, it must be considered as an oxidase rather than a monoxygenase reaction since the actual oxygen atom appearing in the product is not derived from molecular oxygen.

An alternative mechanism for the formation of DOPA-208 could involve a two-electron abstraction by the high valent intermediate to generate a carboxylation instead of a free radical on Tyr-208. However, the two-electron oxidation of a phenol tends to generate a quinone methide intermediate (46–48). This type of intermediate is most susceptible to nucleophilic addition at the C-7 position, with attack ortho to the phenolic...
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Oxygen actually being disfavored. In view of the observed product, it is much more likely that Tyr-208 becomes oxygenated via a free radical intermediate. A similar free radical mechanism has been proposed for the horseradish peroxidase-catalyzed conversion of phenol to catechol (49). Such a pathway for DOPA-208 formation is consistent with the known ability of the R2 protein to generate free radical intermediates. In wild-type R2, at least two different protein-based free radicals have been detected (18, 25) in addition to the kinetically stable Tyr-122" species.

In the final step of the reaction (Fig. 6), the strongly chelating DOPA moiety must displace the bridging oxo group to form a ferric catecholate complex (24). The overall rate of formation of the Fe(III)-catecholate complex is relatively slow, having a $k_{cat}$ of 2.9 s$^{-1}$. In contrast, formation of intermediate II in wild-type and Y122F R2 (Fig. 6) has a $k_{cat}$ of 0.15 s$^{-1}$ and any high valent intermediate would be considerably more short-lived (18, 19). These results suggest that the formation of ferric R2 (DOPA-208) is rapid and that the final Fe chelation with concomitant disruption of the oxo bridge (Fig. 6) is the rate-determining step.

Comparison with Other Iron-Oxo Proteins—RnR belongs to a growing class of evolutionarily related proteins containing a magnetically coupled dinuclear iron center. This class includes the soluble methane monooxygenases (MMO) of methanotrophic bacteria (44, 50), the soluble fatty acid desaturases such as stearoyl-ACP Δ$^5$-desaturase from plant chloroplasts (51), as well as the redox-active rubrerythrin from sulfate-reducing anaerobic bacteria (52). The amino acid sequences of these proteins can be aligned to demonstrate a set of six potential iron ligands in common with RnR (Fig. 1): two sets of EXXX sequences most likely on two separate α-helices and another two Asp or Glu residues contributed by another two helices of a four-helix bundle (44, 51, 52). The structural homology with the R2 protein of RnR is supported by preliminary x-ray crystallographic data on MMO (53) and rubrerythrin (54). Thus, it is likely that all of these proteins have dinuclear iron clusters, but with some variability in the nature of the bridging oxygen species. Resonance Raman spectroscopy has demonstrated that the diferroic forms of stearoyl-ACP Δ$^5$-desaturase$^3$ and rubrerythrin$^3$ have a μ-oxo group similar to RnR, whereas the diferrous form of MMO is more likely to have a μ-OH group (50, 55, 64). The invertebrate oxygen-carrier, hemerythrin (Hr), was the first μ-oxo, diiron protein to be crystallographically characterized (56). It resembles the iron-oxo enzymes in that the Fe-O-Fe moiety is located within a four-helix bundle (57). However, Hr differs significantly from the iron-oxo enzymes in that the Fe-O-Fe moiety is oriented perpendicular rather than parallel to the helix axes, and there is no apparent amino acid sequence identity. Ligands arising from the same helix in Hr (e.g. H$_2$X$^2$H and H$_2$X$^5$E) are separated by 3 rather than 2 intervening residues. The only metalloproteins that show sequence similarity to Hr are the iron-containing purple acid phosphatases (58) and the copper-containing hemocyanins (59). A further difference between Hr and the iron-oxo enzymes is in the chemistry of the reaction with O$_2$. Whereas Hr binds and releases O$_2$ in a reversible fashion, the iron-oxo enzymes catalyze O$_2$-BOND cleavage. Thus, it is highly likely that structure of the peroxo intermediate differs from the end-on, terminal hydroperoxide structure found in oxyHr (56, 57).

In heme enzymes, heterolytic scission of the bond peroxide is believed to be facilitated by a push from the proximal axial

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