Engineering the Diiron Site of *Escherichia coli* Ribonucleotide Reductase Protein R2 to Accumulate an Intermediate Similar to H_{peroxo}, the Putative Peroxodiiron(III) Complex from the Methane Monooxygenase Catalytic Cycle

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The R2 subunit of the ribonucleotide reductase (RNR-R2[1]) from (aerobic) *Escherichia coli* and the hydroxylase component of the soluble methane monooxygenase (MMOH) from the methanotrophs *Methylococcus capsulatus* and *Methylosinus trichosporium* are the best-characterized members of a class of structurally related proteins that use carboxylate-bridged diiron(II) clusters to activate O_2 for diverse oxidation reactions.[2,3] The mechanisms by which the diiron proteins divergently control their O_2 reactions to direct different outcomes have not yet been defined. An adduct between O_2 and the diiron(II) cluster in MMOH (designated H_{peroxo}) has been characterized by stopped-flow optical absorption (broad band centered at ~700 nm, $\epsilon_{700} \approx 1500\ M^{-1}\ cm^{-1}$)[4,5] and rapid freeze-quench Mössbauer spectroscopies ($\delta = 0.66$ mm/s, $\Delta E_Q = 1.51$ mm/s for both irons).[6] A μ-(η:[1]η[1])-peroxodiiron(III) structure has been proposed for this species on the basis of its spectroscopic similarity with a crystallographically characterized model complex.[7] The presumptive peroxodiiron(III) intermediate in the RNR-R2 reaction has not yet been unequivocally identified, though some evidence has been obtained for a very short-lived species that may be similar to H_{peroxo}.[8,9] Herein we report evidence for a much longer-lived peroxodiiron(III) intermediate during O_2 activation by a site-directed mutant of RNR-R2, in which a single aspartate iron ligand (D84) is modified to glutamate (which is present at the corresponding site in MMOH).[10,11] The spectroscopic similarity of the intermediate with H_{peroxo} suggests that the two have homologous structures. Decay of the intermediate leads to production of a stable tyrosyl radical.

Upon addition of O_2 to the diiron(II) cluster of wild-type RNR-R2, an "extra" electron is rapidly ($k \geq 60\ s^{-1}$ at 5 °C[9]) transferred (from excess Fe^{2+}, ascorbate, or, under conditions of limiting reductant, perhaps the protein itself) to the diiron site, producing the formally Fe(III), Fe(IV) cluster **X**,[9,12−15] which can generate the stable radical by one-electron oxidation of tyrosine 122 during conversion to the product μ-oxodiiron(III) cluster.[12,16] We and others have postulated that it may be the unidentified peroxodiiron(III) adduct itself that accepts the extra electron.[17,18] Reductive O−O bond cleavage in RNR-R2 would contrast with the proposed mode of reactivity of H_{peroxo} in MMOH−peroxide bond cleavage without net reduction to yield the (formally) diiron(IV) cluster **Q**.[6,19−21] This mechanistic divergence might reflect (at least in part) divergent inherent reactivities of the diiron(II)−O_2 adducts in RNR-R2 and MMOH, which might arise from "tuning" of each diiron cluster by its protein chelator. Alignment (by comparison of primary[22] and tertiary structures[10,11,23−26]) of the amino acid iron ligands of RNR-R2 from *E. coli* with their cognate ligands in MMOH from *M. capsulatus* reveals a single nonidentity: the counterpart of MMOH ligand E114 is D84 of RNR-R2. We postulated that this "E to D substitution" might be important for tuning the reactivity of the RNR-R2 diiron(II) cluster and its initial adduct with O_2 to favor reductive O−O bond cleavage.[18]

To test this hypothesis, we studied O_2 activation by the diiron(II) cluster in the D84E mutant of RNR-R2, which we obtained as the apoprotein by a procedure which prevents its exposure to Fe^{2+} during overexpression and purification.[27] Reaction of apo RNR-R2-D84E with Fe(II) and O_2, or reaction of the Fe(II)-RNR-R2-D84E complex with O_2, results in the rapid development of a broad optical absorption band centered near 700 nm (Figure 1, dotted line and inset). Decay of this band is associated with development of the sharp peak of the tyrosyl radical at 409 nm[28] and broad bands of the μ-oxo−diiron(III) cluster at ~325 and ~365 nm (Figure 1, solid line). Treatment of the apoprotein with varying quantities of Fe(II) followed by excess O_2 (not shown) yields a maximum of 0.98 ± 0.05 equiv of tyrosyl radical from 3.5 ± 0.2 equiv of Fe(II),[29] for an Fe(II):

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(1) Abbreviations used: RNR-R2, R2 subunit of ribonucleotide reductase from *E. coli*; MMOH, hydroxylase component of methane monooxygenase; k_{obsd}, observed apparent first-order rate constant.

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(28) In RNR-R2-D84E, the sharp peak of the tyrosyl radical maximizes at 409 nm, which is ~1.5 nm less than in the wild-type protein.
(29) The tyrosyl radical in RNR-R2-D84E is quite stable ($k_{decay} \approx 0.0003\ s^{-1}$ at 25 °C), though somewhat less so than in the wild-type protein.

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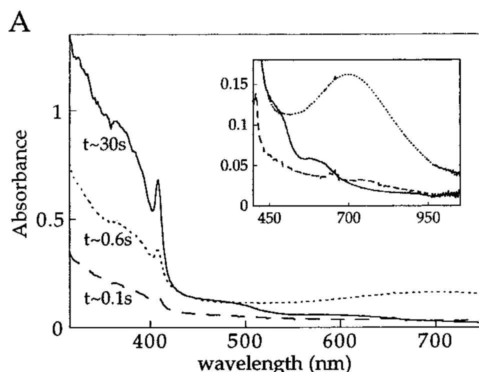

Figure 1. UV−vis absorption spectra acquired after one-to-one mixing at 5 °C of 0.25 mM apoprotein in O_2-saturated 100 mM Na-HEPES (pH 7.6) with 1 mM $FeSO_4$ in O_2-saturated 5 mN H_2SO_4. The experiment was carried out with a Hi-Tech Scientific SFA-20 Rapid Kinetics Accessory (pathlength 1 cm) and an HP8453 diode array spectrophotometer. The deadtime of the SFA-20 is approximately 0.1 s (estimated by comparison to data acquired on a KinTek Corporation Model SF-2001 stopped-flow), and the HP8453 acquires a full spectrum (190−1100 nm) every 0.1 s.

tyrosyl radical stoichiometry of 3.6 ± 0.4, which is indistinguishable from that of the wild-type protein.[16,30] In the reaction of the Fe(II)−RNR-R2-D84E complex with O_2, there is a clear temporal correlation between the rise phase of the 700 nm transient (k_{obsd} = 5.4−10 s^{-1} at 5 °C with O_2 at 33% of saturation)[31] and a lag phase preceding development of the 409 nm feature (k_{obsd} = 5.7−6.4 s^{-1}) and between the decay phase of the 700 nm transient (k_{obsd} = 0.59−0.93 s^{-1}) and the rise phase of the 409 nm feature (k_{obsd} = 0.69−1 s^{-1}).[31] An increase in O_2 concentration from 33 to 67% of saturation (at 5 °C and 1 atm) is associated with ∼1.5-fold increases in the observed first-order rate constants for both the rise phase of the 700 nm transient and the lag phase of the 409 nm feature, but has no significant effect on the rate constants for decay of the 700 nm transient and development of the 409 nm feature. An increase in temperature from 5 to 25 °C is associated with parallel ∼10-fold increases in all four k_{obsd} values, such that the correspondence is maintained. These observations strongly suggest that the 700 nm absorbing species is an intermediate on the pathway to tyrosyl radical formation.

The 4.2 K Mössbauer spectrum of a freeze-quenched sample in which the Fe(II)−RNR-R2-D84E complex was allowed to react with O_2 for 0.44 s (A_{700} maximizes at 0.3−0.4 s under these conditions) exhibits two prominent quadrupole doublets (Figure 2, spectrum A). The doublet with larger ΔE_Q (solid line plotted over the data) is attributable to Fe(II) ions (either bound or in solution). It can be subtracted away to reveal the quadrupole doublet with smaller ΔE_Q (Figure 2, spectrum B), for which the parameters δ = 0.63 mm/s and ΔE_Q = 1.58 mm/s can then be determined (solid line plotted over the data). This doublet contributes 34 ± 2% of the total iron absorption in A (percentage of solid line) which corresponds to 1.4 ± 0.2 equiv of Fe or 0.7 ± 0.1 equiv of a diiron complex. This quantity and reaction time (0.44 s) are consistent with the observed rate constants for formation and decay of the 700 nm absorbing species and the inference that it is a precursor to the 1.0 ± 0.1 equiv of tyrosyl radical. The spectra of samples that were freeze quenched at 0.1 and 1.5 s (not shown) have less intensity attributable to this doublet, and in each case the intensity correlates with the absorbance at 700 nm at that reaction time. Thus, the Mössbauer doublet is almost certainly associated with the 700 nm absorbing intermediate.[32] The δ and ΔE_Q values deduced for this species

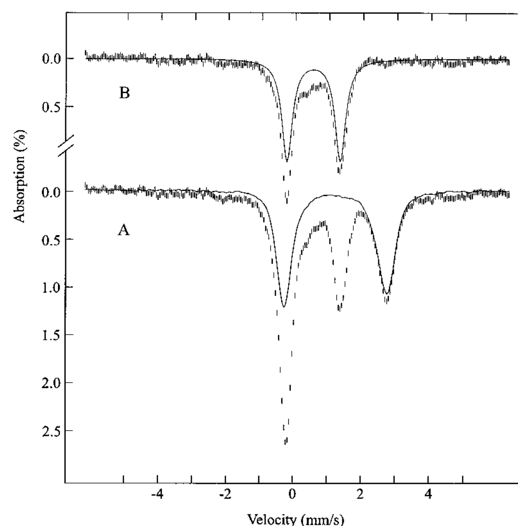

Figure 2. Mössbauer spectral analysis of a sample that was freeze-quenched 0.44 s after equal-volume mixing at 5 °C of 0.83 mM Fe(II)−RNR-R2-D84E complex (Fe(II)/dimer = 4.1) in O_2-free 100 mM Na-HEPES buffer (pH 7.6) with O_2-saturated buffer. A is the experimental spectrum, which was acquired at 4.2 K with a magnetic field of 50 mT applied parallel to the γ-beam (the spectrometer has been described[37]). The solid line over the data is the Fe(II) quadrupole doublet plotted with intensity corresponding to 49% of the total Fe absorption. Spectrum B is the result of subtracting this doublet from spectrum A. The solid line plotted over the data in B is the theoretical spectrum deduced for the intermediate species. It is plotted with intensity corresponding to 34% of the total Fe absorption.

are strikingly similar to those associated with H_{peroxo} in MMOH.[6] Moreover, spectra acquired at high magnetic field (not shown) indicate that the species is diamagnetic, as is H_{peroxo}.[33]

The observation of a diamagnetic, Mössbauer-active intermediate with δ = 0.63 mm/s and ΔE_Q = 1.58 mm/s in association with a broad optical absorption band at ∼700 nm ($\epsilon_{700} \approx 1500$ M^{-1} cm^{-1}) is strong evidence that a species similar to H_{peroxo} accumulates during O_2 activation by RNR-R2-D84E. It is not yet clear whether the D84E mutation *causes* this intermediate to form in preference to a structurally distinct adduct that forms in wild-type RNR-R2[34,35] or simply *stabilizes* (by at least 60-fold, in a kinetic sense) the same adduct as forms in the wild-type protein.[8,9] Irrespective of the answer, the formation of one equivalent of tyrosyl radical in RNR-R2-D84E implies that the pronounced change in reactivity resulting from this substitution is not sufficient to alter the outcome of the reaction to two-electron chemistry. This observation lends support to our contention that outer sphere control of the reaction mechanism, in the form of a specific pathway for transfer of the "extra" electron, is also crucial.[27,36]

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