Mechanism of Assembly of the Diferric Cluster−Tyrosyl Radical Cofactor of *Escherichia coli* Ribonucleotide Reductase from the Diferrous Form of the R2 Subunit

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Ribonucleotide reductases (RNRs) catalyze the conversion of nucleotides to deoxynucleotides, an essential and rate-determining step in DNA biosynthesis.[1] The *Escherichia coli* RNR is composed of two homodimeric subunits: R1 and R2. R1 contains the active site where nucleotide reduction takes place. R2 contains a unique dinuclear ferric cluster−tyrosyl radical cofactor[2] essential for initiation of electron transfer between R2 and R1 and subsequently nucleotide reduction.[1b,3] R2 is one of a growing class of proteins which contain dinuclear ferrous centers capable of reductively activating O_2 to generate diferric centers with distinct structures and chemical reactivities.[4] Reaction of apo R2 with Fe^{2+}, O_2, and excess reductant generates 1.4 diferric clusters[5a] and 1.2 tyrosyl radicals (•Y122) per R2.[6] Recently, this reaction has been studied in detail using stopped-flow absorption spectroscopy (SF-Abs), rapid freeze−quench EPR (RFQ-EPR), and Mössbauer (RFQ-Möss) spectroscopies.[5] The model which has evolved from these studies is shown in Scheme 1. When the extra reducing equivalent is readily available,[7] the data can be modeled by two sequential, irreversible, first-order processes, in which a paramagnetic diiron intermediate, **X**, accumulates with a k_1 of 8 s^{-1} and decays concomitantly with formation of •Y122 and the diferric cluster with a k_2 of 0.8 s^{-1}.[5] The rate constant for the formation of **X** is independent of the absolute concentrations of Fe^{2+}, O_2, and apo R2. Thus, it was postulated that the first-order rate constant of 8 s^{-1} reflects a conformational change of apo R2 required for Fe^{2+} and/or O_2 binding.[5b] This hypothesis is consistent with the available X-ray crystallographic data, which shows that the cluster binding site is >10 Å from the nearest surface, and that there is no obvious channel by which Fe^{2+} can access this site.[8] To test this hypothesis, as well as to further probe the mechanistic function of **X**, apo R2 was preloaded with Fe^{2+}, and the reaction of the diferrous-R2 complex (Fe(II)-R2) with O_2 and excess reductant was monitored by the rapid kinetic methods described previously.[5,9] The results of these studies further support the importance of a conformational change in Fe^{2+} binding and suggest that **X**, in this case as well as in the apo R2 reconstitution experiments, is responsible for oxidation of Y122 to •Y122 with an identical rate constant.

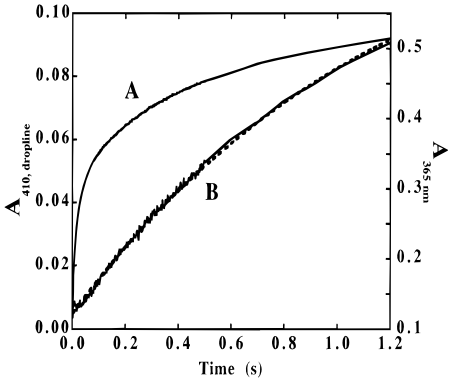
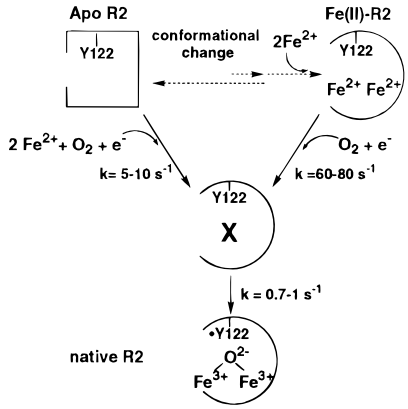


Figure 1. (A) A_{365}-versus-time trace of the reaction of Fe(II)-R2 with O_2. (B) $A_{410,dropline}$-versus-time trace of the same reaction. The theoretical curve (dotted line) is obtained by fitting the data to two sequential, first-order processes with rate constants of ∼60 and 0.85 s^{-1}.

Scheme 1



The time-resolved UV/vis spectra between 320 and 650 nm of the reaction of Fe(II)-R2 with O_2 and excess reductant[9] reveals a broad absorption feature centered at 365 nm, indicative of the formation of **X**.[5b] The A_{365}-versus-time trace exhibits no initial lag phase and gives a rate constant for the formation of **X** of 60−80 s^{-1} (Figure 1).[10] This number is an order of magnitude greater than that observed in the apo R2 reconstitution experiments. Formation of •Y122 can be uniquely monitored

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(7) Extensive studies of the reconstitution of apo R2 have indicated that the cofactor assembly reaction partitions between two pathways.[5] The partitioning depends on the ratio of Fe^{2+}/R2, which determines the availability of the "extra" reducing equivalent (in addition to the three provided by oxidation of Y122 and diferrous R2) that is required to balance the four-electron reduction of O_2 to H_2O. Only the excess Fe^{2+} reaction (Fe^{2+}/R2 = 5) is discussed in this communication.

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(9) Fe(II)-R2 was prepared by incubating apo R2 (140−600 μM) in 100 mM HEPES (pH 7.6) with 5 equiv of Fe^{2+} under anaerobic conditions. SF-Abs experiments were carried out as previously described.[5] The reaction of Fe(II)-R2 with O_2 was initiated by mixing at 5 °C equal volumes of an Ar-saturated solution containing Fe(II)-R2 in 100 mM HEPES (pH 7.6) with O_2-saturated 100 mM HEPES (pH 7.6).

(10) The kinetic modeling of **X** based on monitoring A_{365} is complicated by the fact that 1.4−1.5 diferric clusters are formed for every 1.2 •Y122 per R2. Analysis of the Mössbauer data suggests that **X** is the precursor to the diferric cluster, and therefore 0.2−0.3 equiv of **X** can be converted to diferric cluster independently of the formation of the tyrosyl radical. The rate constant of 60−80 s^{-1} for formation of **X** is based on fits to the initial rates from 2.5 to 50 ms. The rate constants obtained from $A_{410,dropline}$-versus-time traces are the results of fits to a short lag phase followed by the first-order growth in •Y122 (see text).

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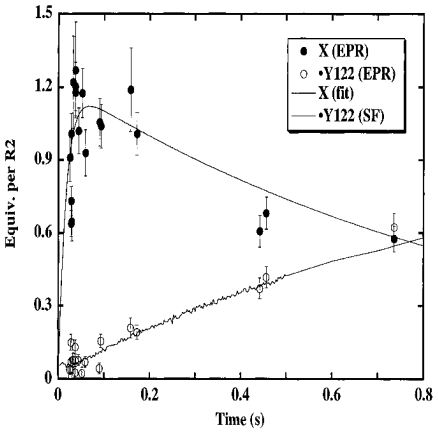

Figure 3. Kinetics of formation of **X** and •Y122 in the reaction of Fe(II)-R2 with O_2. The quantities of **X** and •Y122 are from the RFQ-EPR time course of the reaction. The trace for •Y122 is from the stopped-flow experiment. The trace for **X** is obtained by nonlinear least-squares fitting of the measured quantities of **X** and •Y122 as functions of time to the experimental data corresponding to a k_1 of 60 s^{-1} and a k_2 of 1 s^{-1}.

by $A_{410,\text{dropline}}$ (Figure 1),[5b,10] and the data are well described by two sequential, first-order processes with rate constants of 60 $\pm$ 20 and 0.85 $\pm$ 0.5 s^{-1}. As with apo R2, the •Y122/R2 ratio at completion is 1.2. Thus, the reaction of Fe(II)-R2 with O_2 appears to differ from the reaction of apo R2 with Fe^{2+} and O_2 in the kinetics of formation of **X** and not in the subsequent formation of •Y122 (Scheme 1).

RFQ-EPR and RFQ-Möss experiments confirm the formation of **X** in the Fe(II)-R2 reconstitution reaction.[11] The EPR time course of the reaction of Fe(II)-R2 with O_2 is shown in Figure 2 (supporting information), and the kinetic analysis of this time course is shown in Figure 3. At the early time points, the spectra are dominated by a sharp, isotropic, $g = 2.00$ singlet characteristic of **X**. With time, the doublet signal characteristic of •Y122 appears, while the signal of **X** decays. At completion, the •Y122/R2 ratio is 1.2. Each spectrum can be accounted for as the sum of **X** and •Y122. Kinetic analysis of the EPR results gives a rate constant of 60−80 s^{-1} for the formation of **X** and a rate constant of 1 s^{-1} for its decay with concomitant formation of •Y122 (Figure 3). As expected, the Mössbauer spectrum of the 0.028 s time point (Figure 4, supporting information) reveals a species identical to **X**[5a] accounting for (50 $\pm$ 5)% of total Fe, in addition to a broad quadrupole doublet attributable to Fe(II)-R2 and Fe^{2+} in solution accounting for (32 $\pm$ 2)% of the total iron. The remaining iron is distributed among several species with unusual isomer shifts, and the analysis of this spectrum (Figure 4) in the context of the kinetic analysis of the entire reconstitution process is the subject of ongoing investigation.[12] The results obtained from these three kinetics methods therefore provide support for the model shown in Scheme 1 and reveal that, like the assembly process starting with apo R2, the assembly process starting with Fe(II)-R2 proceeds through intermediate **X**. The kinetics suggest that formation of the diferrous-R2 complex from apo R2 is slow, consistent with the proposal that a conformational reorganization is required for Fe^{2+} binding by apo R2. Subsequent reaction of this complex with O_2 is rapid, giving rise to the intermediate **X**, which then oxidizes Y122 to form •Y122 and the diferric cluster.

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Supporting Information Available: Figure 2 showing the RFQ-EPR time course of the reaction of Fe(II)-R2 with O_2 and Figure 4 showing the 4.2 K Mössbauer spectrum of Fe(II)-R2 mixed with O_2 and freeze quenched at 0.028 s (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(11) The RFQ samples were prepared as previously described.[5] The reaction conditions are identical to those indicated for SF-Abs experiments,[9] and the reaction mixtures were freeze−quenched at time points between 0.025 and 60 s (Figure 2, supporting information).

(12) One of these species ((9 $\pm$ 2)%) appears and disappears during the reconstitution process, suggesting that it may be a kinetically competent intermediate (Figure 4, supporting information). This species can be described by the spectral parameters ($\delta = 0.66$ mm/s and a $\Delta E_q = 1.51$ mm/s) of the diferric peroxide species recently identified in methane monooxygenase.[13]

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