Differing Roles for the Diiron Clusters of Ribonucleotide Reductase from Aerobically Grown *Escherichia coli* in the Generation of the Y122 Radical

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Ribonucleotide reductases (RNR) are a ubiquitous family of enzymes that catalyze the first step in DNA synthesis, namely the reduction of ribonucleotides to deoxyribonucleotides.[1−3] The RNR from aerobically grown *E. coli* is an archetypal class I enzyme, composed of two homodimeric components, R1 and R2. R1 contains the site for substrate reduction,[4] while R2 contains the catalytically essential tyrosyl radical (Y122•) adjacent to a diiron cluster.[5−7] The tyrosyl radical is formed by the reaction of the diiron(II) cluster with O_2, according to eq 1.

$$[\text{Y122, Fe}^{\text{II}}\text{−Fe}^{\text{II}}] + O_2 + e^- \rightarrow [\text{Y122}^{\bullet}, \text{Fe}^{\text{III}}\text{−O−Fe}^{\text{III}}] + OH^- \quad (1)$$

While eq 1 implies the formation of one Y122• per diiron(III) center, the reaction stoichiometry is not so simple. In our preparations, the active enzyme ($R2_{ox}$) as isolated contains 3.5-(2) Fe^{III} (or 1.8(1) diiron(III) centers) and 1.2(1) Y122• per R2 dimer, indicating that only about two-thirds (1.2/1.8) of the diiron-(III) sites have the associated radical; the remaining third of the sites are radical-free and thus equivalent to those of $R2_{met}$, i.e., [Y122, Fe^{III}−O−Fe^{III}]. This observation is consistent with the requirement for about 3 Fe^{II} to produce 1 Y122• found by a number of laboratories.[8−11] The odd stoichiometry for Y122• formation and the source of the extra electron in eq 1 are questions that persist in the field. In this paper, we address these unexplained observations. We show that Y122• formation involves two functionally distinct diiron(II) clusters, one that becomes the [Y122•, Fe^{III}−O−Fe^{III}] site and another that supplies the required extra electron and forms the [Y122, Fe^{III}−O−Fe^{III}] site.

Experiments with methyl viologen (MV) to reduce $R2_{ox}$ indicate that there are two types of diiron(III) clusters, despite the fact that the diiron clusters in $R2_{ox}$ and $R2_{met}$ appear to be spectroscopically indistinguishable.[12−15] Coulometric reductive titrations of $R2_{ox}$ show two phases in the uptake of electrons (Figure 1).[16,17] The faster phase is characterized by the transfer of 2.4(2) electrons with no accumulation of reduced MV (MV_{red}),

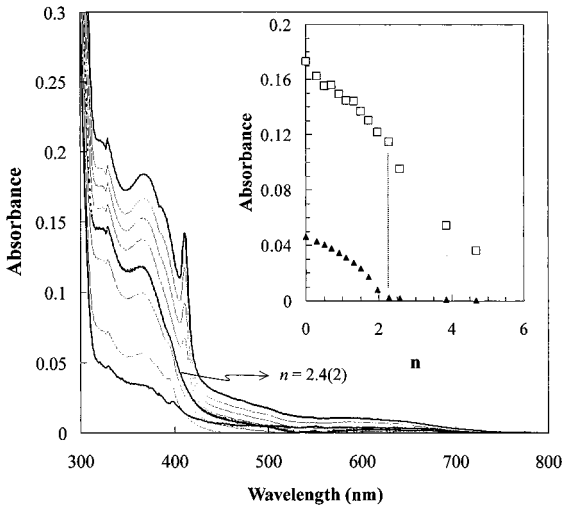

Figure 1. Coulometric reductive titration of $R2_{ox}$ as monitored by UV−vis spectra following stepwise addition of reducing equivalents (0−5 equiv) with use of the methodology of Paulsen et al.,[16] described in further detail in the Supporting Information Conditions: 23 μM $R2_{ox}$ in anaerobic 25 mM HEPES, 5% glycerol buffer at pH 7.5 and 4 °C. The top and bottom traces represent the spectra of $R2_{ox}$ and $R2_{red}$, respectively. The spectrum at $n = 2.4(2)$ represents the point between the fast and slow phases of the reduction at which absorption features of MV_{red} were first observed. Inset: Optical absorbances of the diiron(III) cluster and Y122• versus the number of reducing equivalents added. □, 350 nm absorbance; ▲, 410 nm dropline absorbance. - - -, point at which the *F* cluster is completely reduced and MV_{red} is observed.

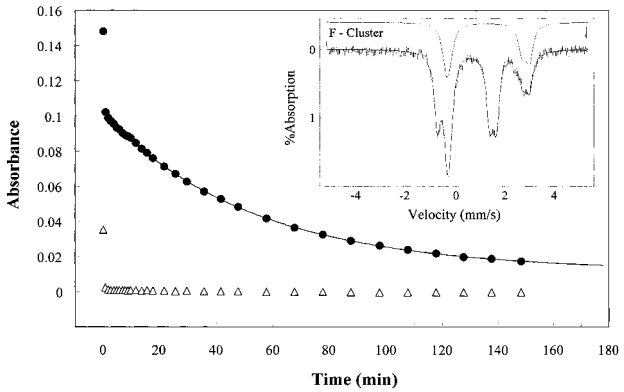

Figure 2. Chemical reduction of $R2_{ox}$ as a function of time. Conditions: 23 μM $R2_{ox}$, 18 μM MV in anaerobic 25 mM HEPES, 5% glycerol buffer at pH 7.5 and 4 °C. The reaction was initiated by the addition of 300 μM dithionite. The contributions due to MV have been subtracted. ●, 370 nm absorbance, △, 410 nm dropline, −, a theoretical line generated from a nonlinear regression fit to the data. Inset: Mössbauer spectrum of partially reduced $R2_{ox}$ or F_{red} S_{ox}. The fraction of F_{red} was determined as described in the caption for Figure S1 (Supporting Information).

indicating fast electron transfer from MV_{red} to the protein. The end of the fast phase corresponds to the spectrum in Figure 1 where 2.4(2) electron equivalents have been added; at this point, all of the Y122• has been reduced, as shown by the disappearance of the sharp band at 410 nm. About $^1/_3$ of the diiron(III) clusters (λ_{max} 350 nm) have also been reduced. The second phase involves the reduction of the remaining diiron(III) clusters as shown in Figure 1. During this phase a buildup of MV_{red} is observed, indicating a much slower transfer of electrons from MV_{red} to the protein. Similarly, chemical reduction of $R2_{ox}$ shows an initial rapid (within seconds)[18] reduction of the radical and approximately $^1/_3$ of the diiron(III) clusters, followed by a slower (in minutes) reduction of the remaining $^2/_3$ of the clusters (Figure 2). $R2_{ox}$ thus

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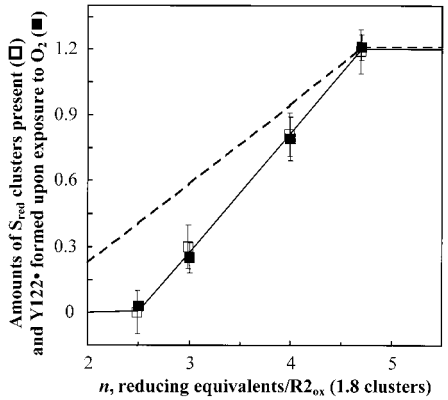

Figure 3. Amount of Y122• formed upon exposure to O_2 from $R2_{ox}$ samples (1.8(1) clusters per R2 dimer) reduced by *n* equivalents of electrons. ■, amount of Y122• generated; □, amount of S_{red} clusters as measured by Mössbauer spectroscopy, assuming prior formation of 0.6 F_{red} clusters; —, amount of Y122• expected based on eq 2, assuming 1.8(1) diiron clusters; - - -, amount of radical predicted from the 1 Y122•/3 Fe model.

consists of a mixture of *F* and *S* clusters, i.e., [Y122, *F*] and [Y122•, *S*], where *F* and *S* designate the fast and slow reducing diiron(III) clusters, respectively.

The difference in reduction rates of *F* and *S* allows us to prepare partially reduced samples of R2 to investigate the role of each diiron center. We have used Mössbauer spectroscopy to monitor the redox states of the diiron clusters and EPR spectroscopy to quantitate Y122•. The Mössbauer spectrum of an $R2_{ox}$ sample treated with 10 equiv of electrons and frozen 15 s after mixing (at the end of the fast phase) shows 31% of the clusters reduced to the diiron(II) state (Figure 2, inset). Furthermore, a sample of $R2_{ox}$ reduced by 2.5 electron equivalents shows that 34% of the clusters have attained the diiron(II) state, while 66% still exhibit a spectrum characteristic of diiron(III) sites.[12,19] The amount of iron reduced (1.2(1) Fe) together with the 1.2(1) Y122• is in agreement with the 2.5 electrons transferred to $R2_{ox}$. The fact that the number of F_{red} clusters corresponds closely to that of the [Y122, $Fe^{III}-O-Fe^{III}$] sites in $R2_{ox}$ suggests that these are the *F* clusters, while the [Y122•, $Fe^{III}-O-Fe^{III}$] sites in $R2_{ox}$ are those designated *S*.

The *F* and *S* clusters also react differently with O_2, showing that they are functionally distinct. $R2_{red}$ (1.8(1) diiron(II) clusters) reacts with O_2 to generate 1.2(1) Y122•/R2, as found for the enzyme as isolated. In contrast, the reaction of O_2 with partially reduced $R2_{ox}$ samples affords significantly less Y122• (Table 1) than expected from the empirically determined 1 Y122•/3 Fe^{II} stoichiometry or $^2/_3$Tyr• per diiron cluster as shown by the dashed line in Figure 3. For example, 2.5-e^--reduced samples containing 0.6(1) diiron(II) cluster/R2 react with O_2 to form the corresponding diiron(III) center but afford almost no Y122•; the 0.03(2) Y122•/R2 formed is more than an order of magnitude lower than the expected value of 0.4 (Figure 3). Additional experiments with 3-e^-- and 4-e^--reduced $R2_{ox}$ show that the amounts of Y122• formed are also lower than expected. The amount of Y122• formed is furthermore unaffected by the addition of excess Fe^{II} or ascorbate (Table 1), both proposed to be capable of providing the extra electron in eq 1.[10,20,21] The low yield of Y122• obtained cannot be ascribed to protein inactivation, since 1.2(1) Y122•/R2 was obtained from a sample that had undergone the following steps (Table 1, entry 8): (a) partial reduction, (b) exposure to O_2 to form $R2_{met}$, (c) degassing, (d) complete reduction to $R2_{red}$, and (e) exposure to O_2. Analysis of the data in Table 1 shows that the amounts of Y122• formed in the various experiments correspond instead directly to the amounts of S_{red} (calculated by the number of diiron(II) clusters minus F_{red} (0.6) as determined by Mössbauer spectroscopy, Table 1, entry 3) present prior to the introduction of O_2 (Figure 3). These results suggest that S_{red} is responsible for generation of Y122•. Furthermore, because no exogenous reductants are available, the only likely source of the required extra electron is F_{red}.

Our observations provide the basis for a scheme ascribing distinct roles for the diiron clusters of $R2_{red}$, a notion proposed by Elgren et al.[8] and more recently by Tong *et al.*[22] Though spectroscopically alike, the *F* and *S* clusters are functionally different. In our proposed scheme, Y122• is generated only by the reaction of S_{red} with O_2, with F_{red} providing the required extra electron in eq 1. Equation 1 can thus be modified as follows:

$$2S_{red} + 1F_{red} + 2O_2 \rightarrow 2[Y122^{\bullet}, Fe^{III}-O-Fe^{III}] + [Y122, Fe^{III}-O-Fe^{III}] + 2OH^- \quad (2)$$

This equation rationalizes why only $^2/_3$ of the diiron(III) sites have a Tyr• (1Tyr• per 3Fe stoichiometry). However, further experiments are required to sort out the intricacies of the Y122• formation mechanism. For example, it is not clear whether the electron-transfer step between *F* and *S* clusters is intra- or intermolecular.[23] The proposed scheme also implies the generation of a mixed-valent *F* cluster ($Fe^{II}Fe^{III}$) that has not yet been observed. Nevertheless, our ability to differentially reduce the diiron(III) clusters of $R2_{ox}$ has allowed us to demonstrate that there are two functionally distinct diiron clusters in R2 which seem to work in concert to form the catalytically essential Y122•.

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Supporting Information Available: Table 1 and further details of the coulometric titration experiments and a Mössbauer spectrum of a 2.5-e^--reduced $R2_{ox}$ sample (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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