Electron Transfer Associated with Oxygen Activation in the B2 Protein of Ribonucleotide Reductase from *Escherichia coli*\(^*\)

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Each of the two β peptides which comprise the B2 protein of *Escherichia coli* ribonucleotide reductase (RRB2) possesses a nonheme dinuclear iron cluster and a tyrosine residue at position 122. The oxidized form of the protein contains all high spin ferric iron and 1.0–1.4 tyrosyl radicals per RRB2 protein. In order to define the stoichiometry of in vitro dioxygen reduction catalyzed by fully reduced RRB2 we have quantified the reactants and products in the aerobic addition of Fe(II) to metal-free RRB2 apo utilizing an oxygraph to quantify oxygen consumption, electron paramagnetic resonance to measure tyrosine radical generation, and Mössbauer spectroscopy to determine the extent of iron oxidation. Our data indicate that 3.1 Fe(II) and 0.8 Tyr\(^{122}\) are oxidized per mol of O\(_2\) reduced. Mössbauer experiments indicate that less than 8% of the iron is bound as mononuclear high spin Fe(III). Further, the aerobic addition of substoichiometric amounts of \(^{57}\)Fe to RRB2 apo consistently produces dinuclear clusters, rather than mononuclear Fe(III) species, providing the first direct spectroscopic evidence for the preferential formation of dinuclear units at the active site. These stoichiometry studies were extended to include the phenylalanine mutant protein (Y122F)RRB2 and show that 3.8 mol-equivalents of Fe(II) are oxidized per mol of O\(_2\) consumed. Our stoichiometry data has led us to propose a model for dioxygen activation catalyzed by RRB2 which invokes electron transfer between iron clusters.

Ribonucleotide reductase (RR)\(^1\) catalyzes the reduction of all four ribonucleotides to their corresponding deoxyribonucleotides, an essential step in the synthesis of DNA in all living cells (1–3). The *Escherichia coli* enzyme contains two proteins, designated B1 (α) and B2 (β). The RRβ1 protein contains the substrate and effector binding sites as well as several redox-active thiols (4–6). The active form of the RRB2 protein contains a stable tyrosine radical, essential for enzymatic activity, and a dinuclear nonheme iron-oxo cluster, involved in the generation and stabilization of the radical (7). Site-directed mutagenesis has been employed (8) to construct a radical-free, inactive mutant in which the native tyrosine at position 122 was substituted with a phenylalanine residue, (Y122F)RRB2, clearly identifying Tyr\(^{122}\) as the position of the tyrosyl radical in the wild-type enzyme.

The radical free dfferic form of wild-type RRB2 (RRB2\(_{\text{apo}}\)) has been crystallized and the x-ray structure determined to 2.2-Å resolution (9). The reported three-dimensional structure supports a stoichiometry of one dinuclear iron-oxo cluster per β polypeptide, as previously proposed by Lynch et al. (10); this raises the possibility of having one tyrosyl radical associated with each dinuclear cluster. However, the highest tyrosyl radical content per RRB2 protein reported (10) is 1.4 as determined by epr spectroscopy.

The fluctuation in the tyrosyl radical concentration in the cell in response to its growth cycle suggests the availability of cellular mechanisms to reduce the radical and then to regenerate it (11). In vitro, various forms of the RRB2 protein have been prepared, as shown in Fig. 1. Reichard et al. (12) have shown that aerobic incubation of RRB2\(_{\text{apo}}\) with a crude extract from *E. coli* will regenerate the tyrosyl radical; however, simple exposure to oxygen will not. Two of the three components from this extract have been isolated and identified as superoxide dismutase (12) and an NAD(P)H:flavin oxido-reductase (13). This observation has led to the proposed mechanism that the oxido-reductase may be capable of reducing RRB2 to a form which could then react with dioxygen followed by oxidation of Tyr\(^{122}\). The tyrosyl radical together with the (μ-oxo)differential cluster may also be generated by exposure of the diferrous (RRB2\(_{\text{apo}}\)) form of the protein to dioxygen (14) or the aerobic Fe(II) reconstitution of metal-free (RRB2\(_{\text{apo}}\)) protein (7, 15). However, the dinuclear site is not reconstituted by the addition of Fe(III) (14). The apparent inefficiency of tyrosyl radical production has led us to further investigate the stoichiometry of dioxygen activation catalyzed by RRB2.

The reduction of dioxygen to water requires four reducing equivalents. RRB2\(_{\text{apo}}\) appears to provide three of these reducing equivalents by the oxidation of one dinuclear ferrous cluster and Tyr\(^{122}\).

\[
2\text{Fe}^{III} + \text{Tyr}^{122} + X + O_2 \rightarrow \text{Fe}^{III}:\text{O}:\text{Fe}^{III} + \text{Tyr}^{122} + X^+ + H_2O
\]

(Reaction 1)

The source of the fourth reducing equivalent (X in Reaction

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1) is unknown. However, in a recent study of mouse ribonucleotide reductase (16), it was speculated that the fourth equivalent is provided by adventitiously bound Fe(II). We have sought to further define the stoichiometry of in vitro dioxygen activation associated with the RRB2 protein. We report here our findings for the stoichiometry of Reaction 1 for wild-type RRB2 and the (Y122F)RRB2 mutant which suggest that the fourth reducing equivalent is not supplied by adventitiously bound iron in the E. coli protein. A model is proposed to account for our stoichiometric data which invokes electron transfer between dinuclear clusters.

**MATERIALS AND METHODS**

RRB2 was isolated from E. coli strain N6405/pSPS2 (17), a heat-inducible overproducer, as previously described (18) and its concentration was determined by absorbance at 280 nm (ε280 = 141 mM⁻¹ cm⁻¹) (10). The procedure used to isolate the mutant (Y122F)RRB2 from E. coli strain K38/pMK5/pGP1-2 (8, 19) was identical to that used for the wild-type. The RRB2 protein was purified to homogeneity as indicated by the presence of a single band on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis. RRB2 isolated by this method displays a specific activity of 4700 determined spectrophotometrically by monitoring the disappearance of NADPH (0.16 mM) in the presence of RRB1 (60 μg/ml), thioredoxin (50 μg/ml), thioredoxin reductase (0.7 μg/ml), CDP (1 mM), and ATP (1.6 mM) as previously described (10). Iron removal was accomplished by dialysis against the lithium salt of 8-hydroxyquinoline-5-sulfonic acid in 1.0 mM imidazole as previously described (7). The concentration of RRB2apo was determined by absorbance at 280 nm (ε280 = 120 mM⁻¹ cm⁻¹). Bovine erythrocyte superoxide dismutase and bovine liver catalase were used as a standard for the g value.

Dioxygen consumption experiments were performed on a Gilson Model K-ICT C Oxygraph with a 1.5-ml water jacketed cell equipped with a Clark electrode. Experiments were conducted at ambient temperature and controlled with a circulating water bath. All solutions were prepared with 25 mM HEPES buffered at pH 7.6, unless otherwise noted. The oxygraph was calibrated by addition of a known concentration of Fe(III), a result which is corroborated by quantitation of the high spin ferric iron bound can be determined by quantification of the double-integrated epr signal at g = 2.12 and 2.44 mm/s and δ = 0.55 and 0.45 mm/s, respectively; these parameters are identical to those observed for the dfferic site in RRB2 (7). The amount of mononuclear high spin ferric iron bound can be determined by quantification of the outer features of its magnetic hyperfine spectrum obtained in 60 kilogauss externally applied field. With this procedure, the mononuclear high spin ferric component accounts for no more than 8% of the total iron content. Aerobic reduction of dioxygen by RRB2apo.

Because Fe(III) has been shown not to be incorporated into the dinuclear binding sites in RRB2 but can bind to the protein adventitiously (14), it is essential to this study to monitor and quantify the fate of the Fe(II) added to RRB2apo upon exposure to O2. Thus, in order to assess the integrity of the dinuclear clusters upon reconstitution and permit the quantification of adventitiously bound Fe(III), oxygraph experiments were run with 57Fe(II) to obtain the O2 stoichiometry and the samples were then subjected to Mössbauer spectroscopic quantification of these iron species. Mössbauer spectra of these samples contain two quadrupole doublets with ΔE0 = 1.62 and 2.44 mm/s and δ = 0.55 and 0.45 mm/s, respectively; these parameters are identical to those observed for the dfferic site in RRB2apo (7). The amount of mononuclear high spin ferric iron bound can be determined by quantification of the outer features of its magnetic hyperfine spectrum obtained in 60 kilogauss externally applied field. With this procedure, the mononuclear high spin ferric component accounts for no more than 8% of the total iron content. Aerobic addition of substoichiometric amounts of 57Fe(II) to RRB2apo consistently produces dinuclear clusters, rather than any mononuclear Fe(III) species.

The Mössbauer data suggest that less than 8% of the Fe(II) is oxidized and ultimately observed as mononuclear high spin Fe(III), a result which is corroborated by quantitation of the epr signal at g = 4.3. Consequently, the oxygen consumption data must be reduced by at most 2% assuming that O2 is reduced to water in generating the mononuclear high spin Fe(III) component. This seems likely since the oxygen consumption results do not change when the reconstitution experiments are run in the presence of catalase, indicating that peroxide is not generated as a result of partial oxygen reduction. Dioxygen consumption is complete 30 s after the addition of Fe(II) to RRB2apo (Fig. 2A). Addition of Fe(II) to the oxygraph cell in the absence of protein requires 4 min to run to completion indicating a much slower oxidation rate (Fig. 2B). The oxygen consumption which results from the addition of Fe(II) to RRB2apo follows a trace similar to that shown in Fig. 2B. Ascorbate is commonly used in the reconstitution of RRB2apo with Fe(II) (7, 14), presumably to keep the iron reduced and to achieve the highest possible degree of iron reconstitution. In order that the number of reducing equivalents may be accurately quantified, we have not used ascorbate in these experiments.

The results for similar stoichiometric measurements are

**RESULTS**

In order to define the stoichiometry of dioxygen reduction catalyzed by RRB2apo (Reaction 1), we have quantified the reactants and products in the aerobic addition of Fe(II) to RRB2apo utilizing an oxygraph to quantify oxygen consumption, epr to measure tyrosine radical generation, and Mössbauer spectroscopy to determine the extent of iron oxidation. Listed in Table I are results from the addition of four equivalents of Fe(II) to an air-saturated solution of RRB2apo. These results indicate that 3.1 mol-equivalents of Fe(II) and 0.8 mol-equivalent of Tyr122 are oxidized per mol of O2 reduced for wild-type RRB2. In vitro reconstitution experiments were run by adding an anaerobic ferrous ammonium sulfate solution to an oxygraph cell filled with an aerobic RRB2apo solution. Aliquots were removed from the oxygraph cell and frozen immediately in liquid nitrogen for epr analysis. The ratios in Table I are not affected by the addition of catalase or superoxide dismutase to the RRB2apo solution, consistent with results reported by Salowe (18), indicating that diffusive peroxide or superoxide species are not produced during the reduction of dioxygen by RRB2apo.
TABLE I

| Sample                  | RRB2/O2  | Fe/O2  | Tyrosyl radicals/O2 | O2/RRB2 | Tyrosyl radicals/RRB2 | Reducing equivalents/O2 |
|-------------------------|----------|--------|---------------------|---------|-----------------------|------------------------|
| A. Wild-type            | 0.8 (±0.05) | 3.1 (±0.18) | 0.8 (±0.06) | 1.3 (±0.08) | 1.0 (±0.07) | 3.9 (±0.22) |
| B. (Y122F) mutant       | 0.9 (±0.05) | 3.9 (±0.19) | NA | 1.1 (±0.05) | NA | 3.9 (±0.19) |
| C. Wild-type model      | 0.8      | 3.2    | 0.8                 | 1.25    | 1.0                   | 4.0                   |

FIG. 2. Oxygraph traces of oxygen consumption. Oxygraph traces of oxygen consumption due to the oxidation of Fe(II) upon reconstitution of the B2 protein of ribonucleotide reductase (A) and due to the oxidation of Fe(II) in the absence of protein (B). Both experiments run at 25 °C in an aerobic 25 mM HEPES solution buffered at pH 7.6.

FIG. 3. Mössbauer spectrum of the oxidized phenylalanine-122 mutant of the B2 protein of E. coli ribonucleotide reductase reconstituted with 57Fe ((Y122F)RRB2). The spectrum was recorded at 4.5 K in a 0.06 T parallel applied magnetic field. The solid line is a simulated spectrum using the Mössbauer parameters of the wild-type protein. The additional absorption between 0 and +1 mm/s double-velocity results from a minor (<8%) impurity, possibly an Fe(III) aggregate in solution.

shown in Table I for the addition of Fe(II) to (Y122F)RRB2apo; this enzyme has a phenylalanine residue substituted at position 122 in place of the native tyrosine residue. These experiments indicate that 3.9 mol-equivalents of Fe(II) are oxidized per mol of O2 consumed. The Mössbauer spectrum of the oxidized form of (Y122F)RRB2, shown in Fig. 3, is indistinguishable from that of the wild-type protein.

DISCUSSION

Listed in Table I are the results from the addition of four equivalents of Fe(II) to an air-saturated solution of RRB2apo, as monitored by epr and Mössbauer spectroscopy and an oxygen selective electrode. These results can be interpreted in light of two observations: O2 consumption is significantly faster for Fe(II) + RRB2apo versus Fe(II) + RRB2nost or Fe(II) alone and Fe(II) is incorporated into RRB2 predominantly, if not almost exclusively, as dinuclear clusters even when substoichiometric amounts are added. The Mössbauer parameters for the diferrous cluster are unique for RRB2. Since addition of Fe(III) to RRB2apo does not generate this unique dinuclear cluster, the diferrous unit must form preferentially at the active site and O2 reduction must occur only at this site. Otherwise, we would observe a greater proportion of mononuclear Fe(III) units, particularly in the substoichiometric additions. Therefore, it seems likely that the formation of the diferrous center facilitates O2 binding and reduction, and the carboxylate-rich nature of the active site (9) would be expected to lower the reduction potential of the diiron center and enhance its oxidation.

Based on the values listed in Table I, we report the following stoichiometry for the reduction of dioxygen by the diferrous clusters in RRB2.

\[ 0.8 \text{RRB2apo} + 3.1 \text{Fe(II)} + \text{O}_2 \rightarrow 0.8 \text{Ty}^{122} \]

This stoichiometry is reported in terms of dioxygen consumption in order to track the number of reducing equivalents, which totals 3.9 per mol of O2 consumed. Therefore, our results appear to be consistent with that reported for the mouse enzyme by Thelander et al. (16) in which three Fe(II) and one tyrosine combine to contribute the four electrons required to reduce O2 to water.

The results for analogous stoichiometry measurements for the addition of Fe(II) to (Y122F)RRB2apo, shown in Table I, indicate that 3.9 mol-equivalents of Fe(II) are oxidized per mol of O2 consumed. This result suggests that the iron clusters are capable of providing all four of the reducing equivalents required to reduce O2. Thus, it appears that one dinuclear cluster is capable of transferring its reducing equivalents to another cluster. This suggests that the activated iron-oxygen species, which results from the binding of O2 and subsequent oxidation of one iron cluster, has a potential high enough that electron transfer from the remaining diferrous cluster takes precedence over the binding and activation of O2 to the latter diferrous cluster.

The data for tyrosyl radical generation in Table I, 1.0 radical per RRB2 protein, is clearly less than the optimum yield of one radical per iron cluster. The value of 0.8 radicals per O2 is also less than the one radical per O2 predicted by a stoichiometry of 3.0 Fe(II) and 1.0 Ty^{122} per O2 consumed. This low tyrosyl radical content, consistent with previous reports for reconstitution experiments (7, 10), may be due to the inefficiency of radical formation, the instability of an intermediate formed during radical generation, or the availability of an alternative mechanism for the reduction of O2 which does not result in the oxidation of Ty^{122}. The mechanism by which O2 reduction is achieved by (Y122F)RRB2 may contribute to the apparent inefficiency of tyrosyl radical production in the wild-type enzyme. Analysis of the wild-type data in Table I, based on a stoichiometry of 3.0 Fe(II) and 1.0 Ty^{122} per O2 consumed, accounts for 2.4 Fe(II) required to generate the 0.8 Ty radicals observed consuming 0.8 mol of O2. The remaining
The mixed valence state has been spectroscopically identified in other dinuclear nonheme iron proteins such as hemerythrin (20), purple acid phosphatase (21), and the hydroxylase component of methane monoxygenase (22). The chemical reduction methods used to generate the mixed valence state in these metalloproteins have not facilitated the isolation of this form of RBB2. However, Hendrich et al. (23) have recently characterized this mixed valence form of RBB2 generated by low temperature x-irradiation of an RBB2 mutant sample. The epr signal associated with this state of the iron cluster disappears when the sample is thawed, indicating that it is a very reactive, unstable intermediate species.

The proposed scheme suggests that the mixed-valence RBB2 intermediate may disproportionate (Reaction 3) similar to that shown to occur for the mixed valence forms of hemerythrin (20, 24). Reaction 4 of this proposed scheme is similar to that proposed for (Y122F)RBB2 in which 4 Fe(II) are consumed per O2 resulting in no radical formation. A balanced stoichiometry for the proposed reaction scheme in Fig. 4 is 3.2 Fe(II), 0.8 Tyr, 0.8 RBB2 consumed per mol of dioxygen reduced and a total of 1.25 mol of O2 consumed per mol of RBB2, assuming equal rates for all processes. These numbers are in good agreement with our experimental data for wild-type RBB2 reported in Table I.

The overall expected yield for the scheme in Fig. 4 is 1.0 tyrosyl radical per RBB2 protein and thus appears to account for the lower than expected tyrosyl radical content consistently observed for the isolated RBB2 protein. Reconstitution of RBB2, in the presence of the crude extract from E. coli has created the highest reported yield of 1.4 tyrosine radicals per RBB2 protein (10). This result implies that the presence of the three protein components of the cell extract described by Reichard et al. (12) must alter the reaction conditions to afford more efficient radical production. We are currently exploring how the stoichiometry of the oxygen consumption associated with oxidation of the diferrous clusters in RBB2 is affected by other protein components and by structural perturbations engendered by appropriate mutations.

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