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Reverse Electron Transfer Completes the Catalytic Cycle in a 2,3,5-Trifluorotyrosine-Substituted Ribonucleotide Reductase

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Supporting Information

ABSTRACT: Escherichia coli class Ia ribonucleotide reductase is composed of two subunits (α and β), which form an α2/β2 complex that catalyzes the conversion of nucleoside 5′-diphosphates to deoxynucleotides (dNDPs). β2 contains the essential tyrosyl radical (Y122α) that generates a thyl radical (C439•') in α2 where dNDPs are made. This oxidation occurs over 35 Å through a pathway of amino acid radical intermediates (Y122 → [W48] → Y356 in β2 to Y731 → Y730 → C439 in α2). However, chemistry is preceded by a slow protein conformational change(s) that prevents observation of these intermediates. 2,3,5-Trifluorotyrosine site-specifically inserted at position 122 of α2 (F3Yα2) perturbs its conformation and the driving force for radical propagation, while maintaining catalytic activity (1.7 s⁻¹). Rapid freeze–quench electron paramagnetic resonance spectroscopy and rapid chemical-quench analysis of the F3Yα2, α2, CDP, and ATP (effector) reaction show generation of 0.5 equiv of Y356•' and 0.5 equiv of dCDP, both at 30 s⁻¹. In the absence of an external reducing system, Y356•' reduction occurs concomitant with F3Y reoxidation (0.4 s⁻¹) and subsequent to oxidation of all α2s. In the presence of a reducing system, a burst of dCDP (0.4 equiv at 22 s⁻¹) is observed prior to steady-state turnover (1.7 s⁻¹). The [Y356•'] does not change, consistent with rate-limiting F3Y reoxidation. The data support a mechanism where Y122•' is reduced and reoxidized on each turnover and demonstrate for the first time the ability of a pathway radical in an active α2/β2 complex to complete the catalytic cycle.

INTRODUCTION

Ribonucleotide reductases (RNRs) catalyze the formation of deoxynucleotides from their corresponding ribonucleotides (Scheme 1) in almost all organisms; allosteric regulation of substrate specificity and activity contributes to fidelity of both DNA replication and repair.1,2 The class Ia RNRs contain two homodimeric subunits, α2 and β2, which form an active α2/β2 complex in the case of the E. coli enzyme.3 The β2 subunit houses a diferric-tyrosyl radical (Y122•') cofactor that reversibly oxidizes C439 in the active site of α2 to a thyl radical.4,5 The C439•' initiates nucleotide reduction by H atom abstraction from the 3' position of the substrate (Scheme 1).6,7 On the basis of in silico docking of the individual X-ray structures of α2 and β2,8,9 the distance between Y122•' and C439 is estimated to be >35 Å. This radical transport (RT) process occurs through a specific path that involves at least three transient aromatic amino acid radical intermediates (proton-coupled electron transfer or PCET through Y122•' → [W48] → Y356 in β2 to Y731 → Y730 → C439 in α2, Figure 1).9,10 During turnover of wild-type (wt) RNR, only the resting state Y122•' is observed. In this paper, we describe the perturbation of PCET kinetics by site-specific incorporation of 2,3,5-trifluorotyrosine (F3Y) at position 122 in β2 resulting in accumulation of a pathway tyrosyl radical intermediate (Y356•') that is kinetically and chemically competent to complete the catalytic cycle of RNR.

In wt RNR, PCET steps are preceded by a rate-limiting protein conformational change(s) (5–10 s⁻¹) that occur(s) upon association of α2, β2, substrate (S, CDP), and allosteric

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Figure 1. Proposed PCET pathway in E. coli class Ia RNR. The pink and blue arrows indicate the movement of electrons and protons through conserved aromatic amino acids (Y356 in β2 and Y731 and Y356 in α2). W356 and its putative proton acceptor D237 are shown in gray, as there is no evidence for their participation in RT. The positions of Y356 and E356 are unknown, as these residues are disordered in all crystal structures of β2.

MATERIALS AND METHODS

Materials. (His)6-wt-α2 (specific activity of 2500 nmol/min/mg) was expressed from pET28a-nrdA and purified using our standard protocol.20 Wt-α2 was pre-reduced by the addition of DTT and hydroxyurea prior to use.24 Tyrosine phenol lyase (TPL) was expressed and purified as described.25 F3Y was enzymatically synthesized from the corresponding phenol using TPL.26 The pBAD-nrdB122TAG and pEVOl-F3YRS-E3 plasmids were generated and isolated as described.27 Apo F3Y-β2 was expressed, purified, and reconstituted as detailed in the Supporting Information (SI). Yields of 10–15 mg of pure apo protein/g cell paste are routinely obtained. Reconstituted F3Y-β2 has a specific activity (750–1000 nmol/min/mg) that varies directly with the radical content (0.6–0.8 F3Y/β2). E. coli TR (40 U/mg) and TRR (1400 U/mg) were purified using established protocols.7,28 [3H]CDP was purchased from Vitrax (Placentia, CA). Hepes, MgSO4, EDTA, 2XT microtial medium, ampicillin (Amp), chloramphenicol (Cm), ATP, CDP, and carrier deoxycytidine (dC) were obtained from Sigma-Aldrich. Promega provided isopropyl β-D-thiogalactopyranoside (IPTG) and DTT. Calf alkaline phosphatase was purchased from Roche. Assay buffer consists of 50 mM Hepes pH 7.6, 15 mM MgSO4, and 1 mM EDTA.
Reaction of F3Y−β2, wt-α2, CDP, and ATP Monitored by RFQ-EPR Spectroscopy. RFQ experiments were performed on an Update Instruments 1019 syringe ram unit and a model 71S Syringe Ram controller (ram speed 1.25−1.6 cm/s) equipped with a Lauda RM6 circulating water bath set at 5 or 25 °C. F3Y/β2 (0.4−0.8 F3Y/β2, 80 μM) and CDP (2 mM) in assay buffer was mixed on a rapid time scale (16 ms−15 s) with an equal volume of wt-α2 (80 μM) and ATP (6 mM) in assay buffer. The reaction was quenched in liquid isopentane (−140 °C), and the crystals were packed into EPR tubes for analysis by EPR spectroscopy. A packing factor of 0.60 ± 0.02 was determined for wt-β2. The reaction at 5 °C was additionally monitored on a longer time scale (20 s−2 min) by mixing all assay ingredients by hand (30 μM wt-α2, 30 μM F3Y/β2, 1 mM CDP, and 3 mM ATP) and quenching in liquid isopentane. EPR spectroscopy was performed at the Department of Chemistry Instrumentation Facility at MIT using wt-β2 (1.2 Y*/β2) as a standard. The concentration of Y* in the wt-β2 standard was previously estimated using a Cu(II)SO4 standard. EPR spectra were recorded at 77 K on a Bruker EMX X-band spectrometer with a quartz finger dewar containing liquid N2. The parameters were as follows: microwave frequency 9.45 GHz, power 30 μW, modulation amplitude 1.50 G, modulation frequency 100 kHz, time constant 5.12 ms, and scan time 41.93 s. From each composite spectrum, residual F3Y* was subtracted by aligning the radical’s distinct features on the high- and low-field sides of the spectrum as previously reported (Figure S1). The subtracted spectrum was reintegrated to quantify the percentage of any observed pathway radical. The complete data sets at 5 and 25 °C were fit to eq 1:

\[ y = A_1(1 - e^{-k_1t}) + A_2(1 - e^{-k_2t}) \]  

where \( A_1 \) and \( A_2 \) are the amplitudes of the two phases and \( k_1 \) and \( k_2 \) are the observed rate constants.

**Reaction of F3Y−β2, wt-α2, CDP, and ATP Monitored by the RCQ Method.** RCQ experiments were performed on a KinTek RQF-3 instrument equipped with a Lauda RM6 circulating water bath set at 5 or 25 °C. Syringe A containing 20 μM wt-α2 and 6 mM ATP in assay buffer was mixed with an equal volume from syringe B containing 20 μM F3Y/β2 (0.85 F3Y/β2) and 1 mM [3H] CDP (22 000 cpnmol) in assay buffer. The reaction was aged for varying times (5 ms−100 s) and quenched with 2% HClO4 in syringe C. The reaction was additionally monitored at >100 s by mixing the contents of the two syringes by hand, incubating the reaction mixture in a circulating water bath for the desired period of time, and manually quenching the reaction with 2% HClO4. All samples were neutralized by the addition of 110−160 μL of 0.5 M KOH and worked up as described.14,30 For the measurement of radioactive background from [3H] CDP, an equal volume of the contents of syringe B was mixed with assay buffer, followed by 2% HClO4 and KOH. The reaction was also performed by hand (100 s at 5 or 25 °C) before and after the entire RCQ time course to account for any air oxidation of wt-α2. The 5 °C data set was fit to eq 1, and the 25 °C data set was fit to eq 2:

\[ y = 0.50(1 - e^{-30\chi}) + A_2(1 - e^{-k_2t}) + k_1t \]  

where \( A_1 \) and \( A_2 \) represent the amplitude and rate constant of the first phase, and \( k_1 \) represents the rate constant for the second phase.

**Reaction of F3Y−β2, wt-α2, CDP, and ATP, TR/TRR/NADPH Monitored by Hand-Quench EPR Spectroscopy.** Reactions were performed in a total volume of 250 μL containing 10 μM wt-α2, 10 μM F3Y/β2 (0.6 F3Y/β2), 1 mM CDP, 3 mM ATP, 40 μM TR, 0.8 μM TRR, and 1 mM NADPH in assay buffer. Samples were incubated in a circulating water bath set at 5 °C and quenched for EPR analysis between 20 and 90 s in liquid isopentane (−140 °C). The reactions were also performed at 25 °C in a final volume of 250 μL containing 30 μM wt-α2, 10 μM F3Y/β2 (0.6 F3Y/β2), 2.5 mM CDP, 3 mM ATP, 80 μM TR, 1.6 μM TRR, and 2.5 mM NADPH.

**Reaction of F3Y−β2, wt-α2, CDP, and ATP, TR, and NADPH Monitored by the RCQ Method.** The reaction was performed in an identical fashion to that described in the absence of a reducing system with minor modifications. For data collected at 5 °C, syringe A contained 20 μM wt-α2, 6 mM ATP, 80 μM TR, and 1.6 μM TRR in assay buffer, while syringe B contained 20 μM F3Y−β2 (0.6 F3Y−β2) and 1 mM [3H] CDP (20 000 cpnmol), and 2 mM NADPH. For the 25 °C reaction, the amount of [3H] CDP in syringe B was increased to 2 mM. Samples were quenched and worked up as described earlier. The time courses of the reactions were fit to eq 3:

\[ y = A(1 - e^{-kt}) + k_2t \]  

Here \( A \) and \( k_1 \) are the amplitude and rate constant for the burst phase, respectively, and \( k_2 \) is the rate constant for the linear phase.

**RESULTS**

"Two or None" Radical Distribution and Half-Sites Reactivity in F3Y−β2. The dифференцированный F3Y* cofactor is self-assembled from apo F3Y−β2 by the addition of Fe2+ and O2 to produce ~0.8 F3Y*/β2 (S1), lower than the 1.2 Y*/wt-β2.31 While the radical distribution in β2 has remained difficult to probe experimentally, evidence collected over the past few years supports that active β2 contains one Y* in each monomer ("two or none", Figure 2A), suggesting that only ~40% of β2 is active

![Figure 2A](https://example.com/figure2a.png)

**Figure 2.** (A) "Two or none" model for radical distribution in F3Y*/β2. The amounts of active (40%) and inactive β2 (60%) are shown for a sample containing 0.8 F3Y*. The amount of radical in F3Y*/β2 is lower than that typically seen in wt-β2 (1.2 Y*/β2).31 (B) Half-sites reactivity in F3Y*/β2. The total amount of Y356* that can accumulate on one turnover is 0.5 equiv/F3Y*.
were performed to quantitate the relative fraction of each radical. The unique hyperfine interactions associated with the fluorine nuclei facilitated spectral deconvolution (Figure S1). The results of the experiment are shown in Figure 3A, and the data were fit to a biexponential equation. Rapid loss of $0.51 \pm 0.02$ equiv of F3Y• (not shown) concomitant with formation of identical amounts of Y356• occurs at 30 ± 5 s−1. Subsequently, reduction of the pathway radical with $k_{app} 0.26 \pm 0.5$ s−1 is accompanied by reformation of F3Y• with the same $k_{app}$ (not shown). These data show for the first time, accumulation of a pathway radical (Y356•) in an active RNR complex that can regenerate the stable radical at position 122 (F3Y•). We note that between 0.1 and 1 s, the concentration of Y356• varies minimally. As shown subsequently, F3Y•-β2 can make multiple dCDPs in the absence of a reducing system, and the reduction of Y356• and reoxidation of F3Y are only visualized after the last turnover when all α2s are oxidized. Finally, regeneration of F3Y• is incomplete with only 0.25 equiv of Y356• reoxidizing F3Y within 10 s.

Table 1. DeoxyCDP Formation Kinetics in the Absence and Presence of TR/TRR/NADPH

<table>
<thead>
<tr>
<th>β2</th>
<th>T (°C)</th>
<th>R</th>
<th>first phase</th>
<th>second phase</th>
<th>total dC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$k_1$ (s−1)</td>
<td>$k_2$ (s−1)</td>
<td>A/radical</td>
</tr>
<tr>
<td>F,Y</td>
<td>25</td>
<td>N</td>
<td>30</td>
<td>0.5 b</td>
<td>0.5 (1)</td>
</tr>
<tr>
<td>F,Y</td>
<td>25</td>
<td>Y</td>
<td>22 (9)</td>
<td>0.40 (5)</td>
<td>2.9 (1)</td>
</tr>
<tr>
<td>F,Y</td>
<td>5</td>
<td>N</td>
<td>3 (1)</td>
<td>0.08 (1)</td>
<td>2.7 (1)</td>
</tr>
<tr>
<td>F,Y</td>
<td>5</td>
<td>Y</td>
<td>6 (3)</td>
<td>0.20 (1)</td>
<td>3.4 (1)</td>
</tr>
</tbody>
</table>

aAll experiments were performed with 10 μM wt-α2 and 10 μM F3Y•-β2. R notes the absence or presence of a reducing system. A represents the amplitude of each phase. bSee description in main text and SI for more details regarding fitting. cNumbers reported reflect the total amount of dC generated within the first two phases. Product generated in the third phase is cytosine.

Figure 3. Reaction of F3Y•-β2, wt-α2, CDP, and ATP at 25 °C monitored by (A) RFQ-EPR spectroscopy and (B) the RCQ method. All data points represent the averages of two independent trials. Data were fit to a (A) two- or (B) three-phase model with the rate constants shown in Table 1. (B) The inset shows dCDP formation during the first 2 s of the reaction. The rate constants measured for Y356• formation and disappearance correlate with the fitted rate constants for dCDP formation in the first two phases.

Figure 4. Reaction of F3Y•-β2, wt-α2, CDP, and ATP at 5 °C monitored by (A) RFQ-EPR spectroscopy and (B) the RCQ method. All data points represent the averages of two independent trials. Black lines represent biexponential fits to the data with the rate constants given in Table 1. (B) The inset shows dCDP formation during the first 5 s of the reaction. The rate constants measured for Y356• formation and disappearance are identical to the rate constants measured for dCDP formation.

were performed to quantitate the relative fraction of each radical. The unique hyperfine interactions associated with the fluorine nuclei facilitated spectral deconvolution (Figure S1). The results of the experiment are shown in Figure 3A, and the data were fit to a biexponential equation. Rapid loss of $0.51 \pm 0.02$ equiv of F3Y• (not shown) concomitant with formation of identical amounts of Y356• occurs at 30 ± 5 s−1. Subsequently, reduction of the pathway radical with $k_{app} 0.26 \pm 0.5$ s−1 is accompanied by reformation of F3Y• with the same $k_{app}$ (not shown). These data show for the first time, accumulation of a pathway radical (Y356•) in an active RNR complex that can regenerate the stable radical at position 122 (F3Y•). We note that between 0.1 and 1 s, the concentration of Y356• varies minimally. As shown subsequently, F3Y•-β2 can make multiple dCDPs in the absence of a reducing system, and the reduction of Y356• and reoxidation of F3Y are only visualized after the last turnover when all α2s are oxidized. Finally, regeneration of F3Y• is incomplete with only 0.25 equiv of Y356• reoxidizing F3Y within 10 s.

Kinetics of Formation and Disappearance of Y356• at 5 °C. The rapid formation of Y356• at 25 °C resulted in generation of 0.25 equiv (50% of total Y356•) prior to the first data point (16 ms, Figure 3A) prompting us to switch to lower temperatures to slow down the reaction. RFQ-EPR experiments were set up at 5 °C as described for 25 °C, and the results are shown in Figure 4A.
A $k_{\text{app}}$ of 3.8 ± 0.5 s$^{-1}$ was measured for formation of Y$_{356}^*$, concomitant with loss of F$_3$Y$^*$ (not shown). In contrast to our observation at 25 °C, only 0.32 ± 0.02 equiv of Y$_{356}^*$ is formed at 5 °C reflecting temperature dependent changes in the rates of formation and decay of the pathway radical. Similar to our observation at 25 °C, the concentration of Y$_{356}^*$ varies minimally between 0.8 and 5 s supporting the proposal that F$_3$Y$^*$-$\beta$2 catalyzes multiple turnovers prior to visualization of reverse RT. Unlike in the 25 °C reaction, Y$_{356}^*$ reduction at 5 °C is accompanied by complete reformation of F$_3$Y$^*$ (0.3 equiv, 0.06 ± 0.01 s$^{-1}$) within ~40 s.

**Kinetics and dCDP Formation with F$_3$Y$^*$-$\beta$2 at 25 °C.** Each dCDP generated by RNR is accompanied by the formation of a disulfide bond in the active site of an α monomer (Scheme 1, Figure 5, step A). Re-reduction of the active site disulfide by a C-terminal cysteine pair on each monomer (step B) facilitates an additional turnover (step C), giving a theoretical maximum of 4 dCDP/α2 can be produced; however, only 3 dCDP/α2 are routinely measured. The reaction mixture contains only 40% active F$_3$Y$^*$-$\beta$2 (Figure 2A) supporting reorganization of active and inactive $\alpha$2/F$_3$Y$^*$-$\beta$2 complexes to oxidize all $\alpha$2s.

![Figure 5](image-url). Amount of dCDP generated in the absence of a reducing system. A theoretical maximum of 4 dCDP/α2 can be produced; however, only 3 dCDP/α2 are routinely measured. The reaction mixture contains only 40% active F$_3$Y$^*$-$\beta$2 (Figure 2A) supporting reorganization of active and inactive $\alpha$2/F$_3$Y$^*$-$\beta$2 complexes to oxidize all $\alpha$2s.

To assess if Y$_{356}^*$ is on-pathway, the kinetics of dCDP formation were determined. Wt-$\alpha$2, F$_3$Y$^*$-$\beta$2, [3H] CDP, and ATP were mixed (5 ms to 300 s) and quenched rapidly with 2% HClO$_4$. CDP and dCDP were separated and analyzed by standard procedures, and the results are shown in Figure 3B. The data are best described by eq 2 with a fixed first exponential phase, a variable second exponential phase, and a very slow third linear phase. We initially attempted to fit the data with an exponential phase and a linear phase with poor results (Figure 2A).

To obtain the fit shown in Figure 3B (black line), we fixed the amplitude and rate constant ($k_1$) of the first phase at 0.5 dCDP/F$_3$Y$^*$ and 30 s$^{-1}$, respectively. Fixing this phase was required due to the scatter in the data at early time points. This scatter is a result of “two or none” and half-sites reactivity associated with RNR (Figure 2A,B). The [3H] dCDP measured between 5 and 100 ms is close to the background measured with [3H] CDP in the absence of $\alpha$2. The range of choices considered for the amplitude and $k_1$ of this phase were based on the amplitude and rate constant measured for Y$_{356}^*$ formation by RFQ-EPR (Figure 3A) and the results obtained in the presence of the reducing system (presented in the next section). The detailed description of data fitting using different parameters for the first kinetic phase is shown in Figure S2A–D. An additional experiment to justify the fixed first phase is shown in Figure S3.

Once the first phase was fixed using eq 2, we obtained an amplitude and rate constant ($k_2$) of 2.9 ± 0.1 dCDP/F$_3$Y$^*$ and 0.5 ± 0.1 s$^{-1}$, respectively, for the second phase and a rate constant ($k_3$) of 0.012 ± 0.001 s$^{-1}$ for the linear phase. This slow linear phase is associated with cytosine release and not dCDP formation. It occurs during the reaction of F$_3$Y$^*$-$\beta$2 with oxidized $\alpha$2 as shown in Scheme S1. In a second manuscript, we show that the inability to monitor complete reverse RT at 25 °C (Figure 3A) is associated with reoxidation of Y$_{356}^*$ (0.25 equiv) by F$_3$Y$^*$-$\beta$2/oxidized $\alpha$2. The fit shown in Figure 3B suggests that the pathway radical is kinetically and chemically competent for nucleotide reduction at 25 °C. These data require that Y$_{356}^*$ accumulates during reverse RT. $k_2$ for product formation correlates well with $k_{\text{app}}$ for Y$_{356}^*$ disappearance at this temperature (0.5 s$^{-1}$ vs 0.4 s$^{-1}$, Figure 3A).

**Kinetics and dCDP Formation with F$_3$Y$^*$-$\beta$2 at 5 °C.** The kinetics of dCDP formation were also measured at 5 °C, and the results are shown in Figure 4B. The data were fit to a bi-exponential equation providing amplitudes of 0.3 ± 0.1 dCDP/F$_3$Y$^*$ and 2.9 ± 0.1 dCDP/F$_3$Y$^*$ with $k_1$ and $k_2$ of 3 ± 1 s$^{-1}$ and 0.08 ± 0.01 s$^{-1}$, respectively (Table 1). $A_1$ and $k_1$ for dCDP formation are very similar to the amplitude and rate constant measured for Y$_{356}^*$ formation by RFQ-EPR spectroscopy at the same temperature (Figure 4A). These data suggest that Y$_{356}^*$ is kinetically and chemically competent for dCDP formation and accumulates during reverse RT. Similar to our observations at 25 °C, $k_2$ of 0.08 s$^{-1}$ for dCDP formation is similar to $k_{\text{app}}$ of 0.06 s$^{-1}$ for reoxidation of F$_3$Y by Y$_{356}^*$ (Figure 4A).

The RCQ data were also analyzed relative to $\alpha$2 to show that 2.7 ± 0.1 dCDPs/α2 are generated. DeoxyCDP formation was monitored for a total of 20 min, and in contrast to the 25 °C data, no third kinetic phase associated with cytosine was observed.

**Kinetics in the Presence of a Reducing System.** EPR Analysis of Y$_{356}^*$ Concentration During Steady-State Turnover. The ability of F$_3$Y$^*$-$\beta$2 to perform multiple turnovers in the presence of a reducing system (3.5 dCDP/F$_3$Y$^*$, Table 1) and the observation of a plateau phase in the RFQ-EPR kinetic traces (Figures 3A and 4A) suggested that reverse RT is visualized subsequent to complete oxidation of $\alpha$2. Thus, we predicted that the concentration of Y$_{356}^*$ would vary minimally in the presence of the reducing system, TR/TPR/NADPH, as oxidized $\alpha$2 is re-reduced. To test this prediction, F$_3$Y$^*$-$\beta$2, wt-$\alpha$2, CDP, and ATP were combined in the presence of TR/TPR/NADPH, and samples were quenched by hand in liquid isopentane between 20 and 90 s. In accordance with our
prediction, the amount of $Y_{356}^{-}$ does not change: 0.26 to 0.28 equiv/F$_3$Y• at 5 °C and 0.40 to 0.46 equiv/F$_3$Y• at 25 °C (Table S2). No reverse RT was visualized during the time frame of the reaction.

**Kinetics of dCDP Formation at 5 and 25 °C.**

The observation of $Y_{356}^{-}$ accumulation during reverse RT (Figures 3 and 4) in the absence of a reducing system and the lack of variation in $[Y_{356}^{-}]$ during steady-state turnover suggest that the rate-limiting step occurs subsequent to dCDP formation and $Y_{356}^{-}$ reformation during reverse RT. This model predicts that RQ experiments in the presence of the reducing system would show a burst of dCDP representing the first turnover by an $\alpha/\beta$ pair. Reverse PCET regenerating F$_3$Y• is rate-limiting during steady-state turnover.

As predicted, a burst of dCDP formation is observed at both temperatures (0.26 ± 0.05 dCDP/F$_3$Y• at 5 °C and 0.40 ± 0.05 dCDP/F$_3$Y• at 25 °C) followed by a linear phase (0.20 ± 0.01 s$^{-1}$ at 5 °C and 1.73 ± 0.04 s$^{-1}$ at 25 °C). The large uncertainties observed in these parameters are associated with low amounts of dCDP arising from the “two or none” model (Figure 2A) and half-sites reactivity (Figure 2B). Unfortunately, we are unable to increase protein concentration in these experiments as studies with wt RNR have revealed kinetic complexities associated with the re-reduction process and potentially quaternary structure interconversions.

The observed rate constant and amplitude for the burst phase are within error similar to the parameters observed for $Y_{356}^{-}$ formation (Figures 3A and 4A) and support our conclusion that $Y_{356}^{-}$ is on-pathway for dCDP formation. These data also correlate well with the kinetics of the first phase measured in the absence of TR/TRR/NADPH (0.3 dCDP/F$_3$Y• at 3 s$^{-1}$ at 5 °C and 0.5 dCDP/F$_3$Y• at 30 s$^{-1}$ at 25 °C) supporting that $k_1$ in the absence of a reducing system and the burst phase in the presence of TR/TRR/NADPH report on the first turnover by one $\alpha/F_3Y^{-}/\beta$ pair in the $\alpha_2/F_3Y^{-}/\beta_2$ complex (Figure 2B). Subsequent to dCDP formation and $Y_{356}^{-}$ regeneration during reverse RT, reoxidation of F$_3$Y and re-reduction of oxidized $\alpha_2$ facilitate further turnovers. We argue subsequently that the rate-limiting step in $F_3Y^{-}/\beta_2$ is reoxidation of F$_3$Y by $Y_{356}^{-}$.

**DISCUSSION**

The rate-limiting protein conformational change(s) that gate(s) *E. coli* class Ia RNR turnover has precluded insight into the forward RT, nucleotide reduction, and reverse RT processes. Our current model for wt RNR based on studies similar to those described herein for F$_3$Y•/β2 is shown in Scheme 2.
Upon association of β2/α2/CDP/ATP, a conformational change (s) (5–10 s⁻¹, Scheme 2, step A) triggers rapid RT into α and nucleotide reduction (>100 s⁻¹, step B). DeoxyCDP formation is rate-limited by the conformational change (s) and occurs at 5–10 s⁻¹ as measured by RCQ methods reported previously and reproduced here under the same conditions utilized for the F3Yβ2 studies (Figure S4). Subsequent to dCDP formation, reverse RT to regenerate Y122 is required to be downhill and rapid (>10⁵ s⁻¹, step C) as modeled by Ge et al. to account for our inability to observe Y122 disappear and reappear during turnover (±TR/TRR/NADPH). The physical steps in wt RNR preclude detection of intermediates in these processes. Thus, studying the chemistry has required engineering specific perturbations to the system initially through site-directed mutagenesis and the use of mechanism-based inhibitors and, more recently, with site-specific incorporation of unnatural amino acids. While with many of these approaches we were able to monitor the disappearance of Y122 concomitant with formation of new radicals, in none of these cases was the catalytic cycle of RNR completed, and no insight was obtained into reverse RT.

Our recent engineering of an orthogonal tRNA-synthetase tRNA pair that can incorporate di- and trifluorotyrosines (F3Y, n = 2, 3) with a range of reduction potentials and pKₐs in RNR allowed us to introduce a tunable thermodynamic perturbation of PCET kinetics with minimal steric perturbations. F3Y is predicted as ~10 mV harder to oxidize than Y, assuming that the first step in forward RT involves PT from the water on the diferric cluster to F3Y concomitant with ET from Y356 to F3Y (Figure 1). F3Yβ2 is capable of catalyzing multiple turnovers but allows detection of Y356 (±TR/TRR/NADPH) due to perturbed reverse RT kinetics. Our current model for F3Yβ2 turnover is shown in Scheme 3. The ability to accumulate Y356 in F3Yβ2 but not in wt RNR is directly related to the differences in the rate-limiting step in the two systems.

In our model, the F3Yβ2/ωt-α2/CDP/ATP complex undergoes a conformational change prior to generation of Y356 in one α/β pair (Scheme 3, step A). The RFQ-EPR data reported in Figures 3A and 4A provide the rate constants for this step and suggest that F3Yβ2 perturbs the conformational gate relative to the wt enzyme (20–30 vs 5–10 s⁻¹). We expect that forward RT into α and dCDP production (step B in Schemes 2 and 3) occur with similar rate constants to wt RNR (>10⁶ s⁻¹). DeoxyCDP formation is rate-limited by the slow, conformationally gated generation of Y356 during forward RT as measured by the first phase in the absence of a reducing system (Figures 3B and 4B) or the burst phase in the presence of TR/TRR/NADPH (Figure 6) in the RCQ studies. Subsequent to product formation, we propose that reverse RT to regenerate Y122 is fast as modeled in wt RNR where reverse RT to regenerate Y122 is 10⁰ s⁻¹. However, unlike in wt RNR, slow reoxidation of F3Y (step C) rate-limits subsequent turnovers.

In the absence of a reducing system, the RFQ-EPR data (Figures 3A and 4A) provide the rate constants for step C. Upon regeneration of F3Yβ2 rapidly dissociates from a partially oxidized α2, associates with a second reduced α2 and cycles through steps A–C until all α2s are completely oxidized. Y356 concentration does not vary significantly during this time as visualized by the plateaus in the RFQ-EPR kinetic traces (Figures 3A and 4A). The second phase of the RCQ studies described in Figures 3B and 4B provides the rate constants for turnover in the absence of TR/TRR/NADPH (Scheme 3, branch I). Altered reverse RT kinetics in F3Yβ2 allow us to observe for the first time the disappearance and reappearance of the radical at position 122 subsequent to complete oxidation of α2. The molecular bases for our ability to observe reverse RT are not well-understood but are likely related to the initiating step in the PCET process. In addition to perturbing the driving force for RT, the fluoro substitutions could alter the distance between the phenolic oxygen and the water on the diferric cluster, thus affecting PT between the two (Figure 1). F3Y also perturbs the pKₐ at position 122 compared to Y (solution pKₐ 6.4 vs 10). Depending on the pKₐ of the water on the diferric cluster, the phenolate F3Y⁻ could be generated instead of the anticipated phenol F3Y.

Additional insight into the differences between wt and F3Yβ2 catalysis is obtained from the amplicity data for dCDP formation in the absence of a reducing system (Table 1 and Figure S4). In the F3Yβ2 system, the first phase (0.5 dCDP/F3Y⁻ at 25 °C) reports on the very first turnover by an α/β pair (Figure 2B),
while the second phase (2.9 dCDP/F3Y*) reports on consumption of all remaining reduced α2s, rate-limited by reverse RT. This result is distinct from our previous\(^1\)\(^1\) and current observations for wt RNR (Figure S4) where two phases are also measured for dCDP formation. The first phase is presumed to report on the conformationally gated generation of 2 dCDPs by all α2s (the experimental observation is 1.3 ± 0.2 dCDP/α2, 6 ± 1 s\(^{-1}\)), and the second phase is interpreted to report on the generation of 2 additional dCDPs subsequent to re-reduction of the active site disulphide (the experimental observation is 1.6 ± 0.2 dCDP/α2, 0.5 ± 0.1 s\(^{-1}\)). The variation in the amplitudes of the two phases between wt and F3Y*/β2 is consistent with different rate-limiting steps in the two systems. However, in both cases the total number of dCDPs generated is the same: 3 dCDP/α2 (Schemes 2 and 3, branch I).

The EPR and RCQ data collected in the presence of a reducing system also lend support to Scheme 3. In F3Y*/β2, a burst of dCDP formation prior to steady-state turnover is observed. The amplitude of this phase (∼0.5 dCDP/F3Y* at 25 °C) again reflects that turnover occurs only on one α/β pair prior to the rate-limiting step and is consistent with slow reverse RT. Upon regeneration of F3Y* after one turnover (Scheme 3, step C), re-reduction of oxidized α2 by TR/TRR/NADPH resets the system for additional turnovers (branch II). Y\(_{550}^{\alpha\beta}\), under these conditions behaves in a similar fashion to Y\(_{122}^{\alpha\beta}\) in wt RNR; i.e., no changes in its concentration are detected during steady-state conditions (Table S2). Slow reoxidation of F3Y followed by rapid re-reduction, forward RT, nucleotide reduction, and regeneration of Y\(_{550}^{\alpha\beta}\) (Scheme 3, steps A–C) precludes observation of its disappearance and reappearance. We interpret the linear phases in Figure 6A,B as representative of the rate constants for reverse RT in the presence of a reducing system (Scheme 3, step C) and the rate constant limits for re-reduction of oxidized α2. Although we set a lower limit for re-reduction from our experiments, we note that the rate constant for this step must be relatively fast, >5–10 s\(^{-1}\), to account for our inability to monitor changes in Y\(_{550}^{\alpha\beta}\) concentration.

A comparison of the burst phase for F3Y*/β2 and that for wt RNR in the presence of TR/TRR/NADPH reveals distinct behavior in the two systems (Figure 6 and Figure S5).\(^11\) Although the conformational change is rate-limiting for dCDP formation in wt RNR, we have previously noted that the rate-limiting step in the presence of a reducing system can switch to re-reduction of oxidized α2 at the high protein concentrations required for RCQ studies (10 μM).\(^11\) In wt RNR, this results in a conformationally gated burst of 2 dCDPs by all α2s (the experimental observation is 1.9 ± 0.1, 9 ± 2 s\(^{-1}\)) prior to steady-state turnover (Figure S5).\(^11\) The burst phase reflects oxidation of all α2 active sites despite the presence of only 60% active wt-β2 (1.2 Y*/β2, Figure 2A) and does not represent a single turnover. In contrast to the wt system, we have isolated the very first turnover by an α2β2 complex with F3Y*/β2 due to rate-limiting reverse RT.

As a final point, the rate constant measured for dCDP formation in the presence of a reducing system is 3-fold faster that that measured in its absence (Scheme 3, branch I vs I). A similar variation has been previously noted for dCDP formation in the wt system.\(^11\) It is possible that re-reduction of the active site disulphide by the C-terminal tail (Figure 5, step B) is reversible and only driven to completion when the TR/TRR/NADPH system is included in the assays. However, we currently cannot rule out other modes by which TR accelerates re-reduction of the α2 active site.

### CONCLUSIONS

Radical initiation in the class I RNRs is proposed to involve long-range PCET through three pathway tyrosines.\(^9\),\(^10\),\(^17\) Using an engineered RNR system, we have observed one of the proposed intermediates and demonstrate for the very first time chemically competent reverse RT that completes the RNR catalytic cycle. We additionally obtained insight into radical stoichiometry within β2, half-sites reactivity, and the ability of β2 to act catalytically during turnover. This work highlights the utility of unnatural amino acids in engineering specific perturbations for the study of redox active tyrosine residues in proteins; F3Y could facilitate understanding of a number of additional tyrosyl radical mediated metabolic processes.\(^45\)–\(^48\)

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b09189.

Detailed experimental procedures, tables, figures, and scheme of cytosine release from CDP (PDF)

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**Notes**

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