A >200 meV Uphill Thermodynamic Landscape for Radical Transport in *Escherichia coli* Ribonucleotide Reductase Determined Using Fluorotyrosine-Substituted Enzymes

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Supporting Information

ABSTRACT: *Escherichia coli* class Ia ribonucleotide reductase (RNR) converts ribonucleotides to deoxynucleotides. A diferric-tyrosyl radical (Y122•) in one subunit (β2) generates a transient thyl radical in another subunit (α2) via long-range radical transport (RT) through aromatic amino acid residues (Y122⇌[W48]{Y356 in β2 to Y731 ⇌ Y730 ⇌ C439 in α2}). Equilibration of Y356•, Y731•, and Y730• was recently observed using site specifically incorporated unnatural tyrosine analogs; however, equilibration between Y122• and Y356• has not been detected. Our recent report of Y356• formation in a kinetically and chemically competent fashion in the reaction of β2 containing 2,3,5-trifluorotyrosine at Y122 (F3Y122•β2) with α2, CDP (substrate), and ATP (effector) has now afforded the opportunity to investigate equilibration of F3Y122• and Y356•. Incubation of F3Y122•β2, Y731F-α2 (or Y731F-α2), CDP, and ATP at different temperatures (2–37 °C) provides ∆E°(F3Y122•β2−Y356•) of 20 ± 10 mV at 25 °C. The pH dependence of the F3Y122•⇌Y356• interconversion (pH 6.8–8.0) reveals that the proton from Y356 is in rapid exchange with solvent, in contrast to the proton from Y122. Insertion of 3,5-difluorotyrosine (F2Y) at Y356 and rapid free-quech EPR analysis of its reaction with Y731F-α2, CDP, and ATP at pH 8.2 and 25 °C shows F2Y356• generation by the native Y122•, F2Y-RNRs (n = 2 and 3) together provide a model for the thermodynamic landscape of the RT pathway in which the reaction between Y122 and C439 is ~200 meV uphill.

# INTRODUCTION

The *E. coli* class Ia ribonucleotide reductase (RNR) contains two homodimeric subunits, α2 and β2, and functions as an α2/β2 complex.1,2 Its active cofactor is a diferric-tyrosyl radical (Y122•) unit buried within β2. This cofactor generates a transient thyl radical (C439•) in α23,4 which initiates reduction of the four nucleotides (CDP, GDP, ADP, and UDP) to their corresponding 2’-deoxynucleotides (dNDP), with the specificity of reduction dictated by the appropriate allosteric effectors (ATP, TTP, dGTP, and dATP).5–8 During each turnover, Y122• reversibly oxidizes C439 via multiple proton-coupled electron transfer (PCET) steps through a pathway involving aromatic amino acid residues Y122⇌[W48]{Y356 in β2 to Y731 ⇌ Y730 ⇌ C439 in α2}. Currently, there is no direct evidence for the involvement of W48 in RT.5–11 In the wild-type (wt) RNR, only Y122• is observed in the presence of substrates (S) and effectors (E); there has been no detectable electron delocalization over the other pathway tyrosines.12 In this paper, we present the first insight into the thermodynamic landscape of the RT pathway within β2. Site-specific replacement of either Y122 or Y356 with fluorotyrosines (F,Y, n = 2 and 3) in combination with pathway-blocked α2 mutants (Y371F-α2 or Y372F-α2)/CDP/ATP and X-band electron paramagnetic resonance (EPR) spectroscopy13 provides evidence for equilibration of Y122• with Y356• as a function of temperature and pH. These studies have allowed estimation of ∆E°(Y356•−Y122•) of ~100 mV.

Detection of low concentrations of any pathway radical in the wt RNR system is challenging due to rate-limiting conformational changes and the substantial overlap in the EPR spectra of the Y•’s.14 Initial attempts to address if Y122• equilibrated with the pathway tyrosines (Y356•, Y731•, and Y730•) utilized the ability to collapse the Y• doublet EPR spectrum into a singlet with β-methylene-deuterated ([β-H2]Y) Y'.12,14 β2 containing globally incorporated [β-H2]Y’s was reacted with α2 containing protonated Y’s, dCDP, and TTP. These conditions promote α2/β2 complex formation1 but prevent turnover, thus potentially allowing equilibration of the pathway Y•’s. Unfortunately, no
unlabeled Y• signal could be detected; the EPR spectrum of Y• in the α2β2 complex was identical to that in free β2.12

Recently, we showed that the reaction of NO2Y122β2 (3-nitrotyrosine at position 122), which is predicted to be 200 mV more difficult to oxidize than Y at pH 7.0,15,16 with wt-α2, CDP, and ATP generates a new Y•, localized to Y356.17 Using 3,5-di-α-fluorotyrosine (F3Y) at Y731 (or Y730) we demonstrated that Y356• equilibrated with F3Y122• or F3Y730•.14 The analysis was facilitated by the unique F3Y• features arising from 19F and 1H-β hyperfine interactions that are observed in both the low- and high-field regions of the EPR spectrum.11,13 This spectroscopic handle gave us the first opportunity to investigate the effect of the protein environment on the reduction potentials of the pathway Y•'s. Quantitation of Y356• in β2 and F3Y731• (or F3Y730•) in α2 by EPR spectroscopy allowed estimation of a ΔE°β′(Y731/Y730−Y356•) of ~100 mV.14 The thermodynamic landscape of the RT pathway constructed from these studies is shown in Figure 1. We proposed that the overall

equilibrate F3Y122• and Y356• with Y731-F-α2 also provided the opportunity to investigate the fate of the Y356• upon oxidation of this pathway Y. A plot of the log([Y356•]/[F3Y122•]) versus pH provides a slope of 1.2 ± 0.2 at 25 °C, consistent with rapid release of the Y356• proton to solvent. With a knowledge of the pH dependence of the F3Y122•/Y356• equilibration, we have implemented an experimental design to determine the thermodynamic difference between Y122 and Y356. Increasing amounts of Y356• are observed with increasing pH. Additionally, by choosing an appropriate pH the reduction potential of F2Y can be tuned to be essentially equal to that of Y356• but oxidized F2Y• has the potential to be spectroscopically observable because of the 19F hyperfine features.13 Thus, the ability of Y122• to oxidize F2Y• incorporated in place of Y356 (F2Y356Fβ2) was tested. Rapid freeze-quench (RFQ)-EPR spectroscopy of the reaction between F2Y356Fβ2, Y731-α2, CDP, and ATP at pH 8.2 and 25 °C revealed F3Y356• at 3 ± 1% of the total radical concentration. This observation provided a ΔE°β′(F3Y122•−Y122•) of 70 ± 5 mV, which along with our recent measurement of the reduction potential of F2Y in a protein environment23,25 gives an estimate of ΔE°β′(Y356•−Y122•) of ~100 mV at pH 7.6. The results of the site specifically incorporated unnatural amino acids described herein together with our previous studies allow us to propose a thermodynamic landscape for the RT pathway in the E. coli class Ia RNR that is ~200 mV uphill between Y122 and C459•.

**Materials and Methods**

**Materials.** (His)6-Y731F-α2,26 (His)6-Y356F-α2,26 wt-α2 (specific activity of 2500 nmol/min/mg),26 tyrosine phenol lyase,27 F2Y,28 and F3Y29 were isolated; apo F1Ydβ2 was expressed, isolated, and reconstituted23 as previously reported. F3Y356Fβ2 (0.7 Y•/β2) was available from an earlier study.29 CDP and ATP were purchased from Sigma-Aldrich. Assay buffer consisted of 50 mM HEPES pH 7.6, 15 mM MgSO4, and 1 mM EDTA unless otherwise specified. In all studies, the temperature was controlled using a Lauda RM6 circulating water bath. The reference spectrum for F1Y122• and its simulation were recently reported.30 The reference spectrum for Y122•, which was obtained as the signal averaged sum of the Y356• difference spectra, is in agreement with the previously reported spectrum.37

**Hand-Quench EPR Analysis of Y356• Formation as a Function of Temperature.** Assay mixtures containing a final volume of 250 μL with 25 μM Y356F-α2, 1 mM CDP, and 3 mM ATP in assay buffer were incubated in a water bath set between 2 and 37 °C. F3Y122•/β2 (0.8 F3Y•/β2) was added to a final concentration of 25 μM to initiate each of the reactions. The reaction mixtures were then transferred to X-band EPR tubes maintained in the water bath, and the samples were frozen in liquid isopentane (~140 °C) at 20 s (or 1 min) and analyzed by X-band EPR spectroscopy. The EPR parameters were as follows: microwave frequency 9.45 GHz; power 30 μW; modulation amplitude 1.50 G; modulation frequency 100 kHz; time constant 40.96 ms; and conversion time 20.48 ms. Three independent sets of experiments were carried out.

**Analysis of EPR Data.** Two different methods, A and B, were used for quantitation of the two radicals due to the small changes in the EPR spectra associated with the changes in T and pH (section described subsequently), the complexity of the spectra, and the half-sites reactivity of RNR (that is, 50% of the starting F3Y•/β2 remains unchanged). The data shown in the Results section were analyzed by method A, chosen for visualization purposes. Both methods of analysis provide similar outcomes and are summarized in Tables S1 and S2. The total spin remained unchanged in all the samples throughout the analyses. The ΔE°β′(F3Y122•−Y356•) was calculated based on the two quantitation methods described below and using

\[ \Delta E^\circ = \frac{RT \ln K_{NS}}{F} \]  

(1)
where $K_{eq} = \frac{[Y_{356}\cdot]}{[F_2Y_{122}\cdot]}$, $R$ is the ideal gas constant, $T$ is the temperature (K), and $F$ is Faraday’s constant.

**Method A: Quantitation of $F_2Y_{122}\cdot$ and $Y_{356}\cdot$ in $\beta2$ as a Function of Temperature.** Each EPR spectrum was normalized to have the same intensity in the low-field features associated with $F_2Y_{122}\cdot$. In this representation of the spectra, the intensity of $F_2Y_{122}\cdot$ remains constant, allowing easier visualization of the $Y_{356}\cdot$ signal that grows in with increasing temperature. Using the low-field features in the spectrum of $F_2Y_{122}\cdot$, $F_2Y_{122}\cdot$ was subtracted from each composite spectrum. The amount of $Y_{356}\cdot$ remaining was determined by double integration.\(^{13}\) The $Y_{356}\cdot$ spectrum observed for each sample was identical by this method.

**Method B.** A detailed description of data analysis by method B is presented in the Supporting Information. In the first step, the baseline was removed from each spectrum with a second-order polynomial fit. In the second step, the 50% signal from $F_2Y_{122}\cdot$ that remains in the composite spectra due to half sites reactivity was subtracted using the $F_2Y_{122}\cdot$ reference spectrum (Figure S1A). The resulting composite spectra show the interconversion between $F_3Y_{122}\cdot$ and $Y_{356}\cdot$ as a function of temperature (Figure S1B), free from the complications caused by half sites reactivity. However, this subtraction increases the noise level of the spectra, so the relative amounts of $F_2Y_{122}\cdot$ and $Y_{356}\cdot$ cannot be determined reliably by eye. Therefore, a script was written in Matlab 2016a to automatically subtract out the remaining $F_2Y_{122}\cdot$. The amount of remaining $F_3Y_{122}\cdot$ was determined by adjusting the intensity of the $F_2Y_{122}\cdot$ reference spectrum (Figure S1C) until the least-squares difference between the reference spectrum and the composite spectra in the g-value interval between 2.0363 and 2.0390 (this defines the highest S/N region of the low-field $F_2Y_{122}\cdot$ features) was minimized. The amount of $Y_{356}\cdot$ after subtracting out the remaining $F_2Y_{122}\cdot$ was determined by double integration. The $Y_{356}\cdot$ spectrum determined by this method was the same in each sample (Figures S1D and S2).

**Temperature-Dependent Equilibration of $F_3Y_{122}\cdot$ and $Y_{356}\cdot$ within the Same Sample.** To support equilibration between $F_3Y_{122}\cdot$ and $Y_{356}\cdot$ in $\beta2$ (at 25°C) as described above in the $Y_{731}F^-\alpha2$ reactions, the EPR spectrum of the 20 s sample was first recorded. Each sample was then thawed by submersion into a room-temperature water bath and was then incubated in a 2°C water bath for 15 s followed by refreezing and reacquisition of the EPR spectra. The samples were thawed again and then placed in a 2°C water bath for 15 s, refrozen, and the EPR spectrum recorded. Quantitation of $Y_{356}\cdot$ and $F_2Y_{122}\cdot$ was performed as described above.

**RFO-EPR Analysis of $Y_{356}\cdot$ Formation as a Function of Temperature.** RFO-EPR experiments were performed on an Update Instruments 1019 syringe ram unit and a model 715 syringe ram controller (ram speed 1.25 cm/s). $F_2Y_{122}\cdot$ ($70 \mu M$, 0.8 $F_2Y_{122}\cdot$,$/β2$ and CDP (2 mM) in assay buffer in one syringe were mixed with $Y_{731}F^-\alpha2$ (70 M$\mu$ and ATP (6 mM) in a second syringe and incubated at varying temperatures (2−37°C) for either 4 or 10 s. The reaction mixture was then sprayed into liquid isopentane, and the crystals were packed into EPR tubes for analysis by X-band EPR spectroscopy. A packing factor of 0.60 ± 0.02 was calculated for $F_2Y_{122}\cdot$. Data acquisition and analysis were performed as described for the hand-quench (HQ) method.

**HQQ-EPR Analysis of $Y_{356}\cdot$ Formation as a Function of pH.** $Y_{731}F^-\alpha2$ (25 M$\mu$), $F_2Y_{122}\cdot$ ($25 M$, 0.6–0.8 $F_2Y_{122}\cdot$,$/β2$), CDP (1 mM), and ATP (3 mM) were combined in 50 mM MES (pH 6.8) or HEPES (pH 7.0–8.0), 15 mM MgSO$_4$, and 1 mM EDTA and incubated at 5 or 25°C. Reaction mixtures were transferred to X-band EPR tubes also maintained in the water bath and frozen in liquid isopentane (−140°C) within 20 s (or 1 min) for analysis by X-band EPR spectroscopy using methods A and B described above. The data were fit to

$$\log K = pH - pK_a$$

where $K = \frac{[Y_{356}\cdot]}{[F_2Y_{122}\cdot]}$.

**RFO-EPR Analysis of the Reaction of $F_2Y_{122}\cdot β2$, $Y_{731}F^-\alpha2$, CDP, and ATP.** $Y_{731}F^-\alpha2$ (80 M$\mu$) and 6 mM ATP in 50 mM TAPS pH 8.2, 15 mM MgSO$_4$, and 1 mM EDTA in one syringe were rapidly mixed at 25°C with an equal volume of $F_2Y_{122}\cdot β2$ (80 M$\mu$, 0.7 $F_2Y_{122}\cdot$,$/β2$ and CDP (2 mM) in the same buffer in the second syringe. The reaction was aged for 10, 20, or 40 s, quenched in liquid isopentane, and analyzed by X-band EPR spectroscopy as described above. The EPR parameters were as follows: microwave frequency 9.45 GHz; power 30 $\mu$W; modulation amplitude 1.50 G; modulation frequency 100 kHz; time constant 163.8 ms; and conversion time 20.48 ms. The total number of scans were 700 (10 s sample), 600 (20 s sample), and 560 (40 s sample). The simulations were carried out using EasySpin v5.0.18\(^{13}\) in Matlab R2015b. The $g$-values (2.0073, 2.0044, and 2.0022) and $\beta$-H hyperfine tensor (54, 52, and 54 MHz) were fixed in the simulations using previously reported values for $Y_{356}\cdot$ in the reaction of NO$_2$Y$_{122}\cdot β2$ with $Y_{731}F^-\alpha2$\(^{14}\) and the $\beta$-H hyperfine values of $F_2Y_{122}\cdot$\(^{22}\).

### RESULTS

**Temperature-Dependent Distribution of $F_3Y_{122}\cdot$ and $Y_{356}\cdot$ in $\beta2$ in the Presence of CDP, ATP, and $Y_{731}F^-\alpha2$ (or $Y_{720}F^-\alpha2$).** We have recently shown that the reaction of $F_3Y_{122}\cdot$ with CDP, $\alpha$-2, ATP, and ATP generates a kinetically and chemically competent $Y_{356}\cdot$ that can reoxidize $F_3Y_{122}\cdot$. We hypothesized that if we carried out the same experiment with a block in the pathway ($Y_{731}F^-\alpha2$ or $Y_{720}F^-\alpha2$) then equilibration of $F_3Y_{122}\cdot$ and $Y_{356}\cdot$ could be measured by EPR spectroscopy as a function of temperature, allowing determination of $\Delta E^{\circ\circ}$($F_3Y_{122}\cdot$,$/Y_{356}\cdot$). $F_3Y_{122}\cdot$,$/β2$, CDP, and ATP were incubated with $Y_{731}F^-\alpha2$ at varying temperatures from 2 to 37°C for 20 s or 1 min. The samples were then frozen in liquid isopentane and examined by X-band EPR spectroscopy. Analysis of the EPR spectra at the chosen times showed no differences between the two time points, suggesting that the reaction mixture had equilibrated. The data from the 20 s incubation time is presented herein. No loss of total spin was observed between the two time points or between the different temperatures.

Interpretation of the EPR data requires consideration of the contributions of each radical and the complexities associated with *E. coli* RNR. First, Figure 2 shows a 1:1 mixture of $F_2Y_{122}\cdot$ and $Y_{356}\cdot$ (pink) and $Y_{356}\cdot$ (blue). All spectra presented subsequently are additive and contain the same concentration of $F_3Y_{122}\cdot$ and increasing amounts of $Y_{356}\cdot$. The dotted lines highlight the regions of the spectrum where the changes that occur upon $Y_{356}\cdot$ formation are most apparent.
with active β2 containing a F3Y122• in each β monomer. Furthermore, while the active form of RNR is αβ2, the enzyme exhibits half-sites reactivity where only one of the two Y122•’s (one α/β pair) is active at a time. A consequence of these phenomena is the presence of 50% of the total spin as residual F3Y122• in all reaction mixtures. Thus, the data shown in Figures 3, S3, and S4 are presented using method A.

Figure 3. Composite EPR spectra of the F3Y122•/β2/Y731F-α2/CDP/ATP reaction as a function of temperature (2–15 °C). The composite spectrum at each temperature was acquired on three independently prepared samples. (A and B) Low- and high-field regions of the spectra for trial 1 are shown here. The color code is described in panel A. Trials 2 and 3 are shown in Figure S3. The composite EPR spectra collected between 15 and 37 °C are shown in Figure S4. (C and D) Low- and high-field regions of a simulated spectrum of a reaction mixture containing 50% each of F3Y122• and Y356•. The spectrum was generated by adding the individual spectra of F3Y122• and Y356• (Figure 2). The dotted lines identify spectral features that are characteristic of Y356•.

As shown by the dotted line in Figure 3A,B, increasing amounts of F3Y122• can then be observed between 2 and 15 °C. Two additional replications of this experiment are shown in Figure S3. The changes in the spectra directly correlate with increasing amounts of Y356• from 17 ± 5% (average of three trials at 2 °C) to 31 ± 2% (average of three trials at 15 °C) of total spin; the quantitation of these data is summarized in Table S1. In contrast with these observations, minimal changes are visualized in the composite EPR spectra recorded between 15 and 37 °C (Figure S4 and Table S1). The average amounts of Y356• in the three experiments are shown in Table S1 (31 ± 2% at 15 °C and 33 ± 1% at 37 °C). The percentage Y356• of total spin as a function of temperature is shown in Figure 4 (pink dots). A break in the curve is observed at ~15 °C, and the amount of Y356• does not appear to change significantly from 15 to 37 °C.

Control Experiments to Support F3Y122•/Y356• Equilibration. Two types of experiments were carried out to provide further support for the equilibration of F3Y122• and Y356•. Previous studies on adenosylcobalamin (AdoCbl) class II RNR have shown that slow quenching of samples by hand shifts the equilibrium relative to rapid freezing methods. Thus, changing ratios of F3Y122• and Y356• by RFQ would support equilibration of the two radical states. Preliminary experiments revealed no spin loss and minimal changes in the EPR spectra of samples quenched at 4 and 10 s using the RFQ method. The time scale for quenching was chosen based on kinetic experiments performed with F3Y122•/β2 and wt-α2.22 Thus, subsequent RFQ samples were quenched at 10 s. The results of these experiments are shown in Figure S5 and summarized in Table S1. The amount of Y356• observed by RFQ is 5–10% higher than that recorded by the HQ method. However, similar trends are observed between the RFQ-EPR and HQ samples. Increasing amounts of Y356• are observed between 2 and 15 °C, whereas the spectra collected between 15 and 37 °C show minimal changes in the percentage of Y356• (Table S1 and Figure S6). The RFQ and HQ methods together support equilibration of F3Y122• and Y356• and the ability to shift the equilibrium between the two radical states based on the quenching method.

A second experiment to support equilibration between F3Y122• and Y356• was carried out as described in the Materials and Methods section. In this experiment, the EPR spectrum of a single sample that was equilibrated at 25 °C was first measured and the sample thawed, equilibrated at 2 °C, and reanalyzed by EPR spectroscopy. The sample was then thawed a final time, shifted back to 25 °C, and the EPR spectrum was recorded. The composite EPR spectra are shown in Figure S7A,B, and the amounts of Y356• ascertained from these spectra are summarized in Table S3. The total spin changed minimally and the ratio of the two radicals shifted with temperature as predicted by the trend observed in Figure 4. The data together support equilibration of F3Y122• and Y356• with an unusual temperature dependence.

Effect of the F Block at Residue 731 in α2 on the F3Y122•/Y356• Equilibrium. Recent high-field (HF)-EPR spectroscopy experiments indicate that the electrostatic environment of Y356• changes in a reaction containing Y731F-.
a2 relative to wt-a2. Differences in reactivity between wt-a2 and Y31F-a2 are also recorded for photo-RNR, which contains a [Re3] photooxidant appended to the C-terminal tail of β2 (S355C). We therefore posited that the block at 731 could perturb the reduction potential of Y356 compared to the wt enzyme. The equilibration experiments were repeated with Y730F-a2, and as seen in Figure 4 (blue dots), variations can be observed between Y730F-a2 and Y730F-a2, with the former construct generating slightly higher amounts of Y356.

Calculation of ∆E°(F3Y122•−Y356•) from the Y731F and Y730F-a2 Studies. To calculate the reduction potential difference between F3Y122• and Y356•, the ln Kq([Y356•]/[F3Y122•]) observed in the Y731F and Y730F-a2 reactions at 25 °C by the HQ method were used (eq 1); ∆E°(F3Y122•−Y356•) at 25 °C is 20 ± 10 and 5 ± 7 mV, respectively. We note again the unusual temperature dependence of the Y356• amounts with a break at 15 °C. A similar temperature dependence has been noted for steady-state dNTP formation in a 1976 study by von Dobeln and Reichard.36 The cause(s) of the break in Figure 4 and in the previous activity studies are unknown but are likely related to RNR conformational changes that rate-limit RT and nucleotide reduction.

Equilibration of F3Y122• and Y356• as a Function of pH and Rapid Proton Exchange with Solvent during Y356 Oxidation. The equilibration of F3Y122• and Y356• described above gave us the opportunity to investigate the fate of the proton released upon Y356 oxidation. Two scenarios for this proton transfer (PT) can be envisioned (Scheme 1). In one case, the proton from Y356 is transferred to an amino acid residue (X) and is not solvent-exchangeable. (B) The proton is in fast exchange with solvent. The initial proton acceptor (Y) is either an amino acid residue or water.

\( F3Y122• + Y356• \rightleftharpoons F3Y122•Y356• + H^+ \)

(A) The proton released from Y356 is accepted by an amino acid residue (X) and is not solvent-exchangeable. (B) The proton is in fast exchange with solvent. The initial proton acceptor (Y) is either an amino acid residue or water.

Figure 5. Composite EPR spectra of the F3Y122•/2/Y31F-a2/CDP/ATP reaction at 25 °C as a function of pH. The composite spectrum at each pH was acquired on two independently prepared samples. (A and B) The low- and high-field regions of the spectra for trial 1 are shown here. The colors represent different pH values as described in panel A. Trial 2 is shown in Figure S8. (C and D) Low- and high-field regions of a simulated spectrum of a reaction mixture containing 50% each of F3Y122• and Y356•. The spectrum was generated by adding the individual spectra of F3Y122• and Y356• (Figure 2). The dotted lines identify spectral features that are characteristic of Y356•.

Figure 6. pH dependence of Y356• formation in the reaction of F3Y122•/2/Y31F-a2/CDP/ATP at 25 °C. (A) Percentage Y356• of total spin as a function of pH. (B) log K as a function of pH where K is the ratio of Y356• to F3Y122•. The observed pH dependence of slope 1.2 ± 0.2 supports that the Y356• proton is in fast exchange with solvent.

To gain insight into the PT pathway at Y356 a series of studies were undertaken. F3Y122•/β2, Y31F-a2, CDP, and ATP were combined in designated assay buffers (pH 6.8–8.0), incubated for 20 s or 1 min at 25 °C (or 5 °C), quenched by hand, and analyzed by EPR spectroscopy. The analysis was first carried out using method A. As with the temperature dependent studies, no variations in total spin were recorded, and no differences were observed between the spectra of samples incubated for 20 s and 1 min, observations consistent with a reaction at equilibrium. The spectral changes are shown in Figure 5A,B, and the dotted line shows an increase in the percentage of Y356• for the pH range 6.8–8.0 at 25 °C (see Figure S10A for data from pH 6.8–7.8 for 5 °C). The percentage of Y356• at pH 6.8 and 7.0 are very low (Table S2), and the percentage of Y356• above pH 8.0 at 25 °C and pH 7.8 at 5 °C does not change. The maximum amounts of Y356• at 25
Although the above studies allowed establishment of ΔE°(F₂Y₁₂₂•β/Y₃₅₆•) in F₂Y₁₂₂•β/2, the ΔE°(Y₁₂₂•−Y₃₅₆•) in wt RNR, which is essential for understanding the thermodynamics of the RT pathway, remains unknown. The pH studies described above show that maximum Y₃₅₆• is generated with F₂Y₁₂₂•β/2 at pH 8.0 or greater and 25 °C. Recent studies suggest that the difference in reduction potential between Y and F₂Y at position 356 at pH 8.2 is small (<10 mV) and that the activity of F₂Y₃₅₆•β/2 at this pH is 50% of the wt activity.7 The pKᵦ of F₂Y₃₅₆• is estimated to be 7.6 at position 356;15 thus, at pH 8.2, >80% of F₂Y₃₅₆• is in the deprotonated state. Due to the ability to detect small amounts of F₂Y utilizing its unique spectroscopic features in the low- and high-field regions of the EPR spectrum, we carried out the following experiment in the hope of obtaining insight about ΔE°(Y₁₂₂•−Y₃₅₆•). F₂Y₅₃₆•β/2, Y₇₃₁F-α2, CDP, and ATP were reacted at pH 8.2 for 10, 20, or 40 s, and the reaction was quenched using the RFQ instrument and analyzed by EPR. Quenching on the millisecond time scale was used to avoid potential shifting of the equilibrium observed with hand quenching (Table S1 and Figure S6).33

The RFQ-EPR data for the reaction at 20 s are shown in Figure 7, and the 10 and 40 s data are shown in Figure S11. A view of the entire spectrum is shown in the inset in Figure 7. The results reveal small features on the low- and high-field sides that suggested the presence of F₂Y₅₃₆•.13 The resolved hyperfine splittings were simulated with the "pepper" module of EasySpin as described in the Methods section. From the initial simulations, it was recognized that the β⁻¹H hyperfine parameters matched the doublet splitting on the high-field side of the spectrum, confirming the identity of this radical species as F₂Y₅₃₆•. The interdoublet splitting was reproduced with two equivalent ¹⁹F couplings having an A₂₂ of 147 MHz.13,30 The sharpness of the 3,5-¹⁹F features are similar to those previously reported for the other pathway residues F₂Y₁₂₂•13 F₂Y₇₃₁•14 and F₂Y₇₃₀• reflecting a rigid conformation constrained by the protein environment. The A₂₂ value for F₂Y₅₃₆• is slightly weaker than those reported previously for the other F₂Y’s (Table S4) and will be of importance when structural insight is obtained.

The amount of F₂Y₃₅₆• was similar at all three time points and was approximated from the simulated spectrum by matching the signal intensities of the wing features in the experimental and simulated spectra and comparing the double integral of the two. The greatest source of error in the analysis comes from the intrinsic line broadening factor (17 ± 4 MHz) used in all simulations.14 The amount of F₂Y₅₃₆• in the 20 s sample was quantitated as 3 ± 1% of total spin. This amount of radical reflects ΔE°(F₂Y₅₃₆•−Y₃₅₆•) of 70 ± 5 mV, which in combination with our reduction potential studies4,25 allows calculation of ΔE°(Y₃₅₆•−Y₁₂₂•) of ~100 mV at pH 7.6 (Figure 8).

![Figure 8](image)

**Figure 8.** Current thermodynamic landscape of the PCET pathway at 25 °C and pH 7.6. (A) Studies performed on F₂Y₁₂₂•β/2 described in this work provided an estimate of the relative reduction potentials of F₂Y₁₂₂• and Y₃₅₆•. (B) Studies performed on F₂Y₅₃₆•β/2 provided an estimate of the relative reduction potentials of Y₁₂₂• and Y₅₃₆•. W₄₅ has been removed from the landscapes for the sake of clarity.

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**DISCUSSION**

RNRs are divided into three classes based on the metallocofactor used for thyrid radical formation.6 All classes of RNR initiate nucleotide reduction by thyrid radical mediated ³⁻H atom abstraction from the substrate.18 The reducing equivalents for the reaction are provided by oxidation of a pair of cysteines in the active site.38-40 with a subtype of the class III enzyme which uses formate as the reductant as the sole cofactor,3 whereas the class III system uses a stable gycyl radical to generate the transient thyrid radical.42 These
observations raise the issue of why and how a 35 Å oxidation process evolved in the class I RNR instead of a direct H atom abstraction process that is used by the other classes. The turnover number for deoxyriribonucleotide formation (2–10 s−1) and the large distance between Y122• and Cys39 in the class Ia RNR require intermediates in the oxidation process and raise the question of how the thermodynamic and kinetic landscape of this process has evolved to maintain balanced dNTP pools and avoid self-inactivation. Investigation of this oxidation process has proven challenging primarily due to the slow rate-limiting conformational changes that occur in the αβ2 complex subsequent to S/E binding and prior to RT. Furthermore, the substantial overlap of the EPR spectra of Y79′s would make identification of these species challenging even if the rate-limiting step could be altered.

**Thermodynamic Landscape of the RT Pathway within the β2 Subunit.** Recently we have assembled the dimeric NO2Y122• cofactor (t1/2 of 40 s at 25 °C) in the β2 subunit of RNR. NO2Y122• is ~200 mV more oxidizing than Y122• and has provided insight about the thermodynamic landscape for the RT pathway in two ways. When NO2Y was substituted in place of each Y in the pathway (Figure 1, where x = 122 and 356 in β2 and 731 and 730 in α2), the resulting mutants were catalytically inactive. Thus, perturbation of the reduction potential by +200 mV is sufficient to shut down the RT pathway. This observation supports previous proposals about the extent to which uphill steps can be accommodated in electron transfer (ET) pathways in general, and in RNR specifically. NO2Y substitution at each position also allowed assessment of the protein environment perturbation of the pKs of the phenol, relative to the pKs in solution. Positions 356, 731, and 730 were found to be minimally perturbed (+0.4, 1.0, and 1.2 units) and position 122 was found to be greatly perturbed (greater than +3 units). We assume that a similar position-dependent perturbation occurs with the F3Y’s incorporated at 356, 731, and 730. However, the unique environment of Y122 (hydrophobic and adjacent to the d ferric cluster), this assumption cannot be made.

The ability to generate NO2Y122• in β2 allowed observation of the equilibration of the pathway tyrosyl radicals: Y356•, F2Y731•, or F2Y730•. This observation was fortuitous as the equilibration arose from several unanticipated consequences of NO2Y122• substitution. First, this mutant uncoupled the conformational gating masking the wt RT process. DeoxyCDP and Y356• formed during reverse RT occurred at 100–300 s−1 much faster than the wt turnover of 5 s−1. Although Y356• was generated rapidly, it was unable to reoxidize the NO2Y• phenolate formed during forward RT (Scheme 2). Thus, a block in the pathway occurred without additional mutations. We note that in wt RNR there is evidence to suggest that a proton is delivered to Y122• from the water on Fe1 in the cluster during forward RT (Scheme 2). In the case of NO2Y, this does not occur, and the phenolate is formed. It is likely that the water on Fe1 remains protonated providing insight into the relative pKs of Y122 and Fe1–H2O. Since the NO2Y phenol has a pK of 7.1, this raises issues about the protonation state of Y122• (pK of phenol is 6.4) on reduction during forward RT (Scheme 2).

Due to the inability to investigate equilibration of Y356• with Y731• and Y730• in wt RNR, F2Y was inserted in place of either Y731 or Y730 providing access to the unique EPR spectroscopic features of F2Y. These experiments showed the presence of 10–15% F2Y731• (or F2Y730•). A knowledge of the pKs

**Scheme 2. First PCET Step in the RT Pathway of E. coli Class Ia RNR**

**Figure 1.** Thermal stability of NO2Y122• in wt RNR. RT initiation in F3Y122•/β2 is proposed to generate the F3Y122• phenolate (Scheme 1).

**(A)** In the wt-β2/α2/CDP/ATP complex, PT from Fe1–H2O to Y122• occurs concomitant with ET from Y356 to Y122•. (B) In the NO2Y122•/β2/α2/CDP/ATP complex, ET from Y356 to Y122• generates the NO2Y• phenolate. RT initiation in F3Y122•/β2 is proposed to generate the F3Y122• phenolate (Scheme 1).
protonation state of F₃Y₁₂₂ while favored to be deprotonated, is unknown and is under investigation.

The potential difference of ~20 mV calculated between F₂Y₁₂₂ and Y₃₅₆ makes generation of F₂Y₁₂₂ an appealing model. We predict that ΔE°(NO₂Y₁₂₂/F₂Y₁₂₂) (at pH 7.6) is ≥200 mV, owing to the inability of Y₃₅₆ to reoxidize NO₂⁻. With these two values, we can estimate ΔE°(NO₂Y₁₂₂/F₂Y₁₂₂) to be greater than or equal to ~184 mV. This calculation agrees with the predicted potential difference between these two analogs based on the solution DPV data collected on the protected amino acids (~180 mV). Unfortunately, we cannot at present directly extrapolate the potential difference calculated between NO₂Y₁₂₂/F₂Y₁₂₂ and Y₃₅₆/Y₃₅₆ to Y₁₂₂/Y₁₂₂. This is primarily due to the unique nature of residue 122’s environment compared to that of the other pathway Y’s. The Y₁₂₂ site is not in equilibrium with solvent at the time course of our experiments (<20 s); its reduction potential is pH-independent and is directly determined by the dielectric constant of the protein environment. Due to these reasons, we turn our attention to an alternate way to monitor equilibration of Y₃₅₆ and Y₃₅₆ where the native Y₁₂₂ remains intact but Y₃₅₆ is replaced with F₂Y₁₂₂.

Our observations with NO₂Y₁₂₂/F₂Y₁₂₂ and the pH-dependent studies reported herein suggest that ΔE°(Y₁₂₂/F₂Y₁₂₂) (∼184 mV) makes generation of F₂Y₁₂₂ an appealing model. We predict that its reduction potential is a good approximation with solvent (Figures 6B and S10B), and at an appropriate pH, NO₂Y₁₂₂ reoxidizes Y₁₂₂ must be downhill and rapid (10³ s⁻¹). In the case of F₂Y₁₂₂, we have measured formation of Y₃₅₆ (20–30 s⁻¹) and demonstrated that reoxidation of F₂Y₁₂₂ by Y₃₅₆ is slow (0.4–1.7 s⁻¹) and rate-limiting for multiple turnovers. In the NO₂Y₁₂₂/F₂Y₁₂₂ system, Y₃₅₆ accumulates (100–300 s⁻¹) due to the inability of this pathway radical to reoxidize NO₂⁻ Y₃₅₆ and is partly a result of the potential difference between Y₁₂₂ and Y₃₅₆. DPV studies have estimated that reduction potential increases in the order Y < F₂Y < NO₂⁻ Y₃₅₆. In accordance with this prediction, the rate constant for forward RT that generates Y₃₅₆ increases with increasing driving force, whereas the rate constant for reverse RT decreases with driving force, reinforcing our model that oxidation of Y₃₅₆ by the native Y₁₂₂ is uphill. We have previously proposed that the conformational change that triggers RT targets the initial PT step from Fe₁–H₂O to Y₁₂₂ (Scheme 2). Uncoupled PT and ET in NO₂Y₁₂₂/F₂Y₁₂₂, and potentially F₂Y₁₂₂/F₂Y₁₂₂, suggest that we may have overcome this conformational gating and obtained direct insight into the thermodynamic effect of replacing Y₁₂₂ with these unnatural analogs. Further support for this model is obtained when the forward RT rate constants in NO₂Y₁₂₂/F₂Y₁₂₂ and F₂Y₁₂₂/F₂Y₁₂₂ are predicted using the Moser-Dutton equation (eq 3) for dependence of k_E on distance (R) and driving force (ΔG).

log k_E = 15 − 0.6R − 3.1(ΔG + λ)²/λ (3)

Assuming identical distances and reorganization energies (λ) for ET in NO₂Y₁₂₂/F₂Y₁₂₂ and F₂Y₁₂₂/F₂Y₁₂₂, the individual expressions for log k_E can be combined to assess the effect of the driving force differences (ΔG, 200 mV vs 20 mV, Figure 8A) on k_E. The net equation requires an estimation of ΔG by varying the reorganization energy from 0.7 to 1.4 eV, which k_E in NO₂Y₁₂₂/F₂Y₁₂₂ was calculated to be 9- to 11-fold faster than k_E in F₂Y₁₂₂/F₂Y₁₂₂. This approximation is similar to our experimental data (5- to 15-fold) supporting the idea that the driving force dictates the kinetics in these mutant RNRs and further that both NO₂Y₁₂₂ and F₂Y₁₂₂ are reduced to the corresponding phenolates during RT.

**Relationship between the Thermodynamic Landscape and Kinetics.** It is important to note that the equilibrium studies described in this work were performed under nonturnover conditions (with Y₇₃¹-Fα2 or Y₇₃₀-Fα2). Thus, a key issue to address is whether the protein environment can alter the thermodynamic landscape to lower ΔE° (Y₃₅₆→Y₁₂₂) and facilitate turnover. Although this is a likely possibility, we argue that oxidation of Y₃₅₆ by Y₁₂₂ must be uphill even under turnover conditions. Evidence for this conclusion is provided by our combined studies with wt RNR. F₂Y₁₂₂/F₂Y₁₂₂ and NO₂Y₁₂₂/F₂Y₁₂₂.

In the case of wt RNR, investigation of RT has been hindered by the inability to monitor Y₁₂₂ disappearance and reappearance during turnover. To account for this observation, we have previously modeled that the reverse RT process in wt RNR in which Y₃₅₆ reoxidizes Y₁₂₂ must be downhill and rapid (10³ s⁻¹). In the case of F₂Y₁₂₂/F₂Y₁₂₂, we have measured formation of Y₃₅₆ (20–30 s⁻¹) and demonstrated that reoxidation of F₂Y₁₂₂ by Y₃₅₆ is slow (0.4–1.7 s⁻¹) and rate-limiting for multiple turnovers. In the NO₂Y₁₂₂/F₂Y₁₂₂ system, Y₃₅₆ accumulates (100–300 s⁻¹) due to the inability of this pathway radical to reoxidize NO₂⁻ Y₃₅₆ and is partly a result of the potential difference between Y₁₂₂ and Y₃₅₆. DPV studies have estimated that reduction potential increases in the order Y < F₂Y < NO₂⁻ Y₃₅₆. In accordance with this prediction, the rate constant for forward RT that generates Y₃₅₆ increases with increasing driving force, whereas the rate constant for reverse RT decreases with driving force, reinforcing our model that oxidation of Y₃₅₆ by the native Y₁₂₂ is uphill. We have previously proposed that the conformational change that triggers RT targets the initial PT step from Fe₁–H₂O to Y₁₂₂ (Scheme 2). Uncoupled PT and ET in NO₂Y₁₂₂/F₂Y₁₂₂, and potentially F₂Y₁₂₂/F₂Y₁₂₂, suggest that we may have overcome this conformational gating and obtained direct insight into the thermodynamic effect of replacing Y₁₂₂ with these unnatural analogs. Further support for this model is obtained when the forward RT rate constants in NO₂Y₁₂₂/F₂Y₁₂₂ and F₂Y₁₂₂/F₂Y₁₂₂ are predicted using the Moser-Dutton equation (eq 3) for dependence of k_E on distance (R) and driving force (ΔG).

log k_E = 15 − 0.6R − 3.1(ΔG + λ)²/λ (3)

Assuming identical distances and reorganization energies (λ) for ET in NO₂Y₁₂₂/F₂Y₁₂₂ and F₂Y₁₂₂/F₂Y₁₂₂, the individual expressions for log k_E can be combined to assess the effect of the driving force differences (ΔG, 200 mV vs 20 mV, Figure 8A) on k_E. The net equation requires an estimation of ΔG by varying the reorganization energy from 0.7 to 1.4 eV, which k_E in NO₂Y₁₂₂/F₂Y₁₂₂ was calculated to be 9- to 11-fold faster than k_E in F₂Y₁₂₂/F₂Y₁₂₂. This approximation is similar to our experimental data (5- to 15-fold) supporting the idea that the driving force dictates the kinetics in these mutant RNRs and further that both NO₂Y₁₂₂ and F₂Y₁₂₂ are reduced to the corresponding phenolates during RT.

Based on our static thermodynamic picture constructed from the studies with NO₂Y₁₂₂/F₂Y₁₂₂ and those reported herein, we propose that the landscape from Y₁₂₂ to Y₇₃₀ is ~200 mV uphill (at 25 °C and pH 7.6, Figure 8B). The landscape between Y₇₃₀ and 3' hydrogen atom abstraction from the nucleotide must further be taken into account to make deoxynucleotides. Electrochemical measurements on the cysteine within glutathione and Y have revealed similar midpoint potentials at pH 7.0, providing an estimation of ~0.04% C₄₅₀ formation in the α2/β2 complex. Given the predicted rate constant for H₂O loss from the 2' position (10⁻⁸–10⁻⁹ s⁻¹) of the nucleotide, the rate of this reaction using 0.04% C₄₅₀ would be ~10⁻² to 10⁻³-fold faster than conformationally gated nucleotide reduction (2–10 s⁻¹). The above calculation assumes that the reaction landscape is isoenergetic subsequent to generation of Y₇₃₁. However, DFT calculations performed on the individual crystal structure of α2 and on model systems have provided an estimate of ~120 mV for ΔE°(C₄₅₀→Y₇₃₀) and ~90–260 mV for 3' H atom abstraction by C₄₅₀. If the measured ΔE°(Y₇₃₀→Y₁₂₂) of 200 mV is reflective of the thermodynamic landscape under turnover conditions, then we estimate that the combined
steps of C439 oxidation and 3° H atom abstraction must be <200 meV uphill to maintain a turnover number of >10 s⁻¹.

The DFT calculations were based on a structure of α2 alone with poor electron density for the substrate and in the absence of allosteric effector. It is likely that the RT pathway and the active site in α2 will be conformationally altered in the active α2/β2/S/E complex. Furthermore, uphill reactions can be partially compensated for by decreasing the ET distance between donor and acceptor 5,45,46 and in the case of PCET reactions by controlling the positioning of the proton acceptor.

The distances between Y122, Y356, and Y731 remain unknown because of the disordered C-terminal tail of β2. Thus, structures of the α2/β2 subunit interface and knowledge of how these structures are altered in the presence of S and E binding to α2 are crucial to understanding the overall landscape of the reaction and the tuning of the individual steps in the RT process. Nonetheless, we believe from the studies described herein, that the overall reaction from Y122• reduction to 3° hydrogen atom abstraction of NDP is uphill and driven forward by rapid and irreversible loss of H₂O from the NDP (Figure 8). 19–21

PCET across the βα Interface Involves Fast Proton Exchange between Y356 and Solvent. The equilibrium between F₁Y122• and Y356• as a function of pH has further provided important insight about the fate of the Y356 proton upon its oxidation. It was originally proposed that a specific sequenced amino acid residue within β2 functioned as the proton acceptor. 10 However, the slope of 1 associated with a plot of log([Y356•][F₁Y122•]) versus pH (Figure 6B) is consistent with the rapid exchange of the Y356 proton with 8) 19, β currently the basis for favoring the latter possibility. 29,35,37,59,60

Accepting (Scheme 1). Three distinct types of experiments are provided important insight about the fate of the Y356 proton by rapid and irreversible loss of H₂O from the NDP (Figure 8). Experiments examining the relative reduction potentials of Y356, Y731, and Y910 provide us with the overall thermodynamic landscape that is uphill by >200 meV and is unprecedented in biology. Why would such a design evolve when other classes of RNases avoid long-range RT by direct hydrogen atom abstraction from the cysteine by their active cofactors? We propose that the enzyme exerts significant kinetic control over radical initiation. RT in class I RNases plays a very important role in the fidelity of DNA replication and repair by regulating the relative ratios of the dNDP (and hence dNTP) pools and the absolute amounts of these species. This process is largely controlled by binding the appropriate S/E pairs in α2, 40–50 Å removed from the site of RT initiation by the diferriç-Y• cofactor. 48 Subtle changes that occur on S/E binding are thus likely to modulate the reduction potential of residues within the RT pathway. All of the experiments conducted to determine the thermodynamic landscape summarized in Figure 8 have been performed with different types of pathway blocks, which are likely to have subtle conformational effects on radical initiation. The proposed uphill nature of the pathway would prevent accumulation of reactive pathway radical intermediates and minimize self-inactivation during the radical initiation process. The connection between our current unprecedented and unexpected thermodynamic measurements and conformational gating of RNR activity by S/E binding is the major focus of our efforts.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b08200.

Temperature dependence of Y356• formation; temperature-dependent equilibration of F₁Y122• and Y356• in the reaction of F₁Y122•-β2, CDP, ATP, and Y910-F-α2 or Y910-F-α2; hyperfine values for β-H and 19F of F₁Y• at different positions on pathway; analysis by method B for one trial of the F₁Y122•-β2/Y351-F-α2/CDP/ATP reaction as a function of temperature and one trial of the F₁Y122•-β2/Y351-F-α2/CDP/ATP reaction as a function of temperature; and one trial of the F₁Y122•-β2/Y351-F-α2/CDP/ATP reaction as a function of temperature (2–15 °C) and the F₁Y122•-β2/Y351-F-α2/CDP/ATP reaction as a function of temperature (15–37 °C); temperature dependence of Y356• formation monitored by RFQ-EPR spectroscopy and in the reaction of F₁Y122•-β2/Y351-F-α2/CDP/ATP as determined by HQ- and RFQ-EPR
spectroscopies; temperature-dependent equilibration of $F_{871}Y_{356}$ and $Y_{356}$ in the reaction of $F_{871}Y_{356}^{-}/2$, CDP, ATP, and $Y_{356}^{-}$, composition EPR spectra of the $F_{871}Y_{356}^{-}/2/Y_{356}^{-}/2$/CDP/ATP reaction at 5 °C and the $F_{871}Y_{356}^{-}/2/Y_{356}^{-}/2$/CDP/ATP reaction at 5 °C as a function of $pH$; pH dependence of $Y_{356}$ formation in the reaction of $F_{871}Y_{356}^{-}/2/Y_{356}^{-}/2$/CDP/ATP at 5 °C; reaction of $F_{871}Y_{356}^{-}/2$, $Y_{356}^{-}/2$, CDP, and ATP monitored by RFQ-EPR spectroscopy (PDF)

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**Notes**

The authors declare no competing financial interest.

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