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 Y122•, Y356•, 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• – 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 (F3Y) at Y356 and rapid freeze-quench EPR analysis of its reaction with Y731F-α2, CDP, and ATP at pH 8.2 and 25 °C shows F3Y356• generation by the native Y122•, F3Y-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 α2β2 complex.12–15 Its active cofactor is a diferric-tyrosyl radical (Y122•) unit buried within β2. This cofactor generates a transient thyl radical (C439•) in α213 which initiates reduction of the four nucleotides (CDP, GDP, ADP, and UDP) to their corresponding deoxynucleotides (dCDP, dGDP, dADP, and dUDP) to their corresponding 2'-deoxynucleotides (dNDP), with the specificity of reduction dictated by the appropriate allosteric effector (ATP, TTP, dGTP, and dATP).12–15 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.9–11 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 (F3Y, n = 2 and 3) in combination with pathway-blocked α2 mutants (Y731F-α2 or Y730F-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 (Y122• Y731•, and Y730•) utilized the ability to collapse the Y• doublet EPR spectrum into a singlet with β-methylene-deuterated ([β-2H2]Y) Y•.14,15 β2 containing globally incorporated [β-2H2]Y’s was reacted with α2 containing protonated Y’s, dCDP, and TTP.12 These conditions promote α2β2 complex formation1 but prevent turnover, thus potentially allowing equilibration of the pathway Y•’s. Unfortunately, no

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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 NO2Y122•β2 (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-dependent (6.8 β-engineered) RNR, we have demonstrated that Y122• equilibrated with F2Y731• or F3Y730•.14 The analysis was facilitated by the unique F2Y• features arising from 19F and 3H-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 F2Y730•) in α2 by EPR spectroscopy allowed estimation of a ∆E°(Y356/ 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

equilibrium of F3Y122• and Y356• with Y731•-α2 also provided the opportunity to investigate the fate of the Y356• proton 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• equilibrium, 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 F3Y can be tuned to be essentially equal to that of Y35615,21 but oxidized F3Y• has the potential to be spectroscopically observable because of the 19F hyperfine features.15 Thus, the ability of Y122• to oxidize F3Y incorporated in place of Y356 (F3Y122•/F2Y356) was tested. Rapid freeze-quench (RFQ)-EPR spectroscopy of the reaction between F3Y122•/F2Y356•-α2, CDP, and ATP at pH 8.2 and 25 °C revealed F2Y356• at 3 ± 1% of the total radical concentration. This observation provided a ∆E°(F3Y122•/F2Y356•) 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 ∆E°(Y356•/ Y356) 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 F3Y28 were isolated; apo F3Y122• was expressed, isolated, and reconstituted22 as previously reported. F3Y122•/F2Y356• (0.7 Y•/β2) was available from an earlier study.20 CDP and ATP were purchased from Sigma-Aldrich. Assay buffer consists 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 F3Y122• and its simulation were recently reported.10 The reference spectrum for Y356•, 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 Y731•F-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 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 analysis. The ∆E°(F3Y122•/F2Y356•) was calculated based on the two quantitation methods described below and using

$$\Delta E^\circ = \frac{RT \ln K_{F2}}{F}$$

(1)

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**Figure 1.** Proposed thermodynamic landscape of the PCET pathway at 25 °C and pH 7.6. The overall reaction is proposed to be thermodynamically uphill and driven forward by the rapid irreversible loss of water from CDP substrate in the active site of α2. No direct evidence is available for the presence of a discrete Wαα radical intermediate. Studies performed on NO2Y122•/β2 determined the relative reduction potentials of Y356, Y731, and Y356•.
where $K_{eq} = [Y_{356•}]/[F_2Y_{122•}]$, $R$ is the ideal gas constant, $T$ is the temperature (K), and $F$ is Faraday’s constant.

Method A: Quantitation of $F_2Y_{122•}$ and $Y_{356•}$ in $\beta 2$ as a Function of Temperature. Each EPR spectrum was normalized to have the same intensity in the low-field features associated with $F_2Y_{122•}$. In this representation of the spectra, the intensity of $F_2Y_{122•}$ remains constant, allowing easier visualization of the $Y_{356•}$ signal that grows in with increasing temperature. Using the low-field features in the spectrum of $F_2Y_{122•}$, $F_2Y_{122•}$ was subtracted from each composite spectrum. The amount of $Y_{356•}$ remaining was determined by double integration. The $Y_{356•}$ 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 $F_3Y_{122•}$ that remains in the composite spectra due to half sites reactivity was subtracted using the $F_3Y_{122•}$ spectrum (Figure S1A). The resulting composite spectra show the interconversion between $F_3Y_{122•}$ and $Y_{356•}$ 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 $F_2Y_{122•}$ and $Y_{356•}$ cannot be determined reliably by eye. Therefore, a script was written in Matlab 2016a to automatically subtract out the remaining $F_2Y_{122•}$. The amount of remaining $F_2Y_{122•}$ was determined by adjusting the intensity of the $F_2Y_{122•}$ 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 $F_3Y_{122•}$ features) was minimized. The amount of $Y_{356•}$ after subtracting out the remaining $F_2Y_{122•}$ was determined by double integration. The $Y_{356•}$ spectrum determined by this method was the same in each sample (Figures S1D and S2).

RESULTS

Temperature-Dependent Distribution of $F_3Y_{122•}$ and $Y_{356•}$ in $\beta 2$ in the Presence of CDP, ATP, and $Y_731F$-α2 (or $Y_730F$-α2). We have recently shown that the reaction of $F_3Y_{122•}$, $F_2Y_{122•}$, $F_3Y_{122•}$, $F_2Y_{122•}$, CDP, and ATP generates a kinetically and chemically competent $F_3Y_{122•}$ that can reoxidize $F_2Y_{122•}$. We hypothesized that if we carried out the same experiment with a block in the pathway ($Y_731F$-α2 or $Y_730F$-α2) then equilibration of $F_3Y_{122•}$ and $Y_{356•}$ could be measured by EPR spectroscopy as a function of temperature, allowing determination of $\Delta E^{\infty}(F_3Y_{122•}-Y_{356•})$. $F_3Y_{122•}$, $F_2Y_{122•}$, CDP, and ATP were incubated with $Y_731F$-α2 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 $F_3Y_{122•}$ (pink) and $Y_{356•}$ (blue). All spectra presented subsequently are additive and contain the same concentration of $F_3Y_{122•}$ and increasing amounts of $Y_{356•}$. The dotted lines highlight the regions of the spectrum where the changes that occur upon $Y_{356•}$ formation are most apparent.

Figure 2. X-band EPR spectra of equimolar concentrations of $F_3Y_{122•}$ (pink) and $Y_{356•}$ (blue). All spectra presented subsequently are additive and contain the same concentration of $F_3Y_{122•}$ and increasing amounts of $Y_{356•}$. The dotted lines highlight the regions of the spectrum where the changes that occur upon $Y_{356•}$ formation are most apparent.
with active β2 containing a F,Y122• in each β monomer.22 Furthermore, while the active form of RNR is αβ2/2, the enzyme exhibits half-sites reactivity where only one of the two Y122•’s (one α/β pair) is active at a time.22 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/γ2F-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•.

described in the experimental section to allow the small changes in the amounts of Y356• as a function of temperature (2, 5, 8, 10, 12, and 15 °C) to be more clearly observable. With method A, the spectra have been manipulated such that the amount of F3Y122• remains constant, while Y356• grows in as a function of temperature. In this analysis method, each spectrum is normalized to have the same intensity in the low-field F3Y122• features. As shown by the dotted line in Figure 3A,B, increasing amounts of Y356• can then be observed between 2 and 15 °C. Two additional replicates 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.

Figure 4. Temperature dependence (2–37 °C) of Y356• formation in the reaction of F3Y122•/β2/γ2F-α2 (pink) or Y730F-α2 (blue). Each data point represents the average of two (blue) or three (pink) independent trials.

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 Y730F-.
α2 relative to wt-α2.14 Differences in reactivity between wt-α2 and Y731F-α2 are also recorded for photo-RNR, which contains a [Re(CO)5] photooxidant appended to the C-terminal tail of β2 (S355C).54,35 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-α2, and as seen in Figure 4 (blue dots), variations can be observed between Y731F-α2 and Y730F-α2, with the former construct generating slightly higher amounts of Y356•.

Calculation of ΔE°(F3Y122•−Y356•) from the Y731F and Y730F-α2 Studies. To calculate the reduction potential difference between F3Y122• and Y356•, the ln Kαβ ([Y356•]/[F3Y122•]) observed in the Y731F and Y730F-α2 reactions at 25 °C by the HQ method were used (eq 1): ΔE°(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. In the second case, the proton is in fast exchange with solvent. The initial proton acceptor (Y) is either an amino acid residue or water.

Scheme 1. Proposed Models for the Fate of the Y356 Proton

(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.

amount of Y356• as the pH is increased. The composite spectra for a second trial are shown in Figure 5B (see Figure 5A for the 5 °C data), and the average amounts of Y356• from the two experiments are reported in Table S2. Figure 6A shows 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

Figure 5. Composite EPR spectra of the F3Y122•/β2/Y331F-α2/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•/β2/Y331F-α2/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.
Although the above studies allowed establishment of the equilibrium of CDP/ATP quenching (Table S1 and Figure S6). This potential shifting of the equilibrium observed with hand quenched using the RFQ instrument and analyzed by EPR.

reacted at pH 8.2 for 10, 20, or 40 s, and the reaction was monitored by RFQ-EPR spectroscopy. Expanded view of the overlay of the EPR spectrum, concomitant with our temperature-dependent distribution between the two radicals (Figure 4).

Equilibration of Y122 and F2Y356 Using F2Y356β2/α2/CDP/ATP. Although the above studies allowed establishment of $\Delta E^\circ(F_2Y_{122}\beta2/Y_{356}\bullet)$ in F$_2Y_{122}\beta2$, the $\Delta E^\circ(Y_{122}\alpha2–Y_{356}\bullet$) 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 F2Y122β2/p2 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 F2Y122β2 at this pH is 50% of the wt activity.7 The $pK_a$ of F2Y356 is estimated to be 7.6 at position 356; 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 the $\Delta E^\circ(Y_{122}\beta2–Y_{356}\bullet$). F2Y356β2, 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).

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 F2Y356•.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 $\beta$-H hyperfine parameters matched the doublet splitting on the high-field side of the spectrum, confirming the identity of this radical species as F2Y356•. The interdoublet splitting was reproduced with two equivalent $^19$F couplings having an $A_{22}$ of 147 MHz.30 The sharpness of the $3.5^-19$F features are similar to those previously reported for the other pathway residues F2Y122, F2Y731, and F2Y730 reflecting a rigid conformation constrained by the protein environment. The $A_{22}$ value for F2Y356• 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 F2Y356• in the 20 s sample was quantitated as 3 ± 1% of total spin. This amount of radical reflects $\Delta E^\circ(F_2Y_{356}\bullet–Y_{122}\bullet)$ of 70 ± 5 mV, which in combination with our reduction potential studies4,25 allows calculation of $\Delta E^\circ(Y_{356}\bullet–Y_{122}\bullet)$ of ∼100 mV at pH 7.6 (Figure 8).

Figure 8. Current thermodynamic landscape of the PCET pathway at 25 °C and pH 7.6. (A) Studies performed on F3Y122β2 described in this work provided an estimate of the relative reduction potentials of F2Y122 and Y356. (B) Studies performed on F2Y356β2 provided an estimate of the relative reduction potentials of Y122 and Y356. $\Delta E^\circ$ has been removed from the landscapes for the sake of clarity.

### DISCUSSION

RNRs are divided into three classes based on the metallo cofactor used for thyl radical formation.6 All classes of RNR initiate nucleotide reduction by thyl 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 substrate. 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 substrate. 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 substrate. 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 substrate.
observations raise the issue of why and how a 35 Å oxidation process evolved in the class I RNR\(^6\) instead of a direct H atom abstraction process that is used by the other classes.\(^8\) The turnover number for deoxynucleotide formation (2–10 s\(^{-1}\))\(^{13}\) and the large distance between Y\(_{122}\) and C\(_{359}\) in the class Ia RNR\(^9,14\) 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 conformation changes that occur in the α2β2 complex subsequent to S/E binding and prior to RT.\(^{43}\) Furthermore, the substantial overlap of the EPR spectra of Y\(_\alpha\)'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 NO\(_3\)Y\(_{122}\) cofactor (t\(_{1/2}\) of 40 s at 25 °C) in the β2 subunit of RNR. NO\(_3\)Y\(_\alpha\) is ~200 mV more oxidizing than Y\(_\alpha\)\(^{16}\) and has provided insight about the thermodynamic landscape for the RT pathway in two ways. When NO\(_3\)Y was substituted in place of each Y\(_x\) in the pathway (Figure 1, where x = 122 and 356 in β2 and 731 and 730 in α2), the resulting mutants were all catalytically inactive.\(^{15}\) 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,\(^{14,46,47}\) and in RNR specifically.\(^{46,47}\) NO\(_3\)Y substitution at each position also allowed assessment of the protein environment perturbation of the pK\(_a\) of the phenol, relative to the pK\(_a\) 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).\(^{15}\) We assume that a similar position-dependent perturbation occurs with the F3Y122α pathway block in order to monitor equilibration. Y731F-ΔE\(_{1/2}\) was a good probe for the reduction potential of both Y731 and Y730.

More recently, we have reported the detailed kinetic analysis of the F3Y122α/β2/α2/CDP/ATP reaction.\(^{22}\) This reaction generates a kinetically and chemically competent Y356α at 20–30 s\(^{-1}\), which in contrast to Y356α generated by NO\(_3\)Y\(_{122}\)/β2 is capable of reoxidizing F3Y122α. The reoxidation process is conformationally gated and rate-limiting for subsequent dCDP formation and only observed after several turnovers upon exhaustion of the reducing equivalents. The observation of both radicals (F3Y122α and Y356α) and activity required that we utilize a pathway block in order to monitor equilibration. Y731F-α2 (Y730F-α2) served that purpose as our previous studies showed that these mutants still allow Y356α generation.\(^{13}\)

To quantitate the reduction potential increase that occurs upon replacement of Y\(_{122}\) with F3Y122α, it is important to determine whether the latter is reduced to the phenol or phenolate (F3Y122α vs F3Y122α-) during RT (Scheme 2).\(^{22}\) We favor the model where F3Y122α- is generated upon RT. In support of this proposal is the observation of NO3Y122α- in the NO3Y122α-/β2 experiments.\(^{17}\) The solution pK\(_a\) of NO3Y is 7.1,\(^{16}\) and the visualization of NO3Y122α- can be rationalized if Fe1–H\(_2\)O has a pK\(_a\) between 8.0 and 10.0. Although ferric iron typically reduces the NO3Y- phenolate formed during forward RT (Scheme 2).\(^{2}\) 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 Y\(_{122}\) from the water on Fe1 in the cluster during forward RT (Scheme 2).\(^{22}\) In the case of NO3Y, this does not occur, and the phenolate is formed. It is likely that the water on Fe1 remains protonated providing insight into the relative pK\(_a\)s of Y\(_{122}\) and Fe1–H\(_2\)O. Since the NO3Y phenol has a pK\(_a\) of 7.1, this raises issues about the protonation state of F3Y122α (pK\(_a\) of phenol is 6.4) on reduction during forward RT (Scheme 2).

Due to the inability to investigate equilibration of Y356α with Y731α and Y730α in wt RNR, F3Y was inserted in place of either Y731 or Y730 providing access to the unique EPR spectroscopic features of F3Y\(_\alpha\).\(^{14}\) These experiments showed the presence of 10–15% F3Y731α (or F3Y730α). A knowledge of the pK\(_a\) perturbation of ~1 unit at these positions,\(^{15}\) in conjunction with differential pulse voltammetry (DPV) studies on the N-acetyl-3,5-difluoro-L-tyrosinamide\(^{24}\) provided an estimate of 85–95 mV for the reduction potential difference between Y731α (or Y730α) and Y356α. This calculation agreed with the results from a second experiment where NO3Y122α/β2 was reacted with [β\(_2\)-H\(_2\)]Y-α2 and probed for variations in the EPR spectrum. Temperature dependent studies provided the ΔE\(_{1/2}\) ([β\(_2\)-H\(_2\)]Y-α2–Y356α) of ~100 mV (Figures 1 and 8). These studies together showed that the RNR protein environment perturbs F3Y and Y in a similar fashion and that F3Y is a good probe for the reduction potential of both Y731 and Y730.
protonation state of F3Y122, while favored to be deprotonated, is unknown and is under investigation.

The potential difference of ~20 mV calculated between F3Y122 and Y356 (Figure 4) makes generation of F3Y122 an appealing model. We predict that $\Delta E^\circ(NO_Y122/NO_Y122^- - Y356/F3Y122)$ is ~200 mV, owing to the inability of Y356 to reoxidize NO_Y122-. With these two values, we can estimate $\Delta E^\circ(NO_Y122/NO_Y122^- - F3Y122/F3Y122^-)$ as 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/NO2Y122- (or F3Y122/F3Y122-) 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 solvent48 over 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 turned our attention to an alternate way to monitor equilibration of Y122- and Y356, where the native Y122 remains intact but Y356 is replaced with F3Y122.

Our observations with NO2Y122/beta214 and the pH-dependent studies reported herein suggest that $\Delta E^\circ(Y122/\beta2$- Y356/F3Y122) can be easily extrapolated from $\Delta E^\circ(Y122/\beta2$- F3Y122/F3Y122-) to Y122/F3Y122. 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 solvent48 over 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 turned our attention to an alternate way to monitor equilibration of Y122- and Y356, where the native Y122 remains intact but Y356 is replaced with F3Y122.

Based on our static thermodynamic picture constructed from the studies with NO2Y122/beta2 and F3Y122/beta2, the individual expressions for log $k_{ET}$ can be combined to assess the effect of the driving force differences ($\Delta G$, 200 mV vs 20 mV, Figure 8A) on $k_{ET}$. The net reaction requires an estimation of $\Delta G$; by varying the reorganizational energy from 0.7 to 1.4 eV, $k_{ET}$ in NO2Y122/beta2 was calculated to be 9- to 11-fold faster than $k_{ET}$ in F3Y122/beta2. 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 F3Y122 are reduced to the corresponding phenolates during turnover.

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 Y730-F122 and Y730-F122). Thus, a key issue to address is whether the protein environment can alter the thermodynamic landscape to lower $\Delta E^\circ(Y356- Y122)$ 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.43 F3Y122/beta224 and NO2Y122/beta2.17

In the case of wt RNR, investigation of RT has been hindered by the inability to monitor Y122 disappearance and reappearance during turnover.39 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 (10^3 s^-1).43 In the case of F3Y122/beta2, we have measured formation of Y356 (20-30 s^-1) and demonstrated that reoxidation of F3Y122 by Y356 is slow (0.4-1.7 s^-1) and rate-limiting for multiple turnovers.22 In the NO2Y122/beta2 system, Y356 accumulates (100-300 s^-1) due to the inability of this pathway radical to reoxidize NO2Y following the first turnover.17 Taken together, these studies suggest that Y356 can be observed during turnover only when reverse RT is slowed down (F3Y122/beta2) or completely inhibited (NO2Y122/beta2) 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 < F3Y < NO2Y.16,24 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).46 Uncoupled PT and ET in NO2Y122/beta2, and potentially F3Y122/beta2, 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/beta2 and F3Y122/beta2 are predicted using the Moser-Dutton equation (eq 3) for dependence of $k_{ET}$ on distance (R) and driving force ($\Delta G$).

$$\log k_{ET} = 15 - 0.6R - 3.1(\Delta G + \lambda)^2/\lambda$$  (3)

Assuming identical distances and reorganizational energies ($\lambda$) for ET in NO2Y122/beta2 and F3Y122/beta2, the individual expressions for log $k_{ET}$ can be combined to assess the effect of the driving force differences ($\Delta G$, 200 mV vs 20 mV, Figure 8A) on $k_{ET}$. The net reaction requires an estimation of $\Delta G$; by varying the reorganizational energy from 0.7 to 1.4 eV, $k_{ET}$ in NO2Y122/beta2 was calculated to be 9- to 11-fold faster than $k_{ET}$ in F3Y122/beta2. 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 F3Y122 are reduced to the corresponding phenolates during turnover.

Based on our static thermodynamic picture constructed from the studies with NO2Y122/beta2 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’ hydroxyl group abstraction from the nucleotide must further be taken into account to make deoxyribonucleotides. Electrochemical measurements on the cysteine within glutathione and Y have revealed similar midpoint potentials at pH 7.0,53 providing an estimation of ~0.04% C439 formation in the a2/b2 complex. Given the predicted rate constant for H2O loss from the 2’ position (10^-10 - 10^-11 s^-1)20-21 of the nucleotide, the rate of this reaction using 0.04% C439 would be ~10^-2 to 10^-3-fold faster than conformationally gated nucleotide reduction (2-10 s^-1).43

The above calculation assumes that the reaction landscape is isoenergetic subsequent to generation of Y730-•. However, DFT calculations performed on the individual crystal structure of a2 and on model systems have provided an estimate of ~120 mV for $\Delta E^\circ(C439- Y730)$44,54 and ~90-260 mV for 3’ H atom abstraction by C439.56-58 If the measured $\Delta E^\circ(Y356- Y122)$ 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 acceptor⁴⁵,⁴⁶ 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 α2β2 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 sequestered 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 partially compensated for by decreasing the ET distance between donor and acceptor and in the case of PCET processes on mutant RNRs containing the radical trap, 3-ffY.⁴⁵,⁶⁰ Together support fast proton exchange between Y₃₅₆ and solvent via water during PCET across the interface.

Summary. Using site specifically incorporated F₁Y and F₂Y in place of β2 residues 122 and 356, respectively, and taking advantage of the unique EPR features of F₁Y• relative to Y•, we have measured the thermodynamic landscape within β2 in the α2β2 complex. These results, when combined with similar types of experiments examining the relative reduction potentials of Y₃₅₆, Y₇₃₁, and Y₇₃₀ 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 RNRs 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 RNRs 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 diferric-Y• cofactor. Subtle changes that occur on S/E binding are thus likely to modulate the reduction potential of residues within the wt 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 Y₇₃₀–F–α2 or Y₇₃₀–F–α2; hyperfine values for β2–H and ¹⁹F of F₂Y• at different positions on pathway; analysis by method B for one trial of the F₁Y₁₂₂•–β2/Y₇₃₁–F–α2/CDP/ATP reaction as a function of temperature and one trial of the F₁Y₁₂₂•–β2/Y₇₃₁–F–α2/CDP/ATP reaction as a function of temperature and one trial of the F₁Y₁₂₂•–β2/Y₇₃₁–F–α2/CDP/ATP reaction as a function of temperature (2–15 °C) and the F₁Y₁₂₂•–β2/Y₇₃₁–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/Y₇₃₁–F–α2/CDP/ATP as determined by Hy- and RfQ-EPR
spectroscopies; temperature-dependent equilibration of F,Y_{125}• and Y_{168}• in the reaction of F,Y_{125}•/β2, CDP, ATP, and Y_{171}•F-α2 or Y_{170}•F-α2; composite EPR spectra of the F,Y_{125}•/β2/Y_{171}•F-α2/CDP/ATP reaction at 25 °C and the F,Y_{125}•/β2/Y_{171}•F-α2/CDP/ATP reaction at 5 °C as a function of pH; pH dependence of Y_{168}• formation in the reaction of F,Y_{125}•/β2/Y_{171}•F-α2/CDP/ATP at 5 °C; reaction of F,Y_{168}•β2, Y_{171}•F-α2, CDP, and ATP monitored by RFQ-EPR spectroscopy (PDF).

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