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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 (Y$_{122}$•) in one subunit (β$_2$) generates a transient thyl radical in another subunit (α$_2$) via long-range radical transport (RT) through aromatic amino acid residues (Y$_{122}$⇌[W$_{48}$]⇌Y$_{356}$ in β$_2$ to Y$_{731}$⇌Y$_{730}$⇌C$_{439}$ in α$_2$). Equilibration of Y$_{356}$•, Y$_{351}$•, and Y$_{730}$• was recently observed using site specifically incorporated unnatural tyrosine analogs; however, equilibration between Y$_{122}$• and Y$_{356}$• has not been detected. Our recent report of Y$_{356}$• formation in a kinetically and chemically competent fashion in the reaction of β$_2$ containing 2,3,5-trifluorotyrosine at Y$_{122}$ (F$_3$Y$_{122}$•/β$_2$) with α$_2$, CDP (substrate), and ATP (cofactor) has now afforded the opportunity to investigate equilibration of F$_3$Y$_{122}$• and Y$_{356}$•. Incubation of F$_3$Y$_{122}$•/β$_2$, Y$_{356}$•/α$_2$ (or Y$_{731}$•/α$_2$), CDP, and ATP at different temperatures (2−37 °C) provides ΔE°([W$_{48}$]⇌Y$_{356}$•) of 20 ± 10 mV at 25 °C. The pH dependence of the F$_3$Y$_{122}$•⇌Y$_{356}$• interconversion (pH 6.8−8.0) reveals that the proton from Y$_{356}$ is in rapid exchange with solvent, in contrast to the proton from Y$_{122}$. Insertion of 3,5-difluorotyrosine (F$_3$Y)$\_\alpha$ at Y$_{356}$ and rapid freeze-quench EPR analysis of its reaction with Y$_{731}$•/α$_2$, CDP, and ATP at pH 8.2 and 25 °C shows F$_3$Y$_{356}$• generation by the native Y$_{122}$•, F$_3$Y-RNRs (α = 2 and 3) together provide a model for the thermodynamic landscape of the RT pathway in which the reaction between Y$_{122}$ and C$_{439}$ is ∼200 meV uphill.

INTRODUCTION

The *E. coli* class Ia ribonucleotide reductase (RNR) contains two homodimeric subunits, α$_2$ and β$_2$, and functions as an α$_2$β$_2$ complex. Its active cofactor is a diferric-tyrosyl radical (Y$_{122}$•) unit buried within β$_2$. This cofactor generates a transient thyl radical (C$_{439}$•) in α$_2$ 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 effector (ATP, TTP, dGTP, and dATP). During each turnover, Y$_{122}$• reversibly oxidizes C$_{439}$ via multiple proton-coupled electron transfer (PCET) steps through a pathway involving aromatic amino acid residues Y$_{122}$⇌[W$_{48}$]⇌Y$_{356}$ in β$_2$ to Y$_{731}$⇌Y$_{730}$⇌C$_{439}$ in α$_2$. Currently, there is no direct evidence for the involvement of W$_{48}$ in RT. In the wild-type (wt) RNR, only Y$_{122}$• is observed in the presence of substrates (S) and effectors (E); there has been no detectable electron delocalization over the other pathway tyrosines. In this paper, we present the first insight into the thermodynamic landscape of the RT pathway within β$_2$. Site-specific replacement of either Y$_{122}$ or Y$_{356}$ with fluorotyrosines (F$_n$Y, n = 2 and 3) in combination with pathway-blocked α$_2$ mutants (Y$_{731}$•/α$_2$ or Y$_{730}$•/α$_2$)/CDP/ATP and X-band electron paramagnetic resonance (EPR) spectroscopy provides evidence for equilibration of Y$_{122}$• with Y$_{356}$• as a function of temperature and pH. These studies have allowed estimation of ΔE°(Y$_{356}$•−Y$_{122}$•) 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. Initial attempts to address if Y$_{122}$• equilibrated with the pathway tyrosines (Y$_{356}$, Y$_{731}$, and Y$_{730}$) utilized the ability to collapse the Y• doublet EPR spectrum into a singlet with β-methylene-deuterated ([β-2H$_2$]Y)•. β$_2$ containing globally incorporated [β-2H$_2$]Y• was reacted with α$_2$ containing protonated Y•’s, dCDP, and TTP. These conditions promote α$_2$/β$_2$ complex formation 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 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-difluorotyrosine (F3Y) at Y731 (or Y730) we demonstrated that Y356• equilibrated with F3Y731• or F2Y731•.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 F2Y731•) in α2 by EPR spectroscopy allowed estimation of a ΔE°(Y731/730−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

to equilibrate F3Y122• and Y356• with Y731-F-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•]/[F2Y356•]) 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 F3Y• has the potential to be spectroscopically observable because of the 19F hyperfine features.15 Thus, the ability of Y122• to oxidize F2Y incorporated in place of Y356• (F3Y356•/F2) was tested. Rapid freeze-quench (RFQ)-EPR spectroscopy of the reaction between F3Y356•/F2, 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°(F3Y356•−F2) of 70 ± 5 mV, which along with our recent measurement of the reduction potential of F2Y in a protein environment24,25 gives an estimate of ΔE°(F3Y356•−

RT pathway in wt RNR is thermodynamically uphill and driven forward by the nucleotide reduction process, specifically the rapid irreversible cleavage of the C2−OH bond18 of the substrate and loss of water (10^5−10^6 s−1)19−21 in the active site of α2. Equilibration of the pathway Y•'s could be measured because oxidation of Y356• by NOY122• is irreversible. Unfortunately, this same feature prohibited use of NOY122• in α2 to monitor equilibration of NOY122• and Y356•.

To obtain insight over the entire thermodynamic landscape of RNR, ΔE°(Y122•−Y356•) must be defined. A recently engineered β2 containing 2,3,5-trifluorotyrosine (F3Y) at position 122 provides an avenue to assess the ΔE°(Y122•−Y356•) energetics.15,22 The reaction of F3Y122•β2, α2, CDP, and ATP results in rapid formation of dCDP concomitant with accumulation of Y356• (20−30 s−1). In contrast to NOY122•β2, however, we have demonstrated that Y356• can reoxidize F3Y122• and that this reoxidation process is rate-limiting for subsequent turnovers.22 The reversible nature of Y356• oxidation in F3Y122•β2 has led to the studies described herein and provided the opportunity to investigate the relative reduction potentials of F3Y122• and Y356•.

In this work, we report the temperature (2−37 °C) and pH-dependent (6.8−8.0) quantitation of F3Y122• and Y356• in the reaction of F3Y122•β2, Y731-F-α2 (or Y356-Fα2), CDP, and ATP by EPR spectroscopy. At pH 7.6 and 25 °C, ΔE°(F3Y122•−Y356•) values of 20 ± 10 and 5 ± 7 mV are observed in the reactions with Y731-F-α2 and Y356-Fα2, respectively. The ability

\[ \Delta E^\circ = \frac{RT \ln K_{eq}}{F} \]
where $K_{eq} = [Y_{356}] / [F_{3}Y_{122}]$, $R$ is the ideal gas constant, $T$ is the temperature (K), and $F$ is Faraday’s constant.

**Method A: Quantitation of F$_{3}$Y$_{122}$• and Y$_{356}$• in β2 as a Function of Temperature.** Each EPR spectrum was normalized to have the same intensity in the low-field features associated with F$_{3}$Y$_{122}$•. In this representation of the spectra, the intensity of F$_{3}$Y$_{122}$• 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$_{3}$Y$_{122}$• and Y$_{356}$• was subtracted from each composite spectrum. The amount of Y$_{356}$• remaining was determined by double integration.$^{13}$ 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$_{3}$Y$_{122}$• that remains in the composite spectra due to half sites reactivity was subtracted using the F$_{3}$Y$_{122}$• reference spectrum (Figure S1A). The resulting composite spectra show the interconversion between F$_{3}$Y$_{122}$• 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$_{3}$Y$_{122}$• and Y$_{356}$• cannot be determined reliably by eye. Therefore, a script was written in Matlab 2016a to automatically subtract out the remaining F$_{3}$Y$_{122}$•. The amount of remaining F$_{3}$Y$_{122}$• was determined by adjusting the intensity of the F$_{3}$Y$_{122}$• 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$_{3}$Y$_{122}$• features) was minimized. The amount of Y$_{356}$• after subtracting out the remaining F$_{3}$Y$_{122}$• was determined by double integration. The Y$_{356}$• spectrum determined by this method was the same in each sample (Figures S1D and S2).

**Temperature-Dependent Equilibration of F$_{3}$Y$_{122}$• and Y$_{356}$• within the Same Sample.** To support equilibration between F$_{3}$Y$_{122}$• and Y$_{356}$• in β2 at 25 °C as described above in the Y731F- and Y730F-α2 reactions, the EPR spectrum of the 20 s sample was rerecorded. Quantitation of Y$_{356}$• and F$_{3}$Y$_{122}$• was performed as described above.

**RFQ-EPR Analysis of Y$_{356}$• Formation as a Function of Temperature.** RFQ 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$_{3}$Y$_{122}$•/β2 (70 mM, 0.8 F$_{3}$Y$_{122}$•/β2) and CDP (2 mM) in assay buffer in one syringe were mixed with Y$_{356}$•/β2 (70 mM) 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 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$_{3}$Y$_{122}$• (pink) and Y$_{356}$• (blue). All spectra presented subsequently are additive and contain the same contribution of F$_{3}$Y$_{122}$• 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.

To support equilibration of F$_{3}$Y$_{122}$• 2o rY 730 F-α2, wt-α2, CDP, and ATP generates a kinetically and chemically competent Y$_{356}$• that can reoxidize F$_{3}$Y$_{122}$•. We hypothesized that if we carried out the same experiment with a block in the pathway (Y731 F-α2 or Y730 F-α2) then equilibration of F$_{3}$Y$_{122}$• and Y$_{356}$• could be measured by EPR spectroscopy as a function of temperature, allowing determination of $\Delta E^\circ$ (F$_{3}$Y$_{122}$•/Y$_{356}$•). F$_{3}$Y$_{122}$•/β2, CDP, and ATP were incubated with Y$_{731}$F-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.

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with active β2 containing a F3Y122• in each β monomer.22 Furthermore, while the active form of RNR is αβ2β2, 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](Image 364x583 to 525x714)

Figure 3. Composite EPR spectra of the F3Y122•/β2/Y356•/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•.

![Figure 4](Image 60x446 to 300x661)

Figure 4. Temperature dependence (2–37 °C) of Y356• formation in the reaction of F3Y122•/β2, CDP, ATP, and Y731F-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 RNR23 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 Y• 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-.
α2 relative to wt-α2.14 Differences in reactivity between wt-α2 and Y731α2 are also recorded for photo-RNR, which contains a [Re(V)] 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 Y730α2, and as seen in Figure 4 (blue dots), variations can be observed between Y731α2 and Y730α2, with the former construct generating slightly higher amounts of Y356.

Calculation of \( \Delta E^\circ (Y_{356}^- - Y_{356}^-) \) from the Y731F and Y730α2 Studies. To calculate the reduction potential difference between F3Y122• and Y356•, the ln \( K_{eq} \) \( ([Y_{356}•]/[F_3Y_{122}•]) \) observed in the Y731F and Y730-α2 reactions at 25°C by the HQ method were used (eq 1): \( \Delta E^\circ (F_3Y_{122}• - Y_{356}^-) \) 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.

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

\[ \begin{align*}
F_3Y_{122}^- & \rightarrow Y_{356}^- \\
X &= \text{amino acid residue} \\
Y &= \text{amino acid residue} \\
\text{solvent} &
\end{align*} \]

(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 F3Y_{122}•/β2/Y731F-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 F3Y_{122}• and Y356•. The spectrum was generated by adding the individual spectra of F3Y_{122}• 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 F3Y_{122}•/β2/Y731F-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 F3Y_{122}•. The observed pH dependence of slope 1.2 ± 0.2 supports that the Y356• proton is in fast exchange with solvent.

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The dependence of log₁₀([Y₃₅₆•]/[F₃Y₁₂₂•]) on pH at 25 and 5 °C is measured in Figures 6B and S10B, respectively. A slope of 1.2 ± 0.2 is measured at 25 °C (1.0 ± 0.1 at 5 °C) supporting the model in which the proton from Y₃₅₆ is in fast exchange with solvent at both temperatures. Y₃₅₆• formation is favored more at 25 °C compared to 5 °C, an observation that is in accordance with our temperature-dependent distribution between the two radicals (Figure 4).

**Equilibration of Y₁₂₂• and F₂Y₃₅₆• Using F₂Y₃₅₆β2α2/CDP/ATP.** Although the above studies allowed establishment of ΔE°(F₂Y₁₂₂•−Y₃₅₆•) in F₂Y₁₂₂•/β2, 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•/β2 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 F₂Y₃₅₆β2 at this pH is 50% of the wt activity. The pKᵣ 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-field regions of the EPR spectrum, we carried out the following experiment in the hope of obtaining insight about ΔE°(Y₁₂₂•−Y₃₅₆•). 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 β•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 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, F₂Y₇₃₁, and F₂Y₇₃₀ reflecting a rigid conformation constrained by the protein environment. The A₁ gracious 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 ΔE°(F₂Y₃₅₆•−Y₁₂₂•) of 70 ± 5 mV, which in combination with our reduction potential studies allows calculation of ΔE°(Y₃₅₆•−Y₁₂₂•) 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 F₂Y₁₂₂•/β2 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 relative to the reduction potentials of F₂Y₁₂₂ and Y₃₅₆.

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**DISCUSSION**

RNRs are divided into three classes based on the metallocofactor 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 Aₐ₉.42 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.40 The turnover number for deoxyribonucleotide formation (2–10 s−1)34 and the large distance between Y122 and C439 in the class I RNR,44,45 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 YF’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 d ferric NO2Y122 cofactor (t1/2 of 40 s at 25 °C) in the β2 subunit of RNR. NO2Y is ∼200 mV more oxidizing than YF, and has provided insight about the thermodynamic landscape for the RT pathway in two ways. When NO2Y was substituted in place of YF, this substitution provided insight about the thermodynamic landscape for the RT pathway during forward RT (Scheme 2).48 In the case of NO2Y, the extent to which uphill steps can be accommodated in the electron transfer (ET) pathways in general,45,46 and in RNR specifically,46,47 NO2Y substitution at each position also allowed assessment of the protein environment perturbation of the pKa of the phenol, relative to the pKa 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 F3Y’s incorporated at 356, 731, and 730. However, given 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−1,17 much faster than the wt turnover of 5 s−1.43 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).18 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 pKa’s of Y122 and Fe1–H2O. Since the NO2Y phenol has a pKa of 7.1, this raises issues about the protonation state of YF• (pKa 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, F3Y was inserted in place of either Y731 or Y730 providing access to the unique EPR spectroscopic features of F3Y.14 These experiments showed the presence of 10–15% F2Y731• (or F2Y730•). A knowledge of the pKa perturbation of ∼1 unit at these positions,15 in conjunction with differential pulse voltammetry (DPV) studies on the N-acetyl-3,5-difluoro-L-tyrosinamide24 provided an estimate of 85–95 mV for the reduction potential difference between Y731• (or Y730•) and Y356•. This calculation agreed with the results from a second experiment where NO2Y122•/β2 was reacted with [β2H2]Y•/α2 and probed for variations in the EPR spectrum. Temperature dependent studies provided the ∆Ea (β2H2Y•/Y356•) 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.

More recently, we have reported the detailed kinetic analysis of the F3Y122•/β2/α2/CDP/ATP reaction.22 This reaction generates a kinetically and chemically competent Y356• at 20–30 s−1, which in contrast to Y356• generated by NO2Y122•/β2 is capable of reoxidizing F3Y122. 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 (F3Y122• and Y356•) 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 Y356• generation.13

To quantitate the reduction potential increase that occurs upon replacement of Y122 with F3Y122, it is important to determine whether the latter is reduced to the phenol or phenolate (F3Y122 vs F3Y122−) during RT (Scheme 2). We favor the model where F3Y122− is generated upon RT. In support of this proposal is the observation of NO2Y122− in the NO2Y122•/β2 experiments.17 The solution pKa of NO2Y is 7.1,16 and the visualization of NO2Y122− can be rationalized if Fe1–H2O has a pKa between 8.0 and 10.0. Although ferric iron typically reduces the pKa of bound water,49 di-iron clusters have been known to shift this value into the physiological pH range (pH > 7.0)50 in a protein-environment-dependent manner. The d ferric cluster environment in the class Ia RNR is unique and as noted above perturbs the pKa of Y122 by >3 units.15 If the pKa of Fe1–H2O is perturbed to >8.0, then initiation of the reaction with F3Y122• would primarily result in the generation of F3Y122−. The
protonation state of F$_2$Y$_{122}$, while favored to be deprotonated, is unknown and is under investigation.

The potential difference of $\sim 20$ mV calculated between F$_3$Y$_{122}$ and Y$_356$ (Figure 4) makes generation of F$_3$Y$_{122}$ an appealing model. We predict that $\Delta G^\circ(F_3Y_{122}^-/F_2Y_{122}^-)$ is $\geq 100$ mV, owing to the inability of Y$_356$ to reoxidize NO$_2$Y$^-$. With these two values, we can estimate $\Delta G^\circ$($NO_2Y_{122}^-/NO_2Y_{122}°$ - F$_3Y_{122}^-/F_2Y_{122}^-) = 100$ 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 ($\sim 180$ mV). Unfortunately, we cannot at present directly extrapolate the potential difference calculated between NO$_2$Y$_{122}^-/NO_2Y_{122}°$ (or F$_3Y_{122}^-/F_2Y_{122}^-) and Y_{356}^-/Y_{356}$ to Y$_{122}^-/Y_{122}$, especially for the native Y$_{122}$, which is partly due to the unique nature of residue 122’s environment compared to that of the other pathway Ys. In Y$_{122}$, site is not in equilibrium with solvent 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 Y$_{122}$ and Y$_{356}$ where the native Y$_{122}$ remains intact but Y$_{356}$ is replaced with F$_2$Y$_{356}$.

Our observations with NO$_2$Y$_{122}^-/Y_{122}$-$β$14 and the pH-dependent studies reported herein suggest that $\Delta G^\circ(Y_{122}°/Y_{122}$-$β$ - F$_2Y_{356}$) can be easily extrapolated from $\Delta G^\circ(Y_{122}°/Y_{122}$-$β$ - F$_2Y_{356}$) in rapid exchange with solvent (Figures 6b and S10B), and at an appropriate pH, we predict that its reduction potential is a good approximation of Y$_{356}$: The reaction of F$_2$Y$_{356}$-$β$2/CDF-A2/ATP was carried out at pH 8.2 to maximize the chances of observing the F$_2$Y$_{356}$ signal and revealed similar amounts of F$_3$Y$_{356}$ at 10, 20 and 40 s, supporting equilibration. The observed percentage of F$_2$Y$_{356}$-$β$ (3%) provides an estimation of $\Delta G^\circ(F_2Y_{356}^-/F_2Y_{356}°-Y_{122}^-/Y_{122})$ of $\sim 70$ mV. At pH 8.2, the reduction potentials of the F$_2$Y$_{356}$-$β$ and Y$_{356}$-$β$ couples are predicted to be roughly the same.24,25 At pH 7.6, the standard assay conditions, the reduction potential of Y$_{356}$ is expected to increase by $\sim 30$ mV, providing a $\Delta G^\circ(Y_{356}°-Y_{122}°)$ of $\sim 100$ mV (Figure 8B). Finally, we note that our data taken together suggest that at 25 °C and pH 7.6 F$_3$Y$_{122}$ is $\sim 120$ mV more oxidizing than Y$_{122}$ within the RNR protein environment. This difference is 10 times greater than we had originally predicted based on the solution DPV data collected on the N-acetyl-fluoro-1-tirosinamide derivatives.11 We note that this original prediction assumed that both F$_3$Y and Y are reduced to the corresponding phenols 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 Y$_{730}$F-$α$2 or Y$_{730}$F-$α$2). Thus, a key issue to address is whether the protein environment can alter the thermodynamic landscape to lower $\Delta G^\circ(Y_{356}°-Y_{122}°)$ and facilitate turnover. Although this is a likely possibility, we argue that oxidation of Y$_{356}$ by Y$_{122}$ must be uphill even under turnover conditions. Evidence for this conclusion is provided by our combined studies with wt RNR.43 F$_3$Y$_{122}$-$β$/22 and NO$_2$Y$_{122}$-$β$/217

In the case of wt RNR, investigation of RT has been hindered by the inability to monitor Y$_{122}$-$β$ disappearance and reappearance during turnover.43 To account for this observation, we have previously modeled that the reverse RT process in wt RNR in which Y$_{356}$-$β$ reoxidizes Y$_{122}$-$β$ is downhill and rapid ($10^3$ s$^{-1}$).43 In the case of F$_3$Y$_{122}$-$β$/2, we have measured formation of Y$_{356}$-$β$ ($20-30$ s$^{-1}$) and demonstrated that reoxidation of F$_3$Y$_{122}$ by Y$_{356}$-$β$ is slow ($0.4-1.7$ s$^{-1}$) and rate-limiting for multiple turnovers.22 In the NO$_2$Y$_{122}$-$β$/2 system, Y$_{356}$-$β$ accumulates ($100-300$ s$^{-1}$) due to the inability of this pathway radical to reoxidize NO$_2$Y$°$ subsequent to the first turnover.17 Taken together, these studies suggest that Y$_{356}$-$β$ can be observed during turnover only when reverse RT is slowed down (F$_3$Y$_{122}$-$β$/2) or completely inhibited (NO$_2$Y$_{122}$-$β$/2) and is partly a result of the potential difference between Y$_{122}$ and Y$_{356}$: DPV studies have estimated that reduction potential increases in the order Y $< F_3Y < NO_2Y$.16,24 In accordance with this prediction, the rate constant for forward RT that generates Y$_{356}$-$β$ increases with increasing driving force, reinforcing our model that oxidation of Y$_{356}$ by the native Y$_{122}$ is uphill. We have previously proposed that the conformational change that triggers RT targets the initial PT step from Fe1–H$_2$O to Y$_{122}$-$β$ (Scheme 2).26 Uncoupled PT and ET in NO$_2$Y$_{122}$-$β$/2, and potentially F$_3$Y$_{122}$-$β$, suggest that we may have overcome this conformational gating and obtained direct insight into the thermodynamic effect of replacing Y$_{122}$ with these unnatural analogs. Further support for this model is obtained when the forward RT rate constants in NO$_2$Y$_{122}$-$β$/2 and F$_3$Y$_{122}$-$β$/2 are predicted using the Moser–Dutton equation12 (eq 3) for dependence of $k_{\text{ET}}$ on (R) and driving force ($\Delta G$).

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

Assuming identical distances and reorganization energies ($\lambda$) for ET in NO$_2$Y$_{122}$-$β$/2 and F$_3$Y$_{122}$-$β$/2, the individual expressions for log $k_{\text{ET}}$ can be combined to assess the effect of the driving force differences ($\Delta G$, 200 mV vs 20 mV, Figure 8A) on $k_{\text{ET}}$. The net equation requires an estimation of $\Delta G$ by varying the reorganization energy from 0.7 to 1.4 eV.43 $k_{\text{ET}}$ in NO$_2$Y$_{122}$-$β$/2 was calculated to be $9-11$-fold faster than $k_{\text{ET}}$ in F$_3$Y$_{122}$-$β$/2. This approximation is similar to our experimental data (5-15-fold) supporting the idea that the driving force dictates the kinetics in these mutant RNRS and further that both NO$_2$Y$_{122}$ and F$_3$Y$_{122}$ are reduced to the corresponding phenolates during RT.

Based on our static thermodynamic picture constructed from the studies with NO$_2$Y$_{122}$-$β$/2 and those reported herein, we propose that the landscape from Y$_{122}$ to Y$_{730}$ is $\sim 200$ mV uphill (at 25 °C and pH 7.6, Figure 8B). The landscape between Y$_{730}$ 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 7.0,53 providing an estimation of $\sim 0.04$% C$_{459}$-$β$ formation in the $α$2/$β$2 complex. Given the predicted rate constant for H$_2$O loss from the 2' position (10$^{-10}$ s$^{-1}$),19-21 of the nucleotide, the rate of this reaction using 0.04% C$_{459}$-$β$ would be $\sim 10^{-2}$ to 10$^{-5}$-fold faster than conformationally gated nucleotide reduction ($2\times 10^{-1}$ s$^{-1}$).43

The above calculation assumes that the reaction landscape is isoeenergetic subsequent to generation of Y$_{122}$-$β$. However, DFT calculations performed on the individual crystal structure of $α$2 and on model systems have provided an estimate of $\sim 120$ mV for $\Delta G^\circ(C_{459}^-/Y_{730})$,54,55 and $\sim 90-260$ mV for 3' H atom abstraction by C$_{459}$-$β$. If the measured $\Delta G^\circ(Y_{356}°-Y_{122})$ of 200 mV is reflective of the thermodynamic landscape under turnover conditions, then we estimate that the combined
steps of C$_{\text{sh}}$ 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$^{54,56}$ and in the case of PCET reactions by controlling the positioning of the proton acceptor. The distances between Y$_{122}$, Y$_{356}$, and Y$_{731}$ 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 Y$_{122}$ reduction to 3’-hydrogen atom abstraction of NDP is uphill and driven forward by rapid and irreversible loss of H$_2$O from the NDP (Figure 8).$^{19-21}$

PCET across the βα Interface Involves Fast Proton Exchange between Y$_{356}$ and Solvent. The equilibrium between F$_1$Y$_{122}$ and Y$_{356}$ as a function of pH has further provided important insight about the fate of the Y$_{356}$ 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([Y$_{356}$]/[F$_1$Y$_{122}$]) versus pH (Figure 6B) is with the rapid exchange of the Y$_{356}$ proton with solvent at the subunit interface either through an amino acid residue or a water cluster functioning as the initial proton acceptor (Scheme 1). Three distinct types of experiments are currently the basis for favoring the latter possibility.$^{29,35,37,59}$

For the most compelling support for this model has been the work of Bennati and co-workers using multifrequency EPR and [3H]-electron–nuclear double resonance (ENDOR) spectroscopic methods on mutant RNRs containing the radical trap, 3-aminotyrosine (NH$_2$Y$_2$). This unnatural amino acid has site specifically replaced Y$_{356}$, Y$_{731}$, or Y$_{730}$, leading to accumulation of NH$_2$Y$_2$ in each case upon incubation with the second subunit, S, and E.$^{26,61}$ HF-EPR studies on NH$_2$Y$_2$ specifically the $g_c$ component of their $g$ tensors, revealed that the electrostatic environment of all three NH$_2$Y$_2$’s are perturbed, but that of NH$_2$Y$_{356}$ is perturbed to a greater extent than either NH$_2$Y$_{731}$ or NH$_2$Y$_{730}$. In contrast with NH$_2$Y$_{731}$ or NH$_2$Y$_{730}$, no moderate hydrogen bonding interactions were observed with NH$_2$Y$_{356}$ by HF-[3H]-ENDOR spectroscopy.$^{56}$ The studies together led to the proposed importance of water clusters in proton removal at the subunit interface.$^{59}$

Using a very different approach, recent studies have been carried out with photo-RNRs in which a photoxidant is attached site specifically to residue 35S in β2 and F$_1$Y (n = 2 or 3) or W replaces Y$_{356}$. In the presence of α2, S, and E and with light initiation, these constructs exert significant control in facilitating PT during oxidation of residue 356, shuttling reactive intermediates between the subunits and in the case of W, rapid PT out of the αβ interface.$^{59}$

Finally, prior to the studies reported herein, the conserved residue E$_{350}$ located on the flexible C-terminal tail of β2 near Y$_{356}$ in sequence space, was considered to be the most likely amino acid candidate that could function as a proton acceptor for Y$_{356}$. Mutation of E$_{350}$ to A abolished RNR activity,$^{11,29}$ an observation we have confirmed.$^{31,32}$ However, using our ability to incorporate F$_1$Y analogs in place of RNR pathway residues, we have shown that E$_{350}$ is likely not the proton acceptor for Y$_{356}$ but that its essentiality stems from its involvement in subunit interaction and in the protein conformational gate for RT initiation.$^{79}$ The experiments presented herein, the E$_{350}$ studies,$^{57}$ the EPR and ENDOR results,$^{40}$ and the photo-RNR experiments$^{35,60}$ together support fast proton exchange between Y$_{356}$ and solvent via water during PCET across the interface.

Summary. Using site specifically incorporated F$_1$Y and F$_2$Y in place of β2 residues 122 and 356, respectively, and taking advantage of the unique EPR features of F$_1$Y relative to Y$_2$, we have measured the thermodynamic landscape within β2 in the α2/β2 complex. These results, when combined with similar types of experiments examining the relative reduction potentials of Y$_{356}$ Y$_{731}$, and Y$_{730}$ 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 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 differric-Y$_2$ cofactor.$^{46}$ 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 Y$_{356}$ formation; temperature-dependent equilibration of F$_1$Y$_{122}$ and Y$_{356}$ in the reaction of F$_1$Y$_{122}$–β2, CDP, ATP, and Y$_{730}$-F-α2 or Y$_{730}$-F-α2; hyperfine values for β'-H and 19F of F$_1$Y at different positions on pathway; analysis by method B for one trial of the F$_1$Y$_{122}$–β2/Y$_{731}$-F-α2/CDP/ATP reaction as a function of temperature and one trial of the F$_1$Y$_{122}$–β2/Y$_{731}$-F-α2/CDP reaction as a function of β2/Y$_{731}$-F-α2/CDP/ATP reaction as a function of temperature of (2–15 °C) and the F$_1$Y$_{122}$–β2/Y$_{731}$-F-α2/CDP/ATP reaction as a function of temperature (15–37 °C); temperature dependence of Y$_{356}$ formation monitored by RFQ-EPR spectroscopy and in the reaction of F$_1$Y$_{122}$–β2/Y$_{731}$-F-α2/CDP/ATP as determined by RFQ-EPR.
spectroscopies; temperature-dependent equilibration of $F_{Y_{731}}$ and $Y_{730}$ in the reaction of $F_{Y_{731}} - F_{Y_{730}}$, CDP, ATP, and $Y_{731}$-F-α2 or $Y_{730}$-F-α2; composite EPR spectra of the $F_{Y_{731}} - F_{Y_{730}}$/CDP/ATP reaction at 5 °C and the $F_{Y_{731}} - F_{Y_{730}}$/CDP/ATP reaction at 5 °C as a function of pH; pH dependence of $Y_{730}$ formation in the reaction of $F_{Y_{731}} - F_{Y_{730}}$/CDP/ATP at 5 °C; reaction of $F_{Y_{731}} - F_{Y_{730}}$, $Y_{731}$-F-α2, 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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