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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 (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• and eC439•) in α2. Equilibration of Y356•, Y731•, and Y730• was recently observed using site specifically incorporated unnatural tyrosine analogs; however, equilibration between Y122• and Y356• has not been detected. Our recent report of Y356• formation in a kinetically and chemically competent fashion in the reaction of β2 containing 2,3,5-trifluorotyrosine at Y122 (F3Y122•/β2) with α2, CDP (substrate), and ATP (effector) has now afforded the opportunity to investigate equilibration of F3Y122•/α2 and Y356•. Incubation of F3Y122•/β2, Y356•, and Y730•, CDP, and ATP at different temperatures (2–37 °C) provides ΔE°(F3Y122•/α2–Y356•) of 20 ± 10 mV at 25 °C. The pH dependence of the F3Y122•/β2 interconversion (pH 6.8–8.0) reveals that the proton from Y356 is in rapid exchange with solvent, in contrast to the proton from Y122. Insertion of 3,5-difluorotyrosine (F2Y) at Y356 and rapid freeze-quench EPR analysis of its reaction with F2Y, CDP, and ATP at pH 8.2 and 25 °C shows F2Y 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 α2/β2 complex.1,2 Its active cofactor is a diferric-tyrosyl radical (Y122•) unit buried within β2. This cofactor generates a transient thyl radical (C439•) in α2,3,4 which initiates reduction of the four nucleotides (CDP, GDP, ADP, and UDP) to their corresponding 2′-deoxynucleotides (dNDP), with the specificity of reduction dictated by the appropriate allosteric effector (ATP, TTP, dGTP, and dATP).5−8 During each turnover, Y122• reversibly oxidizes C439• via multiple proton-coupled electron transfer (PCET) steps through a pathway involving aromatic amino acid residues Y122• ⇌ Y122* (W48•) ⇌ Y356• in β2 to Y731• ⇌ Y730• ⇌ C439• in α2. Currently, there is no direct evidence for the involvement of W48 in RT.5,9−11 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 Y356F-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 ΔE°(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 Ys.1,4 Initial attempts to address if Y122• equilibrated with the pathway tyrosines (Y122, Y731, and Y730) utilized the ability to collapse the Ys doublet EPR spectrum into a singlet with β-methylene-deuterated ([β-2H2]Y)s.12,14 β2 containing globally incorporated [β-2H2]Ys was reacted with α2 containing protonated Ys, dCDP, and TTP. These conditions promote α2/β2 complex formation1 but prevent turnover, thus potentially allowing equilibration of the pathway Ys. 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-difluorotyrosine (F3Y) at Y713 (or Y710) we demonstrated that Y356• equilibrated with F3Y122• or F3Y730•.13 The analysis was facilitated by the unique F3Y• features arising from 19F and 2H/β hydrogen 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. Quantification of Y356• in β2 and F3Y731• (or F3Y730•) 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 reaction of F3Y122• by EPR spectroscopy allowed estimation of a ΔE°(Y356•-β2). The reversible nature of Y356 oxidation provided the opportunity to investigate the relative reduction potentials of Y356, Y731, and Y356•.

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 (102–103 s–1)19–21 in the active site of α2. Equilibration of the pathway Y•s could be measured because oxidation of Y356 by NO2Y122• is irreversible. Unfortunately, this same feature prohibited use of NO2Y122• to monitor equilibration of NO2Y122• into the protein environment23,25 gives an estimate of ΔE°(Y356•-β2) of 70 ± 5 mV, which along with our recent measurement of the reduction potential of F2Y in a protein environment22,25 gives an estimate of ΔE°(F2Y356•-•Y122•) of ~100 mV at pH 7.6. The results of the site specifically incorporated unnatural amino acids described herein together with our previous studies allow us to propose a thermodynamic landscape for the RT pathway in the E. coli class Ia RNR that is ~200 mV uphill between Y122 and C439•.

Materials. (His)6-Y731F-α2,16,26 (His)6-Y356F-α2,26 wt-α2 (specific activity of 2500 nmol/min/mg),26 tyrosine phenol lyase,27 F2Y,14 and F3Y14were isolated; apo F3Y122•β2 was expressed, isolated, and reconstituted22 as previously reported. F3Y122•β2 (0.7 Y•/β2) was available from an earlier study.22 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.30 The reference spectrum for Y122•, which was obtained as the signal averaged sum of the Y356• difference spectra, is in agreement with the previously reported spectrum.27

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

Analysis of EPR Data. Two different methods, A and B, were used for quantitation of the two radicals due to the small changes in the EPR spectra associated with the changes in T and pH (section described subsequently), the complexity of the spectra, and the few 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 ΔE°(F3Y122••Y•) was calculated based on the two quantitation methods described below and using

$$\Delta E^o = \frac{RT \ln K_{eq}}{F}$$

(1)
where $K_{eq} = [Y_{356}] / [F_{2}Y_{122}]$, $R$ is the ideal gas constant, $T$ is the temperature (K), and $F$ is Faraday’s constant.

**Method A:** Quantitation of $F_{2}Y_{122}$ 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 $F_{2}Y_{122}$. This represents the intensity of $F_{2}Y_{122}$ remaining constant, allowing easier visualization of the $Y_{356}$ signal that grows in with increasing temperature. Using the low-field features in the spectrum of $F_{2}Y_{122}$, $F_{2}Y_{122}$ 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 $F_{2}Y_{122}$ that remains in the composite spectra due to half sites reactivity was subtracted using the $F_{2}Y_{122}$ reference spectrum (Figure S1A). The resulting composite spectra show the interconversion between $F_{2}Y_{122}$ 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 $F_{2}Y_{122}$ and $Y_{356}$ cannot be determined reliably by eye. Therefore, a script was written in Matlab 2016a to automatically subtract out the remaining $F_{2}Y_{122}$. The amount of remaining $F_{2}Y_{122}$ was determined by adjusting the intensity of the $F_{2}Y_{122}$ 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 $F_{2}Y_{122}$ features) was minimized. The amount of $Y_{356}$ after subtracting out the remaining $F_{2}Y_{122}$ 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 $F_{2}Y_{122}$ and $Y_{356}$ within the Same Sample.** To support equilibration between $F_{2}Y_{122}$ 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 first recorded. Each sample was then thawed by submersion into a room-temperature E. coli water bath for 15 s and then incubated in a 2 °C water bath for 15 s followed by refreezing and reacquisition of the EPR spectra. The samples were thawed again and then placed in a 25 °C water bath for 15 s, refrozen, and the EPR spectrum rerecorded. Quantitation of Y356 and F2Y122 was performed as described above.

**RFO-EPR Analysis of $Y_{356}$ Formation as a Function of Temperature.** RFO-EPR experiments were performed on an Upgrade Instruments 1019 syringe ram unit and a model 715 syringe ram controller (ram speed 1.25 cm/s), $F_{2}Y_{122}$ (70 μM, 0.8 $F_{2}Y_{122}/β2$), and CDP (2 mM) in assay buffer in one syringe mixture with $Y_{356}$-F-α2 (70 μM) and ATP (6 mM) in a second syringe and incubated at varying temperatures (2°–37 °C) for either 4 or 10 s. The reaction mixture was then sprayed into liquid isopentane, and the crystals were packed into EPR tubes for analysis by X-band EPR spectroscopy. A packing factor of 0.60 ± 0.02 was calculated for $F_{2}Y_{122}$/β2. Data acquisition and analysis were performed as described for the hand-quench (HQ) method.

**HQQ-EPR Analysis of $Y_{356}$ Formation as a Function of pH.** $Y_{356}$-F-α2 (25 μM), $F_{2}Y_{122}$ (25 μM, 0.6–0.8 $F_{2}Y_{122}/β2$), and CDP (1 mM), and ATP (3 mM) were combined in 50 mM MES (pH 6.8) or HEPES (pH 7.0–8.0), 15 mM MgSO4, and 1 mM EDTA and incubated at 5 or 25 °C. Reaction mixtures were transferred to X-band EPR tubes also maintained in the water bath and frozen in liquid isopentane (−140 °C) within 20 s (or 1 min) for analysis by X-band EPR spectroscopy using methods A and B described above. The data were fit to

$$\log K = pH - pK_a$$

Where $K = [Y_{356}] / [F_{2}Y_{122}]$.

**RFO-EPR Analysis of the Reaction of $F_{2}Y_{122}/β2$, $Y_{356}$-F-α2, CDP, and ATP.** $Y_{356}$-F-α2 (80 μM) and 6 mM ATP in 50 mM TAPS pH 8.2, 15 mM MgSO4, and 1 mM EDTA in one syringe was rapidly mixed at 25 °C with an equal volume of $F_{2}Y_{122}/β2$ (80 μM, 0.7 $F_{2}Y_{122}/β2$) and CDP (2 mM) in the same buffer in the second syringe. The reaction was aged for 10, 20, or 40 s, quenched in liquid isopentane, and analyzed by X-band EPR spectroscopy as described above. The EPR parameters were as follows: microwave frequency 9.45 GHz; power 30 μW; modulation amplitude 1.50 G; modulation frequency 100 kHz; time constant 163.8 ms; and conversion time 20.48 ms. The total number of scans were 700 (10 s sample), 600 (20 s sample), and 560 (40 s sample). The simulations were carried out using EasySpin v5.0.183 in Matlab R2015b. The g-values (2.0073, 2.0044, and 2.0022) and β-H hyperfine tensor (54, 52, and 54 MHz) were fixed in the simulations using previously reported values for $Y_{356}$ in the reaction of NO2Y122/β2 with Y731F-α2 and the β1 and β2 hyperfine values of $F_{2}Y_{122}$.

**RESULTS**

**Temperature-Dependent Distribution of $F_{2}Y_{122}$ and $Y_{356}$ in β2 in the Presence of CDP, ATP, and $Y_{356}$-F-α2 (or $Y_{731}$F-α2).** We have recently shown that the reaction of $F_{2}Y_{122}$/β2, wt-α2, CDP, and ATP generates a kinetically and chemically competent $Y_{356}$ that can reoxidize $F_{2}Y_{122}$. We hypothesized that if we carried out the same experiment with a block in the pathway (Y731F-α2 or Y730F-α2), then equilibration of $F_{2}Y_{122}$ and $Y_{356}$ could be measured by EPR spectroscopy as a function of temperature, allowing determination of $\Delta E^\ddagger(F_{2}Y_{122}•–Y_{356}•)$, $F_{2}Y_{122}$/β2, CDP, and ATP incubated with Y731F-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 $F_{2}Y_{122}$ (pink) and $Y_{356}$ (blue). All spectra presented subsequently are additive and contain the same concentration of $F_{2}Y_{122}$ 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.
with active β2 containing a F3Y122• in each β monomer.\textsuperscript{22} Furthermore, while the active form of RNR is α2β2, the enzyme exhibits half-sites reactivity where only one of the two Y122•’s (one α/β pair) is active at a time.\textsuperscript{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/Y731F-α2/CDP/ATP reaction as a function of temperature (2–15 °C). The composite spectrum at each temperature was acquired on three independently prepared samples. (A and B) Low- and high-field regions of the spectra for trial 1 are shown here. The color code is described in panel A. Trials 2 and 3 are shown in Figure S3. The composite EPR spectra collected between 15 and 37 °C are shown in Figure S4. (C and D) Low- and high-field regions of a simulated spectrum of a reaction mixture containing 50% each of F3Y122• and Y356•. The spectrum was generated by adding the individual spectra of F3Y122• and Y356• (Figure 2). The dotted lines identify spectral features that are characteristic of Y356•.

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\textsuperscript{33} 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.\textsuperscript{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-...
alpha2 relative to wt-alpha2. Differences in reactivity between wt-alpha2 and Y731F-alpha2 are also recorded for photo-RNR, which contains a [Re(I)] photooxidant appended to the C-terminal tail of beta2 (S355C). We therefore posited that the block at 731 could perturb the reduction potential of Y356 compared to the wt enzyme. The equilibration experiments were repeated with Y730F-alpha2, and as seen in Figure 4 (blue dots), variations can be observed between Y731F-alpha2 and Y730F-alpha2, with the former construct generating slightly higher amounts of Y356.

Calculation of \( \Delta E^\circ \) of Y356 from Y731F and Y730F-alpha2 Studies. To calculate the reduction potential difference between F3Y122 and Y356, the In(\([\text{Y356}]/[\text{F3Y122}]\)) observed in the Y731F and Y730F-alpha2 reactions at 25 °C by the HQ method were used (eq 1):

\[
\Delta E^\circ(F3Y122 - Y356) = \Delta E^\circ(F3Y122 - Y356) + \Delta E^\circ(Y356) - \Delta E^\circ(F3Y122)
\]

where \( \Delta E^\circ \) values of Y356 from 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 released from Y356 is accepted by 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

\[\text{F3Y122 + Y356} \rightleftharpoons \text{F3Y122 + Y356} + \text{H}^+ \]

Figure 5. Composite EPR spectra of the F3Y122/β2/Y731F-alpha2/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/Y731F-alpha2/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.

The colors represent different pH values as described in panel A. The pH range was 6.8–8.0 at 25 °C. (A) Percentage Y356 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.

The percentage of Y356 for the pH range 6.8–8.0 at 25 °C (see Figure S10A for data from pH 6.8–7.8 for 5 °C). The percentage of Y356 at pH 6.8 and 7.0 are very low (Table S2), and the percentage of Y356 above pH 8.0 at 25 °C and pH 7.8 at 5 °C does not change. The maximum amounts of Y356 at 25...
Although the above studies allowed establishment of 1H hyperfine splitting in the millisecond time scale was used to avoid quenching on the time scale described above show that maximum Y356 is in the deprotonated state. Due to the ability to detect small amounts of the deprotonated Y356, 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 F2Y356β2/α2/CDP/ATP. Although the above studies allowed establishment of ∆E°(F2Y356•→Y356•) in F2Y122•/β2, the ∆E°(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 pKα 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 ∆E°(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 (Figure 7).

The EPR spectra of reaction mixtures quenched at 10 and 40 s are shown in Figure S11. A view of the entire spectrum is shown in the inset on the left in Figure 7. The results reveal small features on the low- and high-field sides that suggested the presence of F2Y356•. The resolved hyperfine splittings were simulated with the “pepper” module of EasySpin as described in the Methods section. From the initial simulations, it was recognized that the β-1H hyperfine parameters matched the doublet splitting on the high-field side of the spectrum, confirming the identity of this radical species as F2Y356•. The interdoublet splitting was reproduced with two equivalent 19F couplings having an Azz of 147 MHz. 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 Azz value for F2Y356• is slightly weaker than those reported previously for the other F2Ys (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. All the amount of F2Y356• in the 20 s sample was quantitated as 3 ± 1% of total spin. This amount of radical reflects ∆E°(F2Y356•→Y122•) of 70 ± 5 mV, which in combination with our reduction potential studies allows calculation of ∆E°(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. Wα2 has been removed from the landscapes for the sake of clarity.

DISCUSSION

RNRs are divided into three classes based on the metallocofactor used for thyl radical formation. All classes of RNR initiate nucleotide reduction by thyl radical mediated atom abstraction from the substrate. The reducing equivalents for the reaction are provided by oxidation of a pair of cysteines in the active site with a subtype of the class III enzyme which uses formate as the reductant as the sole exception. The class II RNR utilizes adenosylcobalamin as a cofactor, whereas the class III system uses a stable glycyl radical to generate the transient thyl radical. These
observations raise the issue of why and how a 35 Å oxidation process evolved in the class I RNR instead of a direct H atom abstraction process that is used by the other classes. The turnover number for deoxynucleotide formation (2–10 s−1) and the large distance between Y122• and Cα39 in the class Ia RNR require intermediates in the oxidation process and raise the question of how the thermodynamic and kinetic landscape of this process has evolved to maintain balanced dNTP pools and avoid self-inactivation. Investigation of this oxidation process has proven challenging primarily due to the slow rate-limiting conformation changes that occur in the αβ2 complex subsequent to S/E binding and prior to RT. Furthermore, the substantial overlap of the EPR spectra of Y•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 diferric-NO2Y122• cofactor (t½ of 40 s at 25 °C) in the β2 subunit of RNR. NO2Y• is ~200 mV more oxidizing than Y• and has provided insight about the thermodynamic landscape for the RT pathway in two ways. When NO2Y was substituted in place of each Y in the pathway (Figure 1, where x = 122 and 356 in β2 and 731 and 730 in α2), the resulting mutants were all catalytically inactive. Thus, perturbation of the reduction potential by +200 mV is sufficient to shut down the RT pathway. This observation supports previous proposals about the extent to which uphill steps can be accommodated in electron transfer (ET) pathways in general, and in RNR specifically. NO2Y substitution at each position also allowed assessment of the protein environment perturbation of the pKα of the phenol, relative to the pKα in solution. Positions 356, 731, and 730 were found to be minimally perturbed (+0.4, 1.0, and 1.2 units) and position 122 was found to be greatly perturbed (greater than +3 units). We assume that a similar position-dependent perturbation occurs with the F2Y• s incorporated at 356, 731, and 730. However, the unique environment of Y122 (hydrophobic and adjacent to the diferric cluster), this assumption cannot be made.

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

Due to the inability to investigate equilibration of Y356• with Y731• and Y730• in wt RNR, F2Y• was inserted in place of either Y731 or Y730 providing access to the unique EPR spectroscopic features of F2Y•. These experiments showed the presence of 10–15% F2Y731• (or F2Y730•). A knowledge of the pKα perturbation of ~1 unit at these positions in conjunction with differential pulse voltammetry (DPV) studies on the N-acetyl-3,5,6-difuoro-L-tyrosinamide 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 ΔE° ([β2H2]Y•–Y•) of ~100 mV (Figures 1 and 8). These studies together showed that the RNR protein environment perturbs F2Y and Y in a similar fashion and that F2Y is a good probe for the reduction potential of both Y731 and Y730.

More recently, we have reported the detailed kinetic analysis of the F2Y122•/β2/α2/CDP/ATP reaction. 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 F2Y122•. 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 (F2Y122• 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.

To quantitate the reduction potential increase that occurs upon replacement of Y122 with F2Y122•, it is important to determine whether the latter is reduced to the phenol or phenolate (F2Y122• vs F2Y122•−) during RT (Scheme 2). We favor the model where F2Y122• is generated upon RT. In support of this proposal is the observation of NO2Y122•− in the NO2Y122•−/β2 experiments. The solution pKα of NO2Y is 7.1, and the visualization of NO2Y122•− can be rationalized if Fe1–H2O has a pKα between 8.0 and 10.0. Although ferric iron typically reduces the pKα of bound water, di-iron clusters have been known to shift this value into the physiological pH range (pH > 7.0) in a protein-environment-dependent manner. The dimeric cluster environment in the class Ia RNR is unique and as noted above perturbs the pKα of Y122 by >3 units. If the pKα of Fe1–H2O is perturbed to >8.0, then initiation of the reaction with F2Y122• would primarily result in the generation of F2Y122•−. The
protonation state of \( F_2Y_{122} \) while favored to be deprotonated, is unknown and is under investigation.

The potential difference of \( \sim 20 \) mV calculated between \( F_2Y_{122} \) and \( Y_{356} \) (Figure 4) makes generation of \( F_2Y_{122} \) an appealing model. We predict that \( \Delta E^o(\text{NO}_2Y_{122}/\text{NO},Y_{122} - Y_{356}/Y_{356}) \) is \( \geq 200 \) mV, owing to the inability of \( Y_{356} \) to reoxidize \( \text{NO},Y \). With these two values, we can estimate \( \Delta E^o(\text{NO}_2Y_{122}/\text{NO},Y_{122} - F_2Y_{122}/F_2Y_{122}) \) as greater than or equal to \( \sim 184 \) 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 (\( \sim 180 \) mV). Unfortunately, we cannot at present directly extrapolate the potential difference calculated between \( \text{NO}_2Y_{122}/\text{NO},Y_{122} \) (or \( F_2Y_{122}/F_2Y_{122} \)) and \( Y_{356}/Y_{356} \) to \( Y_{122}/Y_{122} \). This is primarily due to the unique nature of residue 122’s environment compared to that of the other pathway Y’s. The \( Y_{122} \) site is not in equilibrium with solvent \( \sim 100 \) mV (Figure 8B). Finally, we note that our data taken on model systems have provided an estimate of \( \Delta E^o(Y_{122}/Y_{356}) \)](\( \sim 120 \) mV) more oxidizing than \( Y_{122} \) within the RNR protein environment.

Our observations with \( \text{NO}_2Y_{122}/\text{NO},Y_{122} \) and \( F_3Y_{122}/F_2Y_{122} \) can be easily extrapolated from \( \Delta E^o(Y_{122}/Y_{122} - Y_{356}/Y_{356}) \). The proton from \( F_2Y_{356} \) is in rapid exchange with solvent (Figures 6B and 110B), and at an appropriate pH, we predict that its reduction potential is a good approximation of \( Y_{356} \). The reaction of \( F_2Y_{356}/F_2Y_{122} \) is predicted to be roughly the same.\( \sim 30 \) mV,\( \sim 20 \) mV,\( \sim 40 \) mV, providing a \( \Delta E^o(Y_{122}/Y_{122}) \) of \( \sim 100 \) mV (Figure 8B). Finally, we note that our data taken together suggest that at 25 °C and pH 7.6 \( F_2Y_{122} \) 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-tyrosinamide derivatives. We note that this original prediction assumed that both \( F_2Y \) and \( Y \) are reduced to the corresponding phenols during turnover.

**Relationship between the Thermodynamic Landscape and Kinetics.** It is important to note that the equilibration studies described in this work were performed under nonturnover conditions (with \( Y_{122} \) or \( Y_{356} \) at 0% and 50% of Y2 complex). Given the predicted rate constant for H2O loss from the 2′ position (\( 10^{-8} \) s\(^{-1}\)) of the nucleotide, the rate of this reaction using 0.04% \( C_{439} \) would be \( \sim 10^{-2} \) to \( 10^{-3} \) fold faster than conformationally gated nucleotide reduction (2–10 s\(^{-1}\)). The above calculation assumes that the reaction landscape is isoenergic subsequent to generation of \( Y_{356} \). However, DFT calculations performed on the individual crystal structure of \( \alpha \) and on model systems have provided an estimate of \( \sim 120 \) mV for \( \Delta E^o(C_{439}/C_{439}) \sim 200 \) mV for \( \sim 10^{-2} \) to \( 10^{-3} \) fold faster than conformationally gated nucleotide reduction (2–10 s\(^{-1}\)).

The above calculation assumes that the reaction landscape is isoenergic subsequent to generation of \( Y_{356} \). However, DFT calculations performed on the individual crystal structure of \( \alpha \) and on model systems have provided an estimate of \( \sim 120 \) mV for \( \Delta E^o(C_{439}/C_{439}) \sim 120 \) mV for \( \sim 10^{-2} \) to \( 10^{-3} \) fold faster than conformationally gated nucleotide reduction (2–10 s\(^{-1}\)).
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 effectors. 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 and in the case of PCET reactions by controlling the positioning of the proton acceptor. The distances between Y122, Y356, and Y731 remain unknown because of the disordered C-terminal tail of β2. Thus, structures of the α2/β2 subunit interface and knowledge of how these structures are altered in the presence of S and E binding to α2 are crucial to understanding the overall landscape of the reaction and the tuning of the individual steps in the RT process. Nonetheless, we believe from the studies described herein, that the overall reaction from Y122 reduction to 3'-hydrogen atom abstraction of NDP is uphill and driven forward by rapid and irreversible loss of H2O from the NDP (Figure 8).29,35,59

PCET across the βα Interface Involves Fast Proton Exchange between Y356 and Solvent. The equilibrium between F1Y122 and Y356, as a function of pH has further provided important insight about the fate of the Y356 proton upon its oxidation. It was originally proposed that a specific sequenced amino acid residue within β2 functioned as the proton acceptor. However, the slope of 1 associated with a plot of log([Y356][F1Y122]) versus pH (Figure 6B) is due to the rapid exchange of the Y356 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.29,35,57,59,60

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-amino-tyrosine (NH2Y). This unnatural amino acid has site specifically replaced Y356, Y731, or Y730 leading to accumulation of NH2Y in each case upon incubation with the second subunit, S, and E.26,61 HF-EPR studies on NH2Y’s specifically the gα component of their g tensors, revealed that the electrostatic environment of all three NH2Y’s is perturbed, but that of NH2Y356 is perturbed to a greater extent than either NH2Y731 or NH2Y730. In contrast with NH2Y731 or NH2Y730, no moderate hydrogen bonding interactions were observed with NH2Y356 by HF-[3H] ENDOR spectroscopy.57 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 photooxidant is attached site specifically to residue 355 in β2 and F1Y (n = 2 or 3) or W replaces Y356. In the presence of α2, S, and E 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 α2/β interface.53,60

Finally, prior to the studies reported herein, the conserved residue E350 located on the flexible C-terminal tail of β2 near Y356 in sequence space, was considered to be the most likely amino acid candidate that could function as a proton acceptor for Y356. Mutation of E350 to A abolished RNR activity, an observation we have confirmed.11,28 However, using our ability to incorporate F1Y in place of RNR pathway residues, we have shown that E350 is likely not the proton acceptor for Y356 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 E350 studies, the EPR and ENDOR results, and the photo-RNR experiments 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 α2/β2 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 dNTP (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 differeic-Y• cofactor.69 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
3 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 Y350-Fα2 or Y730-Fα2; hyperfine values for β·H and 19F of F2Y• at different positions on pathway; analysis by method B for one trial of the F1Y122•-β2/Y73F-Fα2/CDP/ATP reaction as a function of temperature and one trial of the F1Y122•-β2/Y73F-Fα2/CDP/ATP reaction as a function of temperature and one trial of the F1Y122•-β2/Y73F-Fα2/CDP/ATP reaction as a function of temperature (2–15 °C) and the F1Y122•-β2/Y73F-Fα2/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/Y73F-Fα2/CDP/ATP as determined by HQ- and RFQ-EPR.
spectroscopies; temperature-dependent equilibration of F$_{Y_{125}}$ and Y$_{198}$ in the reaction of F$_{Y_{125}}$ with CDP, ATP, and Y$_{198}$-F$_{198}$; composite EPR spectra of the F$_{Y_{125}}$ with CDP/ATP reaction at 5 °C and the F$_{Y_{125}}$ with CDP/ATP reaction at 5 °C as a function of pH; pH dependence of Y$_{198}$ formation in the reaction of F$_{Y_{125}}$ with CDP/ATP at 5 °C; reaction of F$_{Y_{125}}$ with CDP, and ATP monitored by RFQ-EPR spectroscopy (PDF)

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**Notes**

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