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

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The *E. coli* class Ia ribonucleotide reductase (RNR) contains two homodimeric subunits, α2 and β2, and functions as an α2β2 complex. Its active cofactor is a diferric-tyrosyl radical (Y122•) unit buried within β2. This cofactor generates a transient thyl radical (C439•′) in α2, which initiates reduction of the four nucleotides (CDP, GDP, ADP, and UDP) to their corresponding 2′-deoxynucleotides (dNDP), with the specificity of reduction dictated by the appropriate allosteric effectors (ATP, dTPP, dCTP, and dATP). During each turnover, Y122• reversibly oxidizes C439 (or W48) via multiple proton-coupled electron transfer (PCET) steps through a pathway involving aromatic amino acid residues Y122•, Y122• (or Y730•), Y356•, and Y731•. This pathway includes a stepwise electron transfer process involving at least four nucleotides and a transient thiyl radical (C439•′) in another subunit (β2) via long-range radical transport (RT) through aromatic amino acid residues (Y122•, Y356•, Y731•, and Y730•) to their corresponding 2′ of the four nucleotides (CDP, GDP, ADP, and UDP) to their corresponding 2′-deoxynucleotides (dNDP), with the specificity of reduction dictated by the appropriate allosteric effectors (ATP, dTPP, dCTP, and dATP). The pH dependence of the F3Y122•F3Y356• reaction with Y731F•† has now afforded the opportunity to investigate equilibration of F3Y122• and Y356•. Incubation of F3Y122•β2, Y356•α2 (or Y730•F2), CDP, and ATP at different temperatures (2−37 °C) provides evidence for equilibration of Y356•α2 (or Y730•F2) with CDP at 25 °C. The pH dependence of the F3Y122•β2 and Y356•α2 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 Y731F•α2, CDP, and ATP at pH 8.2 and 25 °C shows F2Y356• generation by the native Y122•, F2Y-RNRs (n = 2 and 3) together provide a model for the thermodynamic landscape of the RT pathway in which the reaction between Y122• and C439•′ is ~200 meV uphill.

### 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•, Y356•, Y731•, and Y730•). Equilibration of Y122•, Y356•, and Y730• was recently observed using site specifically incorporated unnatural tyrosine analogs; however, equilibration between Y122• and Y356• has not been detected. Our recent report of Y356• formation in a kinetically and chemically competent fashion in the reaction of β2 containing 2,3,5-trifluorotyrosine at Y122• (F3Y122•β2) with α2, CDP (substrate), and ATP (effector) has now afforded the opportunity to investigate equilibration of F3Y122•α2 and Y356•α2. Incubation of F3Y122•α2, Y356•β2 (or Y730•F2), CDP, and ATP at different temperatures (2−37 °C) provides evidence for equilibration of Y356•α2 (or Y730•F2) of 20 ± 10 mV at 25 °C. The pH dependence of the F3Y122•β2 and Y356•α2 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 Y731F•α2, CDP, and ATP at pH 8.2 and 25 °C shows F2Y356• generation by the native Y122•, F2Y-RNRs (n = 2 and 3) together provide a model for the thermodynamic landscape of the RT pathway in which the reaction between Y122• and C439•′ is ~200 meV uphill.
unlabeled Y• signal could be detected; the EPR spectrum of Y• in the α2/β2 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 Y736) we demonstrated that Y122• equilibrated with F2Y731• or F2Y736•.18 The analysis was facilitated by the unique F•Y 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 F2Y736•) 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• upon oxidation of this pathway Y. A plot of the log([Y356•]/[F3Y122•]) versus pH provides a slope of 1.2 ± 0.2 at 25 °C, consistent with rapid release of the Y356• proton to solvent. With a knowledge of the pH dependence of the F3Y122•/Y356• equilibration, we have implemented an experimental design to determine the thermodynamic difference between Y122 and Y356. Increasing amounts of Y356• are observed with increasing pH. Additionally, by choosing an appropriate pH the reduction potential of F2Y can be tuned to be essentially equal to that of Y• –25,26 but oxidized F3Y• has the potential to be spectroscopically observable because of the 19F hyperfine features.12 Thus, the ability of Y122• to oxidize F3Y incorporated in place of Y356 (F2Y356β2) was tested. Rapid freeze-quench (RFQ)-EPR spectroscopy of the reaction between F3Y356β2, 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− Y122) of 70 ± 5 mV, which along with our recent measurement of the reduction potential of F2Y in a protein environment15,25 gives an estimate of ΔE°(Y356− Y122) of ~100 mV at pH 7.6. The results of the site specifically incorporated unnatural amino acids described herein together with our previous studies allow us to propose a thermodynamic landscape for the RT pathway in the E. coli class Ia RNR that is ~200 mV/acid pH between Y122 and C459°.

Figure 1. Proposed thermodynamic landscape of the PCET pathway at 25 °C and pH 7.6. The overall reaction is proposed to be thermodynamically uphill and driven forward by the rapid irreversible loss of water from NDP substrate in the active site of α2. No direct evidence is available for the presence of a discrete Wαα radical intermediate. Studies performed on NO2Y122•/β2 determined the relative reduction potentials of Y356, Y731, and Y356°.

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 (108−1010 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•/β2 to monitor equilibration of NO2Y122• 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.13,22 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 NO2Y122•/β2, however, we have demonstrated that Y356• can reoxidize F3Y122• and that this reoxidation process is rate-limiting for subsequent turnovers.12 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

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

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where $K_{eq} = \frac{[Y_{356}]_{o}}{[F_{3}Y_{122}]}$, $R$ 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 $\beta2$ as a Function of Temperature.** Each EPR spectrum was normalized to have the same area. The EPR parameters were as follows: microwave frequency 9.45 GHz; power 30 mW; 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 was 700 (10 s sample), 600 (20 s sample), and 560 (40 s sample). The simulations were carried out using EasySpin v5.0.18f [22] in Matlab R2015b. The $g$-values (2.0073, 2.0044, and 2.0022) and $\beta$-H hyperfine tensor (54, 52, and 54 MHz) were fixed in the simulations using previously reported values for $Y_{356}$ in the reaction of NOH$_{3}$-F$\beta2$ with Y$_{731}$F-α2$^{14}$ and the $\beta$F and $\beta$-H hyperfine values of $F_{3}Y_{122}$.$^{22}$

### RESULTS

**Temperature-Dependent Distribution of $F_{3}Y_{122}$ and $Y_{356}$ in $\beta2$ in the Presence of CDP, ATP, and Y$_{731}$F-α2 (or Y$_{730}$F-α2).** We have recently shown that the reaction of $F_{3}Y_{122}$-β2, wt-α2, CDP, and ATP generates a kinetically and chemically competent $Y_{356}$ that can reoxidize $F_{3}Y_{122}$.$^{13}$ We hypothesized that if we carried out the same experiment with a block in the pathway (Y$_{731}$F-α2 or Y$_{730}$F-α2)$^{13}$ 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.

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 concentration 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.

![Figure 2. X-band EPR spectra of equimolar concentrations of F$_{3}$Y$_{122}$• (pink) and Y$_{356}$• (blue). All spectra presented subsequently are additive and contain the same concentration 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.](image-url)
with active β2 containing a F3Y122• in each β monomer. 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. 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)

**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•.

described in the experimental section to allow the small changes in the amounts of Y356• as a function of temperature (2, 5, 8, 10, 12, and 15 °C) to be more clearly observable. With method A, the spectra have been manipulated such that the amount of F3Y122• remains constant, while Y356• grows in as a function of temperature. In this analysis method, each spectrum is normalized to have the same intensity in the low-field F3Y122• features. As shown by the dotted line in Figure 3A,B, increasing amounts of Y356• can then be observed between 2 and 15 °C. Two additional replications of this experiment are shown in Figure S3. The changes in the spectra directly correlate with increasing amounts of Y356• from 17 ± 5% (average of three trials at 2 °C) to 31 ± 2% (average of three trials at 15 °C) of total spin; the quantitation of these data is summarized in Table S1. In contrast with these observations, minimal changes are visualized in the composite EPR spectra recorded between 15 and 37 °C (Figure S4 and Table S1). The average amounts of Y356• in the three experiments are shown in Table S1 (31 ± 2% at 15 °C and 33 ± 1% at 37 °C). The percentage Y356• of total spin as a function of temperature is shown in Figure 4 (pink dots). A break in the curve is observed at ~15 °C, and the amount of Y356• does not appear to change significantly from 15 to 37 °C.

![Figure 4](image)

**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 RNR have shown that slow quenching of samples by hand shifts the equilibrium relative to rapid freezing methods. Thus, changing ratios of F3Y122• and Y356• by RFQ would support equilibration of the two radical states. Preliminary experiments revealed no spin loss and minimal changes in the EPR spectra of samples quenched at 4 and 10 s using the RFQ method. The time scale for quenching was chosen based on kinetic experiments performed with F3Y122•/β2 and wt-α2. 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 at 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. Differences in reactivity between wt-α2 and Y730F-α2 are also recorded for photo-RNR, which contains a [ReO3] photooxidant appended to the C-terminal tail of β2 (S355C). We therefore posited that the block at 731 could perturb the reduction potential of Y356• compared to the wt enzyme. The equilibration experiments were repeated with Y730F-α2, and as seen in Figure 4 (blue dots), variations can be observed between Y731F-α2 and Y730F-α2, with the former construct generating slightly higher amounts of Y356•.

Calculation of ΔE°′(F3Y122•−Y356•) from the Y731F and Y730F-α2 Studies. To calculate the reduction potential difference between F3Y122• and Y356•, the ln Ka_q ([Y356•]/[F3Y122•]) observed in the Y731F and Y730F-α2 reactions at 25 °C by the HQ method were used (eq 1) ; ΔE°′(F3Y122•−Y356•) at 25 °C is 20 ± 10 and 5 ± 7 mV, respectively. We note again the unusual temperature dependence of the Y356• amounts with a break at 15 °C. A similar temperature dependence has been noted for steady-state dNDP 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

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

Figure 5. Composite EPR spectra of the F3Y122•/β2/Y351F-α2/CDP/ATP reaction at 25 °C as a function of pH. The composite spectrum at each pH was acquired on two independently prepared samples. (A and B) The low- and high-field regions of the spectra for trial 1 are shown here. The colors represent different pH values as described in panel A. Trial 2 is shown in Figure S8. (C and D) Low- and high-field regions of a simulated spectrum of a reaction mixture containing 50% each of F3Y122• and Y356•. The spectrum was generated by adding the individual spectra of F3Y122• and Y356• (Figure 2). The dotted lines identify spectral features that are characteristic of Y356•.

Figure 6. pH dependence of Y356• formation in the reaction of F3Y122•/β2/Y351F-α2/CDP/ATP at 25 °C. (A) Percentage Y356• of total spin as a function of pH. (B) log K as a function of pH where K is the ratio of Y356• to F3Y122•. The observed pH dependence of slope 1.2 ± 0.2 supports that the Y356• proton is in fast exchange with solvent.

Figure 6A shows the percentage of Y356• for the pH range 6.8–8.0 at 25 °C (see Figure S10A for data from pH 6.8–7.8 for 5 °C). The percentage of Y356• at pH 6.8 and 7.0 are very low (Table S2), and the percentage of Y356• above pH 8.0 at 25 °C and pH 7.8 at 5 °C does not change. The maximum amounts of Y356• at 25

samples incubated for 20 s and 1 min, observations consistent with a reaction at equilibrium. The spectral changes are shown in Figure 5A,B, and the dotted line shows an increase in the
The dependence of $\log([Y_{356}]/[F_3Y_{122}])$ on pH at 25 and 5 °C is measured in Figures 6B and S10B, respectively. A slope of 1.2 ± 0.2 is observed at 25 °C (1.0 ± 0.1 at 5 °C) supporting the model in which the proton from $Y_{356}$ is in fast exchange with solvent at both temperatures. $Y_{356}$ 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 $Y_{122}$ and $F_2Y_{356}$ Using $F_2Y_{356}-\beta 2/\alpha 2/CDP/ATP$. Although the above studies allowed establishment of $\Delta E^\circ(F_3Y_{122}-Y_{356})$ in $F_3Y_{122}-\beta 2$, the $\Delta E^\circ(Y_{122}-Y_{356})$ in wt RNR, which is essential for understanding the thermodynamics of the RT pathway, remains unknown. The pH studies described above show that maximum $Y_{356}$ is generated with $F_3Y_{122}-\beta 2$ at pH 8.0 or greater and 25 °C. Recent studies suggest that the difference in reduction potential between $Y$ and $F_2Y$ at position 356 at pH 8.2 is small (<10 mV)25 and that the activity of $F_2Y_{356}/\beta 2$ at this pH is 50% of the wt activity.7 The $pK_a$ of $F_2Y_{356}$ is estimated to be 7.6 at position 356;15 thus, at pH 8.2, >80% of $F_2Y_{356}$ is in the deprotonated state. Due to the ability to detect small amounts of $F_2Y$ 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 $\Delta E^\circ(Y_{122}-Y_{356})$. $F_2Y_{356/\beta 2}, Y_{731}F-\alpha 2$, CDP, and ATP were reacted at pH 8.2 for 10, 20, or 40 s, and the reaction was quenched using the RFQ instrument and analyzed by EPR. Quenching on the millisecond time scale was used to avoid potential shifting of the equilibrium observed with hand quenching (Table S1 and Figure S6).33

The RFQ-EPR data for the reaction at 20 s are shown in Figure 7, and the 10 and 40 s data are shown in Figure S11. A view of the entire spectrum is shown in the inset in Figure 7. The results reveal small features on the low- and high-field sides that suggested the presence of $F_2Y_{356}$.13 The resolved hyperfine splittings were simulated with the “pepper” module of EasySpin as described in the Methods section. From the initial simulations, it was recognized that the $\beta-^3H$ hyperfine parameters matched the doublet splitting on the high-field side of the spectrum, confirming the identity of this radical species as $F_2Y_{356}$. The interdoublet splitting was reproduced with two equivalent $^3F$ couplings having an $A_{22}$ of 147 MHz.13,30 The sharpness of the $3,5-^19F$ features are similar to those previously reported for the other pathway residues $F_2Y_{122}$,13 $F_2Y_{731}$,14 and $F_3Y_{730}$, reflecting a rigid conformation constrained by the protein environment. The $A_{22}$ value for $F_2Y_{356}$ is slightly weaker than those reported previously for the other $F_2Y$’s (Table S4) and will be of importance when structural insight is obtained.

The amount of $F_2Y_{356}$ 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 $F_2Y_{356}$ in the 20 s sample was quantitated as 3 ± 1% of total spin. This amount of radical reflects $\Delta E^\circ(F_2Y_{356}-Y_{122})$ of 70 ± 5 mV, which in combination with our reduction potential studies24,25 allows calculation of $\Delta E^\circ(Y_{356}-Y_{122})$ 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 $F_3Y_{122}-\beta 2$ described in this work provided an estimate of the relative reduction potentials of $F_2Y_{122}$ and $Y_{356}$. (B) Studies performed on $F_2Y_{356}/\beta 2$ provided an estimate of the relative reduction potentials of $Y_{122}$ and $Y_{356}$. $W_{43}$ 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.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 site38–40 with a subtype of the class III enzyme which uses formate as the reductant as the sole exception.41 The class II RNR utilizes adenosylcobalamin as a cofactor,3 whereas the class III system uses a stable glycyl radical to generate the transient thyl radical.42 These
observations raise the issue of why and how a 35 Å oxidation process evolved in the class I RNR instead of a direct H atom abstraction process that is used by the other classes.18 The turnover number for deoxynucleotide formation (2–10 s−1)43 and the large distance between Y122• and Cα39 in the class Ia RNR9,44 require intermediates in the oxidation process and raise the question of how the thermodynamic and kinetic landscape of this process has evolved to maintain balanced dNTP pools and avoid self-inactivation. Investigation of this oxidation process has proven challenging primarily due to the slow rate-limiting conformation changes that occur in the α2β2 complex subsequent to S/E binding and prior to RT.43 Furthermore, the substantial overlap of the EPR spectra of Y•’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 dioxirano NO2Y122• cofactor (t1/2 of 40 s at 25 °C) in the β2 subunit of RNR. NO2Y• is ~200 mV more oxidizing than Y•16 and has provided insight about the thermodynamic landscape for the RT pathway in two ways. When NO2Y was substituted in place of Y356, 731, and 730 in α2, the resulting mutants were all catalytically inactive.15 Thus, perturbation of the reduction potential by +200 mV is sufficient to shut down the RT pathway. This observation supports previous proposals about the extent to which uphill steps can be accommodated in electron transfer (ET) pathways in general,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 F2Y’s incorporated at 356, 731, and 730. However, the unique environment of Y122 (hydrophobic and adjacent to the dieric 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−1,16 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 pKαs of Y122 and Fe1–H2O. Since the NO2Y phenol has a pKα of 7.1, this raises issues about the protonation state of F2Y122• (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•.14 These experiments showed the presence of 10–15% F2Y731• (or F2Y730•). A knowledge of the pKα perturbation of ~1 unit at these positions,15 in conjunction with differential pulse voltammetry (DPV) studies on the N-acetyl-3,5-difluoro-1-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 ΔE°([β2H2]Y•–Y356•) 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.22 This reaction generates a kinetically and chemically competent Y122• 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. Y731-F-α2 (Y730-F-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 F2Y122• it is important to determine whether the latter is reduced to the phenol or phenolate (F2Y122 or 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 pKα of NO2Y is 7.1,16 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,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 dieric cluster environment in the class Ia RNR is unique and as noted above perturbs the pKα of Y122 by >3 units.13 If the pKα of Fe1–H2O is perturbed to >8.0, then initiation of the reaction with F3Y122• would primarily result in the generation of F2Y122•. The
protonation state of F2Y122, while favored to be deprotonated, is unknown and is under investigation.

The potential difference of ~20 mV calculated between F2Y122• and Y356• (Figure 4) makes generation of F2Y122• an appealing model. We predict that ΔE° (NO2Y122•/NOY122•−− Y356•/Y356•) is ≥200 mV, owing to the inability of Y356• to reoxidize NOY−. With these two values, we can calculate ΔE° (NO2Y122•/NOY122•−− F2Y122•/F2Y122•) as greater than or equal to ~184 mV. This calculation agrees with the predicted potential difference between these two analogs based on the solution DPV data collected on the protected amino acids (~180 mV).24 Unfortunately, we cannot at present directly extrapolate the potential difference calculated between NO2Y122•/NOY122•−− (or F2Y122•/F2Y122•) and Y356•/Y356• to Y122•/Y122•. This is primarily due to the unique nature of residue 122’s environment compared to that of the other pathway Y’s. The Y122 site is not in equilibrium with solvent48 over the time course of our experiments (<20 s); its reduction potential is pH-independent and is directly determined by the dielectric constant of the protein environment. Due to these reasons, we turned our attention to an alternate way to monitor equilibration of Y122• and Y356• where the native Y122• remains intact but Y356• is replaced with F2Y356•.

Our observations with NO2Y122•/Y122•−− are consistent with the pH-dependent studies reported herein that suggest ΔE° (Y122•/Y122•−− Y356•/Y356•) can be easily extrapolated from ΔE° (Y122•/Y122•−− F2Y356•/F2Y356•). The proton from F2Y122• is in rapid exchange with solvent (Figures 6b and 10b), and at an appropriate pH, we predict that its reduction potential is a good approximation of Y356•. The reaction of F2Y356•/F2Y122•−− (or F2Y122•/F2Y122•−−) and Y356•/Y356• couples are predicted to be roughly the same.24,25 At pH 7.6, the standard assay conditions, the reduction potential of Y356• is expected to increase by ~30 mV,24,25,51 providing a ΔE° (Y356•/Y122•−−) of ~100 mV (Figure 8b). Finally, we note that our data taken together suggest that at 25°C and pH 7.6 F1Y122 is ~120 mV more oxidizing than Y122• 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-tyrosinamide derivatives.11 We note that this original prediction assumed that both F1Y 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 Y731F-α2 or Y730F-α2). Thus, a key issue to address is whether the protein environment can alter the thermodynamic landscape to lower ΔE° (Y356•/Y122•−−) and facilitate turnover. Although this is a likely possibility, we argue that oxidation of Y356 by Y122• must be uphill even under turnover conditions. Evidence for this conclusion is provided by our combined studies with wt RNR.43 F1Y122•/β22,24 and NO2Y122•/β217.

In the case of wt RNR, investigation of RT has been hindered by the inability to monitor Y122• 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 Y356• reoxidizes Y122• must be downhill and rapid (103 s−1).43 In the case of F1Y122•/β2, we have measured formation of Y356• (20–30 s−1) and demonstrated that reoxidation of F1Y122 by Y356• is slow (0.4–1.7 s−1) and rate-limiting for multiple turnovers.24 In the NO2Y122•/β2 system, Y356• accumulates (100–300 s−1) due to the inability of this pathway radical to reoxidize NO2Y• subsequent to the first turnover.17 Taken together, these studies suggest that Y356• can be observed during turnover only when reverse RT is slowed down (F1Y122•/β2) or completely inhibited (NO2Y122•/β2) and is partly a result of the potential difference between Y122 and Y356. DPV studies have estimated that reduction potential increases in the order Y < F1Y < NO2Y.16,24 In accordance with this prediction, the rate constant for forward RT that generates Y356• increases with increasing driving force, whereas the rate constant for reverse RT decreases with driving force, reinforcing our model that oxidation of Y356 by the native Y122• is uphill. We have previously proposed that the conformational change that triggers RT targets the initial PT step from Fe1–H2O to Y122• (Scheme 2).26 Uncoupled PT and ET in NO2Y122•/β2, and potentially F1Y122•/β2, suggest that we may have overcome this conformational gating and obtained direct insight into the thermodynamic effect of replacing Y122 with these unnatural analogs. Further support for this model is obtained when the forward RT rate constants in NO2Y122•/β2 and F1Y122•/β2 are predicted using the Moser–Dutton equation12 (eq 3) for dependence of kET on distance (R) and driving force (ΔG).

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

Assuming identical distances and reorganization energies (λ) for ET in NO2Y122•/β2 and F1Y122•/β2, the individual expressions for log kET can be combined to assess the effect of the driving force differences (ΔG, 200 mV vs 20 mV, Figure 8A) on kET. The net equation requires an estimation of λ; by varying the reorganization energy from 0.7 to 1.4 eV,40 kET in NO2Y122•/β2 was calculated to be 9- to 11-fold faster than kET in F1Y122•/β2. This approximation is similar to our experimental data (5- to 15-fold) supporting the idea that the driving force dictates the kinetics in these mutant RNRs and further that both NO2Y122• and F1Y122• are reduced to the corresponding phenolates during RT.

Based on our static thermodynamic picture constructed from the studies with NO2Y122•/β2 and those reported herein, we propose that the landscape from Y122 to Y730 is ~200 mV uphill (at 25°C and pH 7.6, Figure 8B). The landscape between Y730 and 3’ hydroxyl 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 ~0.04% C439• formation in the α2/β2 complex. Given the predicted rate constant for H2O loss from the 2’ position (10−6 s−1),9–21 of the nucleotide, the rate of this reaction using 0.04% C439• would be ~10−2 to 10−3-fold faster than conformationally gated nucleotide reduction (2–10 s−1).43

The above calculation assumes that the reaction landscape is isoenergetic subsequent to generation of Y731•. However, DFT calculations performed on the individual crystal structure of α2 and on model systems have provided an estimate of ~120 mV for ΔE° (C439•/Y730•)45,46 and ~90–260 mV for 3’ H atom abstraction by C439•.56–58 If the measured ΔEp (Y356•= Y122•) of 200 mV is reflective of the thermodynamic landscape under turnover conditions, then we estimate that the combined

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steps of C_{439} 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 acceptor54,56 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_{356}• 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).

PCET across the βα Interface Involves Fast Proton Exchange between Y_{356} and Solvent. The equilibrium between F_{i}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 sequenced amino acid residue within β2 functioned as the proton acceptor. However, the slope of 1 associated with a plot of log([Y_{356}•][F_{i}Y_{122}•]) versus pH (Figure 6B) is consistent with the rapid exchange of the Y_{356} proton with 8).19

Summary. Using site specifically incorporated F_{i}Y and F_{i}Y in place of β2 residues 122 and 356, respectively, and taking advantage of the unique EPR features of F_{i}Y• 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 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 dNDP (and hence dNTP) pools and the absolute amounts of these species. This process is largely controlled by binding the appropriate S/E pairs in α2, 40-50 Å removed from the site of RT initiation by the dифференциальный-фотохимический cofactor.55,59 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_{i}Y_{122}• and Y_{356}• in the reaction of F_{i}Y_{122}•-β2, CDP, ATP, and Y_{730-F-α2} or Y_{730-F-α2}; hyperfine values for β'-H and 19F of F_{i}Y• at different positions on pathway; analysis by method B for one trial of the F_{i}Y_{122}•-β2/Y_{731}F-α2/CDP/ATP reaction as a function of temperature and one trial of the F_{i}Y_{122}•-β2/Y_{731}F-α2/CDP/ATP reaction as a function of temperature; and one trial of the F_{i}Y_{122}•-β2/Y_{731}F-α2/CDP/ATP reaction as a function of temperature (2–15 °C) and the F_{i}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_{i}Y_{122}•-β2/Y_{731}F-α2/CDP/ATP as determined by HQ- and RFQ-EPR.
spectrometers; temperature-dependent equilibration of F$_{Y122}$ and Y$_{95}$ in the reaction of F$_{Y122}$, CDP, ATP, and Y$_{95}$-F$_{α}$-α2; composite EPR spectra of the F$_{Y122}$, CDP, ATP reaction at 25 °C and the F$_{Y122}$, CDP, ATP reaction at 5 °C as a function of pH; pH dependence of Y$_{95}$ in the reaction of F$_{Y122}$, CDP, ATP monitored by RFQ-EPR spectroscopy (PDF)

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**Notes**

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