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Kinetics of Hydrogen Atom Abstraction from Substrate by an Active Site Thiol Radical in Ribonucleotide Reductase

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Supporting Information

ABSTRACT: Ribonucleotide reductases (RNRs) catalyze the conversion of nucleotides to deoxynucleotides in all organisms. Active E. coli class Ia RNR is an αβ2 complex that undergoes reversible, long-range proton-coupled electron transfer (PCET) over a pathway of redox active amino acids (β-Y122 → β-W48 → β-Y156 → α-Y731 → α-Y730 → α-C439) that spans ~35 Å. To unmask PCET kinetics from rate-limiting conformational changes, we prepared a photochemical RNR containing a [ReI] photooxidant site-specifically incorporated at position 355 ([ReI]-β2), adjacent to PCET pathway residue Y356 in β. [ReI]-β2 was further modified by replacing Y356 with 2,5,5-trifluorotyrosine to enable photochemical generation and spectroscopic observation of chemically competent tyrosyl radical(s). Using transient absorption spectroscopy, we compare the kinetics of Y· decay in the presence of substrate and wt-α2-Y731F-α2 or C439S-α2, as well as with 3′-[3H]-substrate and wt-α2. We find that in the presence of wt-α2 and the unlabeled substrate do we observe an enhanced rate of radical decay indicative of forward radical propagation. This observation reveals that cleavage of the 3′-C·H bond of substrate by the transiently formed C439Y· thyl radical is rate-limiting in forward PCET through β and has allowed calculation of a lower bound for the rate constant associated with this step of (1.4 ± 0.4) × 10^4 s⁻¹. Prompting radical propagation with light has enabled observation of PCET events heretofore inaccessible, revealing active site chemistry at the heart of RNR catalysis.

INTRODUCTION

Managing the coupled translocation of protons and electrons is the keystone to energy storage and conversion. 1–3 Biological systems have evolved to capitalize on proton-coupled electron transfer (PCET) to execute energy conversions efficiently and with exquisite control. 4,5 E. coli class Ia ribonucleotide reductase (RNR) maintains reversible, 8,9 PCET over ~35 Å10–13 via a multistep, proton-coupled hopping mechanism and thus serves as a paradigm for the study of PCET in biology. 14,15 RNR catalyzes the conversion of nucleotides to deoxynucleotides, the bottleneck in de novo production of monomeric DNA building blocks. 16 The active form of E. coli class Ia RNR is composed of two homodimeric subunits, α2 and β2 (Figure 1a). 13,15 The active site is located in α, while the diferric-Y122 cofactor required to initiate active site chemistry is buried deep within β (Figure 1a). The rate-determining step in turnover consists of a conformational change triggered by substrate binding. 8 This process initiates radical translocation by way of bidirectional PCET to β-Y122 in which a proton is transferred from a specific water molecule ligated to the diferric cluster, 18 while the electron transfer (ET) results in oxidation of β-Y356. 19 β-Y356 then oxidizes α-Y731 across the α/β subunit interface which subsequently oxidizes α-Y730 and, in turn, α-C439 in sequential PCET steps (Figure 1b). 15,20,21 The C439Y thyl radical initiates active site chemistry by abstracting H· from the C3′-position of substrate. 22,23 Multistep active site radical chemistry follows, 16,24 resulting in reoxidation of C439 and reverse PCET along the same pathway of redox active amino acid residues to restore the radical resting state at β-Y122. 8,9 Despite the fact that this multistep radical transport pathway presents RNR as an ideal system in which to examine biological PCET kinetics, rate-determining conformational changes have largely precluded such studies. In order to disentangle conformational gating from PCET kinetics, we have developed photochemical RNRs. 25 Bypassing conformationally triggered reduction of the Y122 cofactor, we instead initiate PCET events midway through the RNR mechanism by photooxidation of Y356 (Figure 1b). Synchronization in this way has enabled detailed studies of photoinitiated substrate turnover, 26 spectroscopic observation of photogenerated radicals, 27 and direct measurement of radical injection rates into α2. 28 For initial

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More recently, we have developed a $\beta$-over the PCET pathway in $[\text{ReI}]$ appended to unnatural 2,3,5-tri mutations (C268S, C305S, and S355C) render a single cysteine Y731F-

$[\text{ReI}]$ is placed in a hypothetical position (dashed green line) brown; $\text{C}$ encompassing the 20 transfer during the generation of Y356 by circumventing the requirement for concomitant proton direct were prevented by fast charge recombination and thus a However, attempts to measure radical propagation kinetics chemical constructs, the $\alpha$-

$[\text{ReI}]$-Br crystal structure$^{29}$ is placed in a hypothetical position (dashed green line) intended for illustrative purposes only.

Figure 1. (a) Pseudoatomic model of the active $E.\text{coli}$ class Ia RNR $\alpha_2\beta_2$ complex$^{16,13,32}$ using PDB files 1MXR$^{33}$ and 4R1R.$^{34}$ $\alpha_2$ (purple and violet) binds substrate (blue), and effector (slate), and crystallizes with the 20-mer peptide corresponding to the C-terminal tail of $\beta_2$ (forest and light green) contains the $\mu$-O-Fe$^{II}$/Y$_{356}$- cofactor (Fe, brown; $\mu$-O, red). (b) Illustration of photoinitated radical transport over the PCET pathway in $\alpha$ (purple), and $\beta$ (green) via excitation of $[\text{Re}^I]$ appended to $\beta$-S$_{355}$C and with 2,3,5-F$_3$Y replacing $\beta$-Y$_{356}$. Residues 340–360 are disordered in the $\beta_2$ structure, and the $[\text{Re}^I]$-C$_{355}$S-Y$_{356}$ fragment (constructed from the $[\text{Re}^I]$-Br crystal structure$^{30}$) is placed in a hypothetical position (dashed green line) intended for illustrative purposes only.

constructs, the $\beta_2$ subunit was replaced by a short peptide encompassing the 20 C-terminal residues of the $\beta_2$ protein. More recently, we have developed a $\beta_2$ in which three mutations ($C_{268}S$, $C_{305}S$, and $S_{355}C$) render a single cysteine residue surface-exposed, facilitating site-specific conjugation of a bromomethylpyridyl rhenium(I) tricarbonyl phenanthroline complex to position 355 ([Re$^I$], Figure 1b).$^{29}$ By measuring the $[\text{Re}^I]$ excited-state lifetimes of $[\text{Re}]-\beta_2$ and $[\text{Re}]-\text{Y$_{356}$F}$-$\beta_2$ under different conditions we have shown that this photochemical $\beta_2$ is capable of reporting on Y$_{356}$ oxidation.$^{30}$ However, attempts to measure radical propagation kinetics directly were prevented by fast charge recombination and thus a low yield of photochemically produced radical. We have now achieved direct observation of Y$^\bullet$ propagation by circumventing the requirement for concomitant proton transfer during the generation of Y$_{356}$. Installation of an unnatural 2,3,5-trifluorotyrosine$^{31}$ in place of Y$_{356}$ ([Re$^I$]-F$_3$Y$_{356}$-$\beta_2$, Figure 1b) has successfully boosted the yield of the photochemically generated radical, allowing spectroscopic resolution of downstream radical propagation kinetics by transient absorption (TA) spectroscopy. We have shown that this construct is competent for photoinitiated enzymatic turnover and present the first direct measure of Y$^\bullet$ propagation kinetics through the active $\alpha_2\beta_2$ RNR complex. By comparing the kinetics of Y$^\bullet$ decay in the presence of substrate and wt-$\alpha_2$, $Y_{356}$F-$\alpha_2$, or C$_{355}$S-$\alpha_2$, as well as with [3′-2H]-substrate and wt-$\alpha_2$, we find that only in the presence of wt-$\alpha_2$ and substrate with natural isotopic abundance are radical decay kinetics enhanced. These data support that cleavage of the 3′–C–H bond of substrate by the transiently formed C$_{355}$S thyl radical is rate-limiting in forward PCET through $\alpha$. We report a lower limit for the rate constant associated with this step of (1.4 ± 0.4) × 10$^4$ s$^{-1}$. Unmasking PCET events in the active $\alpha_2\beta_2$ RNR has provided a first direct measure of active site kinetics in the class Ia enzyme, yielding new evidence for a long-standing model and shedding light on the mechanism by which RNR maintains control and specificity during long-range PCET.

MATERIALS AND METHODS

Materials. Wt-$\alpha_2$ (2000 nmol mol$^{-1}$min$^{-1}$) was expressed from pET28a-nrdA and purified as previously described.$^{35}$ Glycerol stocks of Y$_{356}$F-$\alpha_2$ and C$_{355}$S-$\alpha_2$ were available from a previous study$^{36}$ and were expressed and purified as wt-$\alpha_2$. All $\alpha_2$ proteins were prereduced prior to use.$^{21}$ [5′-3H]-cytidine 5′-diphosphate sodium salt hydrate ([5′-3H]-CDP) was purchased from ViTrax (Placentia, CA). 3′-Deuterated cytidine 5′-diphosphate ([3′-2H]-CDP) was available from a previous study,$^{36}$ in which it was synthesized as reported$^{22,23}$ Tricarbonyl[1,10-phenanthroline](4-bromomethyl-pyridine)rhenium- (I) hexafluoropropionate ([Re$^I$]-Br) was available from a previous study.$^{30}$ $E.\text{coli}$ thioredoxin (TR, 40 μmol/min/mg) and thioredoxin reductase (TRR, 1800 μmol/min/mg) were prepared as previously described.$^{37,38}$ 2,3,5-Trifluorotyrosine was synthesized enzymatically from pyruvate, ammonia, and 2,3,6-trifluorophenol with tyrosine phenol lyase as the catalyst.$^{39}$ Assay buffer consists of 50 mM HEPES, 15 mM MgSO$_4$, and 1 mM EDTA adjusted to the specified pH.

Preparation of $[\text{Re}]-F_3Y_{356}\beta_2$. Construction of C$_{268}S$/C$_{305}S$/S$_{355}C$/Y$_{356}$Z-pBAD-nrdB was achieved by site-directed mutagenesis using pBAD-nrdB as a template, and primers listed in the Supporting Information. $E.\text{coli}$ TOP10 cells were cotransformed with the newly constructed nrdB plasmid and pEVOL-Y$_{356}$-aARS obtained from a previous study$^{31}$ plated on LB-agar plates supplemented with 100 μg/mL ampicillin and 35 μg/mL chloramphenicol and incubated at 37°C overnight. A 1 mL culture containing the same antibiotics was inoculated with a single colony, incubated at 37°C for 10 h, and then used to inoculate a small culture grown overnight at 37°C. This starter-culture was used to inoculate 4 × 2 L cultures of 2xYT at a 100-fold dilution. The cells were grown in the presence of 1.5 mM 2,3,5-F$_3$Y until reaching an OD$_{600}$ of 0.5, at which point the F$_3$Y-aARS and nrdB genes were induced with arabinose (0.05% w/v). The cells were grown for an additional 4 h to a final OD$_{600}$ of ~1.5, and then harvested by centrifugation (3000 × g, 10 min, 4°C). Yields of ~2 g/L were obtained. Success of expression was assessed by 10% SDS-PAGE. The protein was purified by anion-exchange chromatography following a previously reported protocol,$^{40}$ to give 10–15 mg per g of cell paste. Holo-S$_{355}C$/2,3,5-F$_3$Y$_{356}$Z$-\beta_2$ contained 0.6 Y$^\bullet/\beta_2$ and exhibited no enzymatic activity. This variant is inactive due to the presence of a thiol/thiolate in the tricysteine mutant, which may be oxidized by F$_3$Y. This quenching process is not a concern in the photoRNR experiments because conjugation to [Re$^I$] results in a thiocysteine, which is difficult to oxidize. Purified material contained ≤5% ββ heterodimer resulting from the presence of protein truncated at position 356 (a consequence of the method used for unnatural amino acid incorporation). Treatment with hydroxyurea to quantitatively reduce Y$_{356}$Z and labeling with [Re$^I$]-Br were achieved as reported previously$^{29}$ to yield met-[Re$^I$]-F$_3$Y$_{356}$Z$-\beta_2$ exhibiting >95% labeling efficiency.

Steady-State Emission $pK_a$ Titration. The steady-state emission intensity of 5 μM [Re$^I$]-F$_3$Y$_{356}$Z in the presence of 1 mM CDP, 3 mM ATP, and 20 μM wt-$\alpha_2$ was measured in buffer containing 50 mM of either MES (pH 5.2–6.8) or HEPES (pH 7.0–7.6). 15 mM MgSO$_4$, and 1 mM EDTA. Excitation at 315 nm using a 420 nm long-pass cutoff filter allowed spectra to be recorded over 450–650 nm, scanning 3 times per sample at a rate of 0.1 nm/s and detecting in 0.5 nm steps. Samples were held at 25°C for 2 min prior to scan and
throughout the duration of the measurement. Integrated emission intensity was plotted versus pH and fit to eq 1 (Figure S1). Here, $I$ corresponds to integrated emission intensity and $I_{\text{max}}$ and $I_0$ correspond to $I$ at pH 7.6 and 5.2, respectively.

$$10^{(pH + pK_a)} = \frac{I - I_0}{I_{\text{max}} - I_0}$$

(1)

Photochemical Turnover. Single turnover experiments under photochemical conditions were performed by mixing 10 μM each of met-[Re]-F3Y-β2 with wt-α2, Y122F-α2, or Cys356S-α2 in the presence of 0.2 mM [5-3H]-CDP (specific activity 26,700 cpm/nmol), 1 mM ATP, and 10 mM Ru(NH3)6Cl3 in assay buffer at pH 7.6. Samples were placed in a 4 mm × 4 mm quartz cuvette and held at 25 °C under illumination for 10 min with white light powered at 800 W (35 V and 24 A DC) in conjunction with a 313 nm long-pass cutoff filter. Quantitation of radioactive products by scintillation counting and confirmation of product identity (Figure S2) were performed as previously described.28 Equivalents of dCDP per α2 were calculated in the presence of [Re]-F3Y-β2 were normalized to dCDP production in the presence of wt-α2 under the photochemical conditions, which produces 1.2 equiv/α2 of a theoretical maximum of 4 equiv/α2. Photochemical activity for 3–5 independent sets of unique samples was determined, and the standard deviation associated with both wt-α2 and [Re]-F3Y-β2 dCDP production was propagated during normalization.

Nanosecond Laser Flash Photolysis. Samples were prepared in a total volume of 750 μL and recirculated through a 1 cm path length flow cell to reduce sample decomposition. An inline filter (Acrodisc 13 mm 0.2 μM Supor Membrane, Pall Corporation) was used to collect solid photoproducts. Optical long-pass cutoff filters (λ > 375 nm) were used to filter probe light before detection to remove scattered 355 nm pump light. Samples contained 50 μM [Re]-F3Y-β2; 75 μM of wt-α2, Y122F-α2, or Cys356S-α2; 3 mM ATP; and either 1 or 0.5 mM CDP or [3-3H]-CDP. Laser experiments were performed using a system that has previously been described.34 Single wavelength kinetics data were collected at 412.5 nm using slit widths corresponding to 0.7 nm resolution and recorded over 100 laser shots for each sample. TA spectra were collected over 500 four-spectrum sequences where two of four conditions result in exposure to the pump beam.

Lifetimes were obtained by averaging three sets of decay traces from each unique sample of a single protein preparation (both wt-α2 and [Re]-F3Y-β2 expression, purification, and [Re]-labeling) (Trial 1 in Table S1), according to eq 2:

$$y = y_0 + Ae^{-t/τ}$$

(2)

Lifetimes from the decay traces for another three sets of unique samples were then obtained using a second protein preparation (Trial 2 in Table S1). Table 1 lists the propagated error for the six measurements across the two trials with weighted averages provided by error associated with the fit for each data set within a trial, compounded with the standard deviation between the two trials. An exemplary data set, along with fits to eq 2, and associated residuals analysis are included in Figure S3.

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<td>CDP</td>
<td>18 (1)</td>
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<tr>
<td>Y122F</td>
<td>CDP</td>
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<tr>
<td>wt</td>
<td>[3-3H]-CDP</td>
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*Photogenerated Y- lifetimes monitored by TA spectroscopy at 412.5 nm and fit to monoeXponential decay from 3 to 76.5 μs (Figure S3). Weighted averages represent duplicate sets of three measurements each, on two separate protein preparations of [Re]-F3Y-β2 and wt-α2. Samples contain 50μM met-[Re]-F3Y-β2; 75 μM α2 variant, 1 mM or 0.5 mM CDP or [3-3H]-CDP, 3 mM ATP, 10 mM Ru(NH3)6Cl3, and assay buffer at pH 8.2.

**RESULTS**

pKα of 2,3,5-F3Y356 within the α2/β2 Complex. Photochemical generation of an observable population of F3Y356 is enhanced when the amino acid resides in its deprotonated state. This enhanced radical generation is a direct consequence of the ability to generate the radical by removal of only an electron as opposed to removal of an electron and proton (i.e., PCET). To determine the optimum pH for photochemical radical generation, we measured the pKα of F3Y356 within the α2/[Re]-β2 complex. This measurement was accomplished by monitoring the steady-state emission from the rhenum complex excited state ([ReI*]), which is quenched much more effectively when the adjacent F3Y is deprotonated.41 Plotting emission intensity as a function of pH revealed a pKα of 6.2 ± 0.1 (Figure S1). This value is in line with the pKα of 6.4 measured for the free amino acid derivative,42 particularly in light of the fact that a positively charged [ReI] complex is present. The pKα measured here is also in line with the value of 6.8 predicted by titrations using 3-NO2Y356β2, in which, by comparison with free NO2Y, the ΔpKα due to perturbations arising from the protein environment at position 356 was inferred.40 All subsequent spectroscopy was conducted at pH 8.2 such that ~99% of F3Y356 is deprotonated.

Observation of Transient Y-. In order to observe photogenerated Y-‘s, charge recombination within the initially formed charge-separated state ([ReI]-F3Y356-β2) must be prevented. Therefore, we applied flash-quench methodology by including an excess of Ru(NH3)6Cl3 in reaction mixtures. Bimolecular quenching of the [ReI]* excited state furnishes the [ReII] complex via reduction of Ru(NH3)63+ to Ru(NH3)62+. This [ReII] species is capable of driving rapid oxidation of trifluorotyrosinate (Figure 2a). Figure 2b shows the TA spectrum of Y- collected 3 μs after excitation, at which point nearly all photochemistry is completed and F3Y- and/or Y- are the only transient species contributing to the absorption feature centered at 412 nm. The overall photochemical yield of Y- is ~1.9% (calculation included in the Supporting Information).

Experiments were performed at protein concentrations such that >97% of [Re]-F3Y356β2 is complexed to α2 (based on the previously reported $K_0$ of 0.7 ± 0.1 μM, measured for [Re]-β2 binding to α2 under the same conditions).30 The control experiment performed with [Re]-Y356F-β2 (black, Figure 2b) shows a minor TA signal, which we have previously observed and ascribed to off-pathway generation of Y-30. In the absence of an adjacent redox active amino acid, off-pathway radical generation is maximized. Thus, this spectrum (black, Figure 2b) represents a maximum possible contribution to the observed signal and is likely greater than any off-pathway contributions operative when F3Y or Y is present.

Photochemical Competence for Turnover. To evaluate the relevance of photochemically generated Y- in RNR chemistry, we sought to verify chemical competence for enzymatic turnover via photochemical initiation. Steady-state illumination under single turnover conditions in the presence of radiolabeled substrate ([5-3H]-CDP), effector (ATP), Ru(NH3)6Cl3, and α2 allows quantitation and identification of photogenerated products.30,41,43 Of note, the μ-O-FeIII/Y122 cofactor of [Re]-F3Y356β2 has been reduced with inhibitor hydroxurea to form met-[Re]-F3Y356β2 thus the normal mechanism for turnover is not viable with this construct.

Table 1. Y- Lifetimes for α2- Variant/Substrate Combinations

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preparation of both wt-α and [Re]-F3Yβ2 data sets from two protein preparations were measured. Accordingly, the data in Table 1 are the propagated error for the six measurements across the two trials. Of note, the initiation process in which F3Y356− is oxidized by [ReII] simply generates the radical on the PCET pathway. All fits to kinetics data begin at 3 μs, after the F3Y− has formed. Thus, by removing the proton dependence of the initiation step, we are able to increase the yield of the radical (by relying only on an ET vs PCET for radical initiation), and we do not alter the PCET mechanism at play during the steps of interest that follow. With the exception of τ measured in the presence of Y731F-α2, the lifetime in each case corresponds to a total signal composed of contributions from F3Y356− in β1·Y731 and Y730′ in α2. Similar signal amplitudes were observed at t = 0 within each trial (Table S1), revealing that Y′-generation was similar to the different α2 variants and substrates.

We found that the measured lifetime of τ = 18 ± 1 μs for wt-α2 is extended to 24 ± 1 μs for the Y731F-α2 variant, which cannot produce a radical in α2 (Table 1). This observation suggests that the PCET pathway in α introduces an additional route for Y′-decay. The significant difference between these two values provides a means of differentiating on- and off-pathway radical decay. The relative kinetics of the productive, on-pathway contribution to the total decay is calculated according to eq 3, where τo represents the lifetime measured in the presence of Y731F-α2. The resultant rate constant (kobs) is (1.4 ± 0.4) × 104 s−1.

\[ k_{\text{obs}} = \frac{1}{\tau} - \frac{1}{\tau_o} \]  

(3)

In order to understand which processes in α limit the rate of radical transport, we next blocked the interior end of the PCET pathway by using C439S-α2. A lifetime identical to that measured in the presence of Y731F-α2 was observed (Table 1), revealing that the rate-determining step operative in the presence of wt-α2 occurs either during or after oxidation of residue C439. We note, however, that this observation does not reveal whether or not radical injection into the α subunit occurs in the presence of this variant. It could be envisioned that the downstream perturbation to the PCET pathway imposed by the C439S amino acid substitution precludes radical injection entirely. Thus, either radical injection followed by rapid reverse PCET and quenching occurs or injection is precluded. Both possibilities provide evidence for strict conformational control over PCET events. Throughout our analysis we have assumed that, in the presence of wt-α2 and CDP, new routes for off-pathway Y′-decay have not been introduced. In all cases, τo is unrestricted in its definition and simply represents the Y′-lifetime via any avenue of nonproductive decay. Thus, in calculating kobs we apply the same τo value for nonproductive decay in the case where the productive pathway (turnover) has also been enabled.

Once C439 is oxidized, the ensuing radical abstracts a hydrogen atom from the 3′-position of substrate (Scheme 1). Subsequent loss of a molecule of water from the 2′-position represents the first irreversible step of turnover. This irreversibility renders any subsequent steps inconsequential to the Y′-decay rate. Thus, the rate-determining step with respect to Y′-decay in our system (namely, where the native pathway through β is overridden) must be either oxidation of C439 or H-abstraction from C3′ of substrate. To differentiate between these two possibilities we next measured τ in the presence of

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**Pathway and Isotope Dependence of Y′-Lifetime.** We set out to explore individual PCET steps by measuring the kinetic behavior of Y′ under different conditions. We compared the lifetime of transiently formed Y′ (τ) in the presence of CDP, ATP, and wt-α2 with that of τ in the presence of CDP, ATP, and α-variants containing redox-inactive pathway substitutions, as well as in the presence of [3′-H]-CDP, ATP, and wt-α2. Lifetime data were determined from measurements of three unique samples from a single protein preparation of both wt-α2 and [Re]-F3Yβ2.
wt-α₂ and [3′-2H]-CDP. Here, a lifetime similar to that observed in the presence of wt-α₂ and CDP would suggest that oxidation of C₄₃⁹ by Y₇₃₀ is rate-determining, because slowing down the subsequent step has no effect on τ. Alternatively, a lifetime intermediate between that observed in the presence of wt-α₂ and CDP and that observed in the presence of pathway-blocked variants would indicate that cleavage of the 3′-C=H bond by C₄₃⁹ is rate-limiting. We found that the latter case prevails, and to its extreme amount.

Introduction of deuterium at the 3′-position of substrate completely alters the relative kinetics of the system, resulting in τ statistically identical to τ₀ (Table 1). Further, unlike in the case with C₄₃⁹-S-α₂, here we are using wt-α₂ and so no perturbation to the PCET pathway is incurred. Therefore, in this case we do assume that radical injection into α occurs. Observation of this isotope effect (IE) suggests that we have successfully uncoupled radical translocation from conformational gating, thus allowing for the first direct measure of PCET kinetics within the αβγ complex.

## DISCUSSION

More than 30 years ago [3′-2H]-NDPs were used to investigate the mechanism of RNR. Small amounts of RNR-mediated ³H₂O release and ³[H/V/K] isotope effects provided strong evidence that 3′-C=H bond cleavage occurs during NDP reduction. This study also established that the first irreversible step during a single turnover occurs after hydrogen atom abstraction from substrate and formed the underpinning for the mechanistic model shown in Scheme 1 (intermediates 2–4) and Figure S4.

Stepwise PCET between Tyr-O· → Cys-S· → R₅C· akin to the interconversion of 1 → 2 → 3 in Scheme 1 seems contrary to thermodynamic favor (bond dissociation energies of PhO–H, RS–H, and HOCH₂–H are ~86, 91, and 94 kcal/mol, respectively). However, a central tenet of the model proposed in Scheme 1 is that enzymatic coupling of endergonic steps to an irreversible reaction can provide a means of overcoming thermodynamic hurdles. The irreversible and entropically favored release of a molecule of water from the 2′-position of substrate (e.g., 3 → 4 in Scheme 1) is postulated to drive the RNR reaction forward as and when small amounts of intermediate 3 are formed from the reversible steps leading up to it. The loss of a rate enhancement for Y· decay in the presence of [3′-2H]-CDP reveals a primary IE on the cleavage of the substrate 3′-C=H bond, providing direct evidence in support of this model.

The steps in the RNR mechanism that are relevant to the current experiment are outlined in Scheme 1. We note that radical translocation in our system is initiated midstream along the PCET pathway; thus steps within the β subunit are inconsequential. Here, the step between A and B accounts for our experimental observable, a composite signal of unknown relative contributions from F₂Y₃₅₆ in β, Y₇₃₁, and Y₇₃₀ in α₂. Reoxidation of F₂Y₃₅₆ by Y₇₃₁ is predicted to be ~110 mV uphill at pH 8.2 based on the relative potentials of these amino acids in solution. Yet, experiments in RNR suggests that Y₃₅₆ is ~100 mV easier to oxidize than Y₇₃₁ within the subunit interface. Similarly, Y₇₃₁ and Y₇₃₀ have been predicted, and experimentally shown, to be isoenergetic within the enzyme complex. Thus, all of these radical species have been drawn as reversibly interconverting and reduction of intermediate 1 by an off-pathway quencher, Q, can potentially occur by way of any of these radical intermediates.

On-pathway Y· decay occurs by oxidation of C₄₃⁹ to give intermediate 2. Upon oxidation, C₄₃⁹ reversibly abstracts H· from the 3′-position of substrate to generate 3. General base (E₄₄₁) and acid (C₂₂₅) catalysis facilitate dehydration from the 2′-position to yield the 2′-αketyl radical intermediate (4). Despite the fact that active site chemical transformations following the formation of 4 ultimately result in the regeneration of the C₄₃⁹ and reverse PCET to reform 1 (Figure S4), these processes occur on a much slower time scale (~100 s⁻¹) than under examination here. Accordingly, 1 is not expected to reform during the time course of the experiment. Thus, 4 represents a terminal product with respect to Y· decay and the mechanistic steps relevant to our experimental conditions are limited to the interconversion of species 1–4 in Scheme 1.

Our results comparing τ in the presence of [3′-2H]-CDP versus CDP reveal that the rate enhancement for nonlabeled CDP occurs after oxidation of C₄₃⁹. However, the implications of the reversibility of the oxidation of C₄₃⁹ on the radical lifetimes of 1 must be considered. With little kinetic information regarding the equilibrium between 1 and 2, we turn to small molecule model studies in which the kinetics of bimolecular oxidation of Cys by Tyr· were examined by pulse radiolysis. These studies report a rate constant of 2 × 10⁶ M⁻¹ s⁻¹ and that the reverse reaction (oxidation of Tyr by Cys·) is significantly faster. These results suggest that a pre-equilibrium may be established between intermediates 1 and 2 in our system and facilitates an estimate for the magnitude of the corresponding equilibrium constant, Kₛ⁻¹ (kᵤ⁻¹/kₛ), from thermodynamic values. Electrochemical measurements of tyrosine and glutathione reveal that cysteine and tyrosine have approximately equal midpoint potentials at pH 7.0 (0.94 ± 0.04 V and 0.93 ± 0.02 V, respectively). However, calculations based on a trapped form of the active RNR complex suggest that oxidation of C₄₃⁹ by Y₇₃₀ is endergonic by 3–4 kcal/mol, resulting in Kₛ⁻¹ ≈ 10⁻².⁴⁷ Taken together, these
studies support that $2$ is formed reversibly from $1$ and that the resultant equilibrium constant is $\leq 1$. We note that $2$ has never been observed or trapped in a class I enzyme, indicating that this intermediate is likely consumed upon its formation. This scenario renders $k_{\text{obs}}$ a lower bound for $k_2$ where the extent to which the actual $k_2$ is greater than $k_{\text{obs}}$ increases as $K_{2,1}$ decreases from unity.

The reversibility of H atom abstraction may also lead to a case where $k_2$ is greater than $k_{\text{obs}}$. The extent to which this is manifest depends upon the efficiency with which $3$ proceeds forward as a fraction of its total decay. This efficiency can be described by a net rate constant equal to $k_2/(k_{-2} + k_2)$, which has a maximum value of $1$. Reactions similar to both of the processes described by $k_{-2}$ and $k_2$ have been studied extensively, producing rate constants ranging from $10^6$--$10^8$ M$^{-1}$ s$^{-1}$, $52$--$56$ and $10^8$--$10^9$ s$^{-1}$, $57$--$59$ for $k_{-2}$ and $k_2$, respectively. Though it is difficult to compare bimolecular reactions in solution to those within an enzyme active site, we note that these values are all $10^1$--$10^4$-fold faster than the corresponding bimolecular rate constants akin to conversion of alcohols and ethers by cysteine, glutathione, and penicillin amine radicals have been measured by pulse radiolysis, $52$--$56$ laser flash photolysis, $50$ and NMR $51$ and EPR $52$ competition experiments. Rate constants for C--H bond cleavage in deoxyribose, tetrahydrofuran, 2-propanol, glucose, and other $2^\text{nd}$ alcohols and ethers by cysteine, glutathione, and penicillin amine radicals have been measured by pulse radiolysis to give second-order rate constants in the range $1.2$--$1.8 \times 10^4$ M$^{-1}$ s$^{-1}$, $52$--$56$. These studies bolster our interpretation that hydrogen atom transfer from C$3'$ to C$4_{35}'$ is rate-limiting within the context of Scheme 1 and provide values that are consistent with the rate constant reported here.

Previous success in unmasking radical translocation kinetics in RNR is limited to only three examples. Replacing $\beta$-Y$_{122}$ with NO$_2$Y, whose high reduction potential and low pK$_a$ decouple PT from ET, allowed examination of $\beta$-NO$_2$Y$_{122}$ reduction by stopped-flow absorption and rapid freezeframe EPR spectroscopies. $39$ This work, though still limited by mixing times and possibly by further conformational gating, allowed the rate constant for ET to NO$_2$Y$_{122}$ to be bracketed with a lower limit of $300$ s$^{-1}$. $49$ The limitations imposed by mixing times are completely eliminated with photoRNRs. Modification of the C-terminal tail of $\beta$ with an appended [ReI]$^2$-2,3,6-F$_3$Y revealed a rate constant of $(3 \pm 2) \times 10^5$ s$^{-1}$ for radical injection into $\alpha_{\text{sub}}$ and this observation was further corroborated by our measurement of the rate constant for charge separation ($k_{\text{CS}}$) in a photo-$\beta_\text{CS}$ containing the native Y residue at position 356 of $\beta$, where a $k_{\text{CS}} = (4.1 \pm 0.1) \times 10^5$ s$^{-1}$ in the presence of wt-$\alpha_{\text{sub}}$, CDP, and ATP was observed. $50$ All of these results are in line with the conclusions presented here: radical injection into the $\alpha_{\text{sub}}$ subunit is faster than subsequent PCET and substrate activation steps.

The fact that C--H bond cleavage from substrate by the transiently formed C$_{45}'$ occurs at $10^5$ s$^{-1}$, along with previous results, reveals that PCET events occur rapidly during radical translocation. Together with recent findings that implicate alignment of the PCET pathway as a target of conformational gating, $13$ $18$ $47$, these results suggest that the reaction profile of the active $\alpha_2\beta_2$ complex remains locked in place as radical translocation and subsequent active site chemical steps transpire. This ability to lock the PCET pathway indicates that RNR capitalizes on the constraints imposed by PT distances in achieving acute control over long-range ET.

A number of studies indicate that the PCET pathway of RNR runs slightly thermodynamically uphill in the forward direction, $14$ $15$ $31$ $46$ $49$ and active site chemistry is driven forward by the rapid and irreversible loss of water from the $2'$-position of substrate. $24$ $57$--$59$. This reaction landscape presents a mechanism by which RNR avoids the buildup of reactive amino acid radical intermediates over the course of its $\sim$70 Å round-trip traverse between $\alpha$ and $\beta$. Our observation that HAT from C$3'$ of substrate to C$_{45}'$ is rate-limiting in forward PCET through $\alpha$ provides further evidence that an uphill PCET pathway generates the initial substrate radical.

### CONCLUSIONS

Jump-starting radical propagation with light has enabled the direct observation of PCET events previously inaccessible, revealing active site chemistry at the heart of RNR catalysis. Despite the fact that RNR turnover is rate-limited by conformational changes occurring at $\sim$2--$10$ s$^{-1}$, radical propagation steps are rapid. To unmask PCET events we have constructed a photochemically competent $\beta_2$ subunit capable of generating observable transient Y' species within the $\alpha_2\beta_2$ complex. With this construct, we have observed an IE on cleavage of the substrate $3'$-C--H bond, revealing that this step is rate-limiting with respect to Y' propagation through $\alpha$ and allowing us to report a lower bound for the rate constant associated with this step of $(1.4 \pm 0.4) \times 10^5$ s$^{-1}$. Unmasking PCET events in the active $\alpha_2\beta_2$ RNR has provided a first measure of active site kinetics in the class Ia enzyme, yielding new evidence for a long-standing model and shedding light on the mechanism by which RNR maintains control and specificity during long-range radical transport.

### ASSOCIATED CONTENT

5 Supporting information

Experimental methods and instrumentation; calculation of photochemical Y' yield; complete table of data including r and $\Delta$OD amplitudes for two trials; pK$_a$ titration; HPLC analysis of radiolabeled photoproducts; single wavelength kinetics data; fitting, and residuals analysis; scheme depicting the entire model for RNR-mediated substrate turnover. This material is available free of charge via the Internet at http://pubs.acs.org.

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